Divergent Potentials for Cytoplasmic Inheritance within the Genus Syringa. A New Trait Associated with Speciogensis

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Epifluorescence microscopic detection of organelle DNA in the mature generative cell is a rapid method for determining the potential for the mode of cytoplasmic inheritance. We used this method to examine 19 of the known 22 to 27 species in the genus Syringa. Organelle DNA was undetectable in seven species, all in the subgenus Syringa, but was detected in the 12 species examined of the subgenera Syringa and Ligustrina. Therefore, species within the genus Syringa display differences in the potential cytoplasmic inheritance. Closer examination revealed that the mature generative cells of the species in which organelle DNA was detected contained both mitochondria and plastids, but cells of the species lacking detectable organelle DNA contained only mitochondria, and the epifluorescent organellar DNA signals from the mature generative cells corresponded to plastid DNA. In addition, semiquantitative analysis was used to demonstrate that, during pollen development, the amount of mitochondrial DNA decreased greatly in the generative cells of the species examined, but the amount of plastid DNA increased remarkably in the species containing plastids in the generative cell. The results suggest that all Syringa species exhibit potential maternal mitochondrial inheritance, and a number of the species exhibit potential biparental plastid inheritance. The difference between the modes of potential plastid inheritance among the species suggests different phylogenies for the species; it also supports recent conclusions of molecular, systematic studies of the Syringa. In addition, the results provide new evidence for the mechanisms of maternal mitochondrial inheritance in angiosperms.

Extranuclear genomes carried by plastids and mitochondria are inherited according to non-Mendelian genetics. The majority of angiosperm species display maternal inheritance of the plastid genome (for review, see Kirk and Tilney-Bassett, 1978; Sears, 1980; Kuroiwa, 1991; Mogensen, 1996). However, because few visible phenotypes are encoded by organellar genes, the mode of cytoplasmic inheritance has been determined genetically for only a few species (Smith, 1988). Since the 1970s, electron microscopy has been successfully used in a number of species to reveal the mechanisms of the impedance or enhancement of paternal plasts prior to fertilization, demonstrating the possible modes of plastid inheritance (Whatley, 1982; Hagemann and Schröder, 1989). Epifluorescence microscopy, combined with 4',6-diamidino-2-phenylindole (DAPI) staining of organellar DNA, has been used more recently in a similar fashion (Miyamura et al., 1987). There is a positive correlation between the appearance of organellar DNA fluorescence in the mature sperm or generative cells and a biparental mode of cytoplasmic inheritance (Miyamura et al., 1987). Recently, fluorometry-equipped microscopy with DAPI staining has revealed that the amount of organellar DNA increases in male reproductive cells of species that display biparental cytoplasmic inheritance but decreases in cells of species that display maternal cytoplasmic inheritance (Nagata et al., 1999). Because cytological techniques such as electron and epifluorescence microscopy detect the prerequisites for paternal transmission, we have termed the results determined by these methods the potential cytoplasmic inheritance. During the past few years, the potential cytoplasmic inheritance has repeatedly been shown to be strongly correlated with the actual modes of cytoplasmic inheritance (for examples, see Miyamura et al., 1987; Kuroiwa et al., 1993; Nagata et al., 1999; Nishimura et al., 1999).

Epifluorescence microscopy, which gives rapid and reliable results, has been used for large-scale screening of angiosperm species to determine the mode of cytoplasmic inheritance. Of the more than 600 species examined, nearly 80% exhibit potential maternal cytoplasmic inheritance, and the rest exhibit potential biparental inheritance (Corriveau and Coleman, 1988; Zhang et al., 2003). In general, the potential cytoplasmic inheritance is consistent within a genus (Corriveau and Coleman, 1988; Zhang et al., 2003). For this reason, very few species have been used to represent individual genera during such studies. In the study by Corriveau
and Coleman (1988), one species, Syringa vulgaris, was examined for the genus Syringa, and the species was reported to display maternal inheritance. However, another Syringa species, Syringa pekinensis, was later found to have a strong potential for biparental inheritance (Zhang et al., 2003). These contrasting data suggest an unusual divergence with respect to the mode of cytoplasmic inheritance situation within the genus Syringa.

