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Cytological basis for a tetraspory in *Cupressus sempervirens* L. megagametogenesis and its implications in genetic studies

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Abstract The processes of megasporogenesis and early megagametogenesis were cytologically investigated in *Cupressus sempervirens* L. in order to elucidate, at the cellular level, the origin of the megagametophyte. After pollination, sporogenous tissue developed in the chalazal region of the nucellus, but only one megaspore mother cell differentiated and divided meiotically without cell-wall formation. This led to the development of a cell with four nuclei which directly functioned as a megaspore. The *C. sempervirens* megagametophyte is thus tetrasporic, in contrast to the majority of conifers where the megagametophyte is monosporic. The consequenses of this observation are discussed from a genetics point of view.

Key words *Cupressus sempervirens* · Cytology · Megasporogenesis · Megagametogenesis · Genetics

Introduction

In the Mediterranean region, *Cupressus sempervirens* L. (Mediterranean Cypress) is a well-adapted indigenous conifer. It can be found in limited natural stands on the eastern part of the Mediterranean basin, but it is also extensively planted as windbreaks and ornamentals (Papageorgiou et al. 1993, 1994), and more rarely as a forest tree, throughout the whole Mediterranean region. However, *C. sempervirens* has interesting properties, such as adaptability to drought and poor soils, fast and continuous growth, good wood quality and some fire tolerance. This could make it a suitable conifer for reforestation and economic upgrading of low-elevation degraded sites. Unfortunately, this species suffers serious damage from bark canker disease caused by *Seiridium cardinale* Wag., and only limited information is available on its biology, physiology and genetics.

In the field of genetics, Papageorgiou et al. (1993) analysed isozyme variability of *C. sempervirens* Greek provenances. As in many other isozyme analyses carried out in conifers, these authors expected to take advantage of the salient reproductive feature of gymnosperms, i.e. the presence in the seed of a haploid megagametophyte surrounding the diploid embryo. Using the embryo-surrounding tissue for his analyses, Papageorgiou (1995) obtained the unexpected result of zymograms with a diploid pattern that were in all respects identical to those of the mother tree. This genetic particularity was attributed to the presence, in this tissue, of remnants of the diploid sporophytic nucellus, i.e. perisperm. However, it is commonly assumed that in mature conifer seeds, the nucellus is reduced to a thin pellicle composed of collapsed cells (Tillman-Sutela et al. 1996). It is thus likely that this characteristic cannot be attributed to perisperm, but to a true megagametophyte, which is the only visible tissue surrounding the embryo in cypress seeds (El Maâtaoui, unpublished observations).

In a previous report (Pichot and El Maâtaoui 1997) we showed that the *C. sempervirens* megagametophyte contained nuclei of different ploidy levels. As a second step towards a better understanding of the genetic behaviour of this species, we examined the cytological process of megasporogenesis and early stages of megagametogenesis. The main objective of the study presented here was to determine the cellular origin(s) of the megagametophyte and relate it to previous isozyme analyses. Furthermore, while the basic sexual

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reproductive cycle has been described in some Cupressaceae with special attention to pollination, fertilization and embryogenesis (Singh 1978), megagametophyte ontogenesis has been poorly investigated.

Materials and methods

Developing female cones were collected at intervals of 2–3 days from the end of January (beginning of pollination) until mid-May (megagametophyte at free nuclear stage) 1996. At each date, five to seven open-pollinated cones were randomly collected from one *C. sempervirens* adult tree growing in a windbreak near Avignon (France). Small groups of ovules attached to a portion of the ovuliferous scale were excised from the cones and immediately immersed in FAA as a fixative solution (formalin : glacial acetic acid : 50% ethanol, 1 : 1 : 18 by volume). Samples were vacuum-infiltrated and stored in the same solution until needed. They were then washed overnight in tap water, dehydrated in a graded ethanol series (70, 80, 90, 95, 100% v/v) and embedded in glycol methacrylate Technovit 7100 (Kultzer) according to the manufacturer's instructions. Samples were longitudinally sliced at a 3-μm thickness using a Reichert-Jung Supercut 2065 microtome equipped with disposal Histoknife H (Kultzer). The sections were smoothed out on a distilled waterbath, serially collected on microscope slides, dried to affix and stored until needed for staining. Starch and other polysaccharides were stained using the periodic acid-Schiff (PAS) reaction (Mac Manus 1948), and total proteins by naphtol blue black (Fisher 1968). Slides were then rinsed in distilled water, dried and mounted in Micromount (SURGIPATH) before observation using an Olympus BH2 microscope. For each collection date, approximately 13 ovules were observed.