Although different potential modes of cytoplasmic inheritance have been detected in the above two Syringa species, little is known about the inheritance mechanisms and the significance of diverging modes occurring within one genus. For these reasons, the

Table 1. Plastids, mitochondria, plastid DNA, and mitochondrial DNA in mature generative cells

<table>
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<th>Species</th>
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Figure 1. Epifluorescence micrographs of pollen cells of S. pinnatifolia (a), S. oblata (b), S. pubescens (c), S. villosa (d), and S. pekinensis (e) stained with DAPI. Fluorescent granules (aggregates) corresponding to cytoplasmic DNA appear to be associated with the generative nuclei of S. pubescens (c), S. villosa (d), and S. pekinensis (e; indicated by arrows) but not with the generative nuclei of S. pinnatifolia (a) or S. oblata (b). These results indicate that the potential for paternal cytoplasmic inheritance varies among these species. GN, Generative nucleus; VN, vegetative nucleus. Bar = 10 μm.
behavior of organelles during pollen development was examined in 19 species that represent a majority of the Syringa species. The amount of plastid DNA was found to be enhanced in the generative cells of 12 of the species, resulting in biparental inheritance, and plastid DNA was excluded from the cells of 7 of the species, resulting in maternal inheritance. In all of the species examined, the mitochondrial genome was found to be degraded during pollen development; it tends, therefore, to be transmitted maternally. Although multiple modes of plastid transmission exist within this genus, the modes appear to be consistent within the systematic series. These results suggest that there are different phylogenetic distances between the species and series in the Syringa.

RESULTS

Multiple Potentials for Cytoplasmic Inheritance within the Genus Syringa

As described above, the presence or absence of cytoplasmic DNA in mature male reproductive cells is a visual trait that indicates the potential for cytoplasmic inheritance. We routinely use epifluorescence microscopy for initial inspections of the species for this trait. Mature pollen grains of the species listed in Table I were squashed, stained with DAPI, and examined under an epifluorescence microscope. All of the species were binucleate, with one generative and one vegetative nucleus. However, as shown in Figure 1, no fluorescence was associated with the generative nuclei of certain species, such as Syringa pinnatifolia and Syringa oblata, but many fluorescent granules were associated with the generative nuclei of other species, such as Syringa pubescens, Syringa villosa, and S. pekinensis. Since these fluorescent granules, which correspond to cytoplasmic DNA in male reproductive cells, are characteristic of paternal cytoplasmic transmission, different Syringa species exhibit the potential for either maternal or biparental cytoplasmic inheritance. Of the 19 species examined, 7 exhibited the trait for maternal inheritance and the other 12 exhibited the trait for biparental cytoplasmic inheritance (Table I). The traits of the first 7 species were defined as pattern I and those of the remaining 12 species as pattern II.

Plastid Behavior Reveals Potential Biparental Inheritance

To determine whether the above fluorescent granules correspond to plastid and/or mitochondrial DNA, we subjected pollen sections of the various species to DAPI-DiOC₇ double staining. The results are summarized in Table I, and examples of the results in S. oblata and S. pekinensis are shown in Figure 2. The sections were first observed under blue excitation to reveal the mitochondrial fluorescence (Fig. 2, b, d, f, and h) and then under UV excitation to detect fluorescent spots corresponding to cytoplasmic DNA (Fig. 2, a, c, e, and g). Mitochondria in the pollen cells were stained by DiOC₇ as distinct spherical granules, but plastids were not stained with this dye. Both the early generative cell that attaches to the intine just after the first pollen mitosis and the mature generative cell were examined. In general, mitochondrial granules were detected in both the early and mature generative cells of all of the species in the two groups (Fig. 2, b, d, f, and h). However, in contrast to the overlaying of the mitochondrial granules with the fluorescent DNA granules observed in the early generative cells (Fig. 2, a, b, e, and f), no detectable fluorescent DNA signals appeared at the positions of the corresponding mitochondrial granules in the mature generative cells (Fig. 2, c, d, g, and h), suggesting that the mitochondrial DNA may have degraded during pollen development. This behavior of the mitochondrial DNA is a common trait that suggests maternal mitochondrial inheritance in the genus Syringa.

Since DiOC₇ stains mitochondria but not plastids, fluorescent DNA granules that do not correspond to
the mitochondrial granules are indicative of plastid DNA. In the pattern I species, no plastid DNA granules were detected in either the early or the mature generative cells (Fig. 2, a–d). This suggests that in this group, plastids may not be apportioned into the generative cell during the first mitosis. However, in the pattern II species, plastid DNA granules were detected in both the early and the mature generative cells (Fig. 2, e–h). Thus, both plastids and mitochondria are likely to be maintained in the generative cells of the pattern II species, and the fluorescent DNA granules appeared to correspond to plastid DNA but not mitochondrial DNA.

Electron microscopy confirmed the proposal that plastids are excluded from the early generative cell during the first pollen mitosis in *S. oblata* (Fig. 3a) but that they are apportioned to the generative cell in *S. pekinensis* (Fig. 3b). Plastids were not observed in mature generative cells of *S. oblata* (Fig. 3c) but were evident in the generative cells of *S. pekinensis* (Fig. 3d). Therefore, the pattern II species in the genus Syringa exhibit Pelargonium-type biparental plastid inheritance, and those in the pattern I group display Lycopersicon-type maternal plastid inheritance (to be discussed below).

Mitochondrial DNA Is Degraded in All Syringa Species

Epifluorescence microscopy failed to detect mitochondrial DNA in the mature generative cell (Figs. 1 and 2), despite the preservation of mitochondria in the cell (Fig. 3). This suggests that the mitochondrial DNA in the generative cell may be degraded during pollen development. However, since epifluorescence microscopy has limited sensitivity and the fluorescence of small amounts of mitochondrial DNA fades easily, the above proposal was also investigated using the more robust technique of immunoelectron microscopy. The *S. oblata* early generative cells showed strong and consistent localization of gold particles on mitochondria (Fig. 4, a–e), suggesting that mitochondria at this stage contain remarkable amounts of DNA. By contrast, the mitochondria of mature generative cells consistently showed much less labeling (Fig. 4, f–k). Serial sectioning was used to examine 15 mitochondria from different early generative cells and 70 mitochondria from different mature generative cells, revealing that the average number of gold particles per mitochondrion in mature cells was 97% less than on early cells (Fig. 7a).

A similar phenomenon was observed in *S. pekinensis*, a representative of the pattern II species. Mitochondria were consistently labeled in early generative cells of this species (Fig. 5, a–c), whereas those in the mature generative cells labeled poorly (Fig. 5, d–g). Treatment of serial sections of 11 and 66 mitochondria of early and mature cells, respectively, showed that labeling of mitochondria in the mature cells was reduced by 90%, as compared to that in the early cells (Fig. 7b). The relative amounts of particles indicate that mitochondrial DNA degrades during pollen development in all Syringa species.

The Amount of Plastid DNA Increases in the Pattern II Species

As expected, immunoelectron microscopy successfully revealed plastid DNA in both early and mature generative cells of *S. pekinensis* (Fig. 6). This technique also revealed differences in the amounts of labeling in the two stages. In the early generative cells, small clusters of particles localized to plastids (Fig. 6, a–c), but many more particles appeared on plastids in mature generative cells (Fig. 6d). Inspection of serial sections of 10 and 35 plastids of early and mature generative cells, respectively, revealed a 3-fold increase in the average amount of labeling per plastid in mature generative cells (Fig. 7c). This suggests that a selective increase in the amount of plastid DNA occurs in the generative cells of the pattern II species.
**DISCUSSION**

A consistent potential mode of cytoplasmic inheritance within a genus was first noted by Corriveau and Coleman (1988), based on epifluorescence microscopic examination of 235 angiosperm species in 187 genera, some of which were represented by only one species. With the exception of a small number of rare cases, this observation is in agreement with subsequent results of studies, such as that by Zhang et al. (2003), which was performed on 295 species in 254 genera. Since all of the species in a genus are thought to originate from the same progenitor, it is reasonable for the species to display consistency in a biological trait such as the potential mode of cytoplasmic inheritance. However, this study revealed an unusual discrepancy in the potential mode of plastid inheritance in a genus. Our study demonstrates that plastids are not maintained in the generative cells of pattern I species, which belong to the series Syringa and Pinnatifoliae in the subgenus Syringa (Table I), the plastids and plastid DNA were maintained in the generative cell. Therefore, these species exhibit the potential for biparental plastid inheritance. By contrast, in this study, the pattern II species, which belong to the series Villosae and Pubescentes in the subgenus Syringa and the subgenus Ligustrina (Table I), the plastids and plastid DNA were maintained in the generative cell. In addition, the amount of DNA per plastid was observed to increase significantly during pollen development. This finding is consistent with the report by Nagata et al. (1999) that the amount of organelle DNA increases in male reproductive cells of species that display biparental cytoplasmic inheritance but decreases in cells of species that display maternal cytoplasmic inheritance.

Immunogold labeling revealed a more than 90% reduction in the amount of mitochondrial DNA in the generative cells of *S. oblata* and *S. pekinensis* during pollen development, suggesting that a selective degradation of mitochondrial DNA takes place. In angiosperms, plastids are excluded from the male reproductive cells in maternal inheritance, but mitochondria are always preserved in these cells (Hagemann and Schröder, 1989; Sodmergen et al., 2002), and the mitochondrial genome is more strictly inherited from the maternal side (Mogensen, 1996). The common phenomenon of maternal inheritance seems to conflict with the constant presence of mitochondria in the male reproductive cells. Although mechanisms must exist to impede the paternal transmission of mitochondria, little is known about these mechanisms, except for the degradation of mitochondrial DNA observed in a few species (Nagata et al., 1999; Sodmergen et al., 2002). In this study, we again found evidence for the degradation of mitochondrial DNA in the generative cell. This degradation would undoubtedly contribute to the prevention of paternal mitochondrial DNA transmission and is therefore a possible mechanism for the maternal inheritance of mitochondria. Although immunoelectron microscopy was not used with all of the species studied because the presence of mitochondria and the absence of a corresponding epifluorescence of mitochondrial DNA in the mature generative cell have been confirmed in most of the species, the degradation of mitochondrial DNA in the generative cell is probably a common trait in the genus Syringa.

However, in this study, the degradation of mitochondrial DNA in the generative cell was incomplete, at least at the flowering stage in which mature pollen grains were sampled. The average amount of labeling per mitochondrion in both *S. oblata* and *S. pekinensis* was obviously higher than the average background labeling (Fig. 7, a and b). In addition, the degradation was not strongly synchronized within the cell. In one case, two mitochondria with strong labeling were

![Figure 4. Immunogold electron micrographs showing the labeling of mitochondrial DNA in early (a–e) and mature (f–k) *S. oblata* generative cells. Mitochondria in the outlined areas in a and f are shown in serial sections (b–e and g–k, respectively). Note that the localization of gold particles to the mitochondria greatly decreases during pollen development. M, Mitochondrion; GN, generative nucleus. Bars = 0.5 μm.](https://www.plantphysiol.org/content/136/4/2766/F4)

In angiosperms, most common mechanism for maternal plastid inheritance (Lycopersicon-type maternal plastid inheritance; for review, see Hagemann and Schröder, 1989) is the exclusion of plastids during the first pollen mitosis. The pattern I species clearly displayed maternal plastid inheritance. By contrast, in this study, the pattern II species, which belong to the series Villosae and Pubescentes in the subgenus Syringa and the subgenus Ligustrina (Table I), the plastids and plastid DNA were maintained in the generative cell. Therefore, these species exhibit the potential for biparental plastid inheritance. In addition, the amount of DNA per plastid was observed to increase significantly during pollen development. This finding is consistent with the report by Nagata et al. (1999) that the amount of organelle DNA increases in male reproductive cells of species that display biparental cytoplasmic inheritance but decreases in cells of species that display maternal cytoplasmic inheritance.
observed in a cell in which most of the mitochondria were nearly free of labeling (data not shown). This result indicates that DNA degradation occurs to a lesser extent in some mitochondria and explains why the mean variances at this stage were as high as or higher than the mean value (Fig. 7, a and b). In each cell, at least, mitochondrial DNA remnants that could be transmissible should exist. In animals, very small amounts of mitochondrial DNA in the sperm cells are transmitted to the zygote (Ankel-Simons and Cummins, 1996). Therefore, in angiosperms, in which mitochondria are strictly maternally inherited, mechanisms probably exist that are additional to the degradation of paternal mitochondrial DNA during pollen development. Recent evidence indicates that mitochondrial DNA of sperm cell origin is selectively transferred to the zygote.

Figure 5. Immunogold electron micrographs showing the labeling of mitochondrial DNA in early (a–c) and mature (d–g) S. pekinensis generative cells. Serial sections neighboring a (b and c) and d (e–g) are presented to show the localization of DNA in the mitochondria. Note that the localization of gold particles to the mitochondria greatly decreases during pollen development. M, Mitochondrion; P, plastid; GN, generative cell nucleus. Bar = 1 μm.

Figure 6. Immunogold electron micrographs showing the labeling of plastid DNA in early (a–c) and mature (d) S. pekinensis generative cells. Note that the localization of gold particles to the plastids increases greatly during pollen development. P, Plastid; GC, generative cell; GN, generative cell nucleus; VC, vegetative cell. Bar = 1 μm.
degraded in the zygote (Shalgi et al., 1994; Kaneda et al., 1995; Sutovsky et al., 1996; Sutovsky and Schatten, 2000). This type of mechanism may also be present in angiosperms.

As listed in Table I, species in the genus Syringa have been traditionally grouped into two subgenera, the Syringa and the Ligustrina (Rehder, 1945; Harborne and Green, 1980). This classification is mainly based on the morphology of the species, including leaf shape and floral size. The species in the subgenus Syringa have been further grouped into four series: the Syringa, Villosae, Pubescentes, and Pinnatifoliae. In general, traditional classification reflects plant morphological similarities more than the genetic relationships of the species. Further studies are usually necessary to better understand the phylogeny of the species. For example, failures to obtain interseries hybrids in the Syringa suggest greater genetic distances between the species than within each series (for review, see Pringle, 1981). However, hybrids can be obtained between members of the series Syringa and the monotypic series Pinnatifoliae (Anderson and Rehder, 1935), suggesting that these two series have a sister relationship. Recently, based on sequence similarities in the nuclear ribosomal DNA, Li et al. (2002) suggested that the series Syringa and the monotypic series Pinnatifoliae (Anderson and Rehder, 1935), suggesting that these two series have a sister relationship. Recently, based on sequence similarities in the nuclear ribosomal DNA, Li et al. (2002) suggested that the series Syringa and the monotypic series Pinnatifoliae are divergent from the main Syringa group. This indicates that the series Syringa and Pinnatifoliae are paraphyletic to other species in the Syringa. By contrast, data suggest that Ligustrum, traditionally classified as an independent genus within the Oleaceae, is a monophyletic group derived from within the Syringa (see Fig. 9 for explanation). These suggestions have dissolved the former boundary between the two subgenera, as well as the defined genus Syringa. Another study of chloroplast DNA sequences has provided similar results (Wallander and Albert, 2000).

The species for which the potential cytoplasmic inheritance was examined in this study cover both subgenera and all of the series in Syringa. Our results show that the species in the series Syringa and the monotypic series Pinnatifoliae exhibit maternal inheritance for both plastids and mitochondria, whereas the species in the series Villosae and Pubescentes and in the subgenus Ligustrina exhibit maternal mitochondrial inheritance but biparental plastid inheritance (Table I). In addition, the species within these series, and the species within the subgenus Ligustrina, display consistent modes of cytoplasmic inheritance. Based on the potential modes of cytoplasmic inheritance determined in this study, it is likely that (1) the species within a series and the species in the subgenus Ligustrina are monophyletic; (2) the series in the subgenus Syringa are paraphyletic; (3) the species in the series Syringa and Pinnatifoliae are monophyletic and have a close phylogenetic relationship; and (4) the series Villosae and Pubescentes and the subgenus Ligustrina are monophyletic and distant from the series Syringa and Pinnatifoliae. These presumptions, based solely on similarities in the potential modes of cytoplasmic inheritance (Table I), are consistent with

![Figure 7](image)

**Figure 7.** Changes in the amounts of immunogold labeling per mitochondrion (a and b) and plastid (c) in *S. oblata* (a) and *S. pekinensis* (b and c). Data were collected from serial sections of mitochondria and plastids. Black columns show the background level of gold particles in equal areas of the sections. Note the remarkable decrease in the amount of labeling of mitochondrial DNA and the increase in the amount of labeling of plastid DNA during pollen development. EGC, Early generative cell; MGC, mature generative cell.

![Figure 8](image)

**Figure 8.** Epifluorescence micrographs of pollen cells of *L. lucidum* (a), *L. quihoui* (b), and *L. valgare* (c) stained with DAPI. Fluorescent granules (aggregates) corresponding to cytoplasmic DNA appear to be associated with the generative nuclei. These results indicate the potential for paternal cytoplasmic transmission in these species. GN, Generative nucleus; VN, vegetative nucleus. Bar = 10 µm.

![Figure 9](image)

**Figure 9.** Traditional and recently proposed classifications of speciogenesis in Syringa and Ligustrum. The DNA sequence similarity (Wallander and Albert, 2000; Li et al., 2002) and the divergence of the potential mode of cytoplasmic inheritance suggest the same conclusion.
findings from other recent studies (Wallander and Albert, 2000; Li et al., 2002). To address the suggestion that species in the genus Ligustrum (an independent genus within the Oleaceae) form a monophyletic group derived from within the Syringa (Wallander and Albert, 2000; Li et al., 2002), we used epifluorescence microscopy to examine cytoplasmic DNA in generative cells in *Ligustrum lucidum*, *Ligustrum quihoui*, and *Ligustrum vulgare*. Coincidentally, the results show potential biparental plastid inheritance in these species (Fig. 8). Using the trait of potential cytoplasmic inheritance, we came to the same conclusion as the molecular studies (Fig. 9). Therefore, the potential mode of cytoplasmic inheritance may be a new trait associated with speciogenesis.

The potential cytoplasmic inheritance in angiosperms describes the control of the fate of organelles from the paternal side. The evolution of the mechanisms that control this phenomenon must have begun after the appearance of angiosperms, as the male gametophyte, in which the paternal control takes place, was not differentiated in earlier plants. Since the development of the mechanisms occurred as early as the time of establishment of angiosperms, it is reasonable to propose that the divergent mechanisms of cytoplasmic inheritance are associated with speciogenesis. In this study, we have shown that the potential plastid inheritance varies and is associated with phylogeny within the Syringa. This suggests that independent development of the controls of plastid inheritance occurred in angiosperms. As suggested by Liu et al. (2004), it is possible that maternal inheritance may have become dominant before the appearance of angiosperms and that the development of maternal control of cytoplasmic inheritance in angiosperms is an extension of the mechanism that existed in early eukaryotic cells. The maternal inheritance of the chloroplast genome in the unicellular green alga *Chlamydomonas* (see Kuroiwa et al., 1982; Nishimura et al., 1999; Nakamura et al., 2003) is mechanisms similar to that in animals (Shalgi et al., 1994; Kaneda et al., 1995; Sutovsky et al., 1996; Sutovsky and Schatten, 2000). We have shown that the paternal control of mitochondria is not complete and that there might also be maternal control in the zygote, resulting in strict maternal inheritance and indicating an animal-like mechanism for mitochondrial inheritance in angiosperms. It appears that unicellular eukaryotic cells, animals, and angiosperms share a similar mechanism for mitochondrial inheritance. However, for plastids, the paternal control in angiosperms and the maternal control in *Chlamydomonas* are distinctly different mechanisms. This difference supports the idea that the mechanisms for the control of plastid inheritance in angiosperms developed independently and may have developed later than those for mitochondrial inheritance. These presumptions are based on the fact that the potential mode of plastid inheritance varies considerably in angiosperms and that it is associated with speciogenesis.

**MATERIALS AND METHODS**

**Plant Materials**

Pollen grains were collected from plants grown in the Beijing Botanical Garden, Institute of Botany, at the Chinese Academy of Sciences, Beijing or from plants growing on the campus of Peking University.

**Epifluorescence Microscopy**

Epifluorescence microscopic examination of pollen cell cytoplasmic DNA was performed according to Kuroiwa and Suzuki (1980). In brief, mature pollen grains were placed on a glass slide and immersed in a drop of TAN buffer (Nemoto et al., 1988) supplemented with 3% glutaraldehyde and 1 μg/mL DAPI. Immediately after being covered with a coverslip, the pollen grains were squashed by exerting an appropriate degree of pressure through the coverslip. Next, to ensure adequate fixation and staining, the slides were incubated for about 10 min. Finally, the samples were examined under an Olympus BHS-RFK epifluorescence microscope (Tokyo). Photomicrographs of the cells were captured with a cooled CCD camera (Spot; Diagnostic Instruments, Sterling Heights, MI) attached to the microscope.

Double staining of pollen cells with DAPI and DiOC₆ was based on the method of Nagata et al. (1999). Pollen grains of each species were fixed in 3% glutaraldehyde in cacodylate buffer, pH 7.4, for at least 24 h at 4°C, dehydrated through an ethanol series, and then embedded in Technovit 7100 resin (Kulzer and Company, Wehrheim, Germany). The samples were cut in 0.5-μm sections on a Ultracut Microtome (Leica, Wien, Austria) and dried on coverslips. The sections were stained with 100 μg/mL DiOC₆ in ethanol, washed with 50% ethanol and distilled water, and then further stained with 1 μg/mL DAPI in TAN buffer. To prevent fading, 1 mg/mL n-propyl gallate in 50% glycerol was added to the samples before the epifluorescence microscopic examination. Photomicrographs were captured with a cooled CCD camera.

**Electron Microscopy**

For transmission electron microscopy, pollen grains were fixed in 3% glutaraldehyde in cacodylate buffer, pH 7.4, for at least 24 h at 4°C, and then overnight in 1% osmium tetroxide at 4°C. The fixed pollen grains were dehydrated through an alcohol series and embedded in Spurr’s resin. Ultrathin sections were stained in 1% uranyl acetate and lead citrate and examined with a JEOL electron microscope (Tokyo).

Immunoelectron microscopy for stable detection of cellular DNA was based on the method of Johnson and Rosenbaum (1990). Pollen grains were fixed with glutaraldehyde as described above, except for omitting the postfixation step, and embedded in LR White resin (Sigma-Aldrich Chemie, Steinheim, Germany). Continuous sections were carefully collected in single-slot grids. The grids were incubated with a mouse monoclonal antibody that recognizes single- and double-stranded DNA (Boehringer Mannheim, Mannheim, Germany). After washing, the grids were incubated with a goat anti-mouse IgM conjugated to 10-nm colloidal gold (British BioCell International, Cardiff, UK). Finally, the samples were stained with 1% uranyl acetate and examined with a JEOL electron microscope. As a negative control, sections were pretreated with DNase and processed as above. The localization of immunogold particles in these sections was routinely compared to that of control sections.

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**LITERATURE CITED**

Liu et al.