Results

In gross morphology the *C. sempervirens* post-pollination ovule (Fig. 1a) conformed to the general description reported for other Cupressaceae (Owens and Molder 1974 ; Cecchi Fiordi and Maugini 1976 ; Singh 1978). The dome-shaped nucellus was surrounded by integuments and presented a large micropyle. Its lower part exhibited collapsing cells, the remnant of each probably contributes to the composition of the pollination drop. This led to the formation of a concave corroded tip where pollen grains depose and germinate (Fig. 1a). The ovules sectioned during March showed in their innermost region (chalaza) numerous sporogenous cells (archespores) surrounded by a tapetum-like layer (Fig. 1b). The sporogenous cells became distinguishable by their spherical section, their dense cytoplasm rich in starch grains and their prominent and centrally positioned nucleus (Fig. 1b). However, in all of the ovules observed, only one megaspore mother cell (MMC) differentiated and underwent meiosis. Prior to this, the fertile MMC progressively elongated and accumulated copious starch in large amyloplastes randomly distributed throughout the cytoplasm. The ovules collected during April showed different stages of megasporogenesis from mature MMC to megaspore. All steps of the meiotic process were found (Fig. 1c-f)., but surprisingly no cell wall was built to isolate the nuclei produced at the dyad (Fig. 1e) and tetrad stages (Fig. 1g). As result of the first meiotic division two nuclei were formed (Fig. 1e). They immediately entered the second meiotic division, producing a tetranucleated cœnomegaspore (Fig. 1g). No nuclei degeneration was observed during the meiotic process. The resulting four nuclei aggregated in the central part of the megaspore before they migrated to the periphery (Fig. 1h). The section performed on ovules collected during May showed the progressive development and individualization of the megagametophyte in the detriment of the surrounding tissues. In fact, immediately after meiosis the cœnomegaspore expanded (Fig. 1h) by breaking down and depleting the constituents of the neighbouring nonfunctionnal archespores. Through successive waves of division, the four meiotic nuclei participated in the initiation of the free nuclear megagametophyte. Later, sections showed enlargement of the megagametophyte accompanied by progressive depletion of the encompassing nucellar cells (Fig. 1i). The megagametophyte then reached the cellular stage before archegonia differentiation, fertilization, embryogenesis and seed maturation (data not shown).

Discussion

In seed plants, it is a well known fact that in female reproductive organs (ovules) specialized sporogenous cells differentiate and undergo meiosis to produce haploid megaspores in a linear tetrad (Bouman 1984 ; Willemse and Van Went 1984). This sporogenic process is immediately followed by megagametogenesis via successive mitotic divisions of the functional megaspore(s). Although in most of the angiosperms studied, the outer three megaspores degenerate while the innermost (chalazal) megaspore initiates the megagametophyte or embryo sac (monospory), bisporic or tetrasporic embryo sacs are also known to occur (Willemse and Van Went 1984). With respect to conifers, a monosporic origin of the megagametophyte seems to be the rule (Singh 1978). In this group, triads containing an undivided upper dyad cell, and tetrads have been reported (Owens and Molder 1975; Cecchi Fiordi and Maugini 1976 ; Singh 1978), but nothing is known about the genus *Cupressus*. The cytological study reported here shows that *C. sempervirens* megasporogenesis resulted in the production of an apparently tetranucleated cell which functions directly as a megaspore. A similar feature was reported for two evoluted gymnosperms, *Gnetum* and *Welwitschia* (Waterkeyn 1954; Martens 1963), in which the megagametophyte has been reported to be tetrasporic. Based on the developmental process of the megagametophyte, *C. sempervirens* would be one of the more evoluted conifers. This is in accordance with our previous conclusions based on the

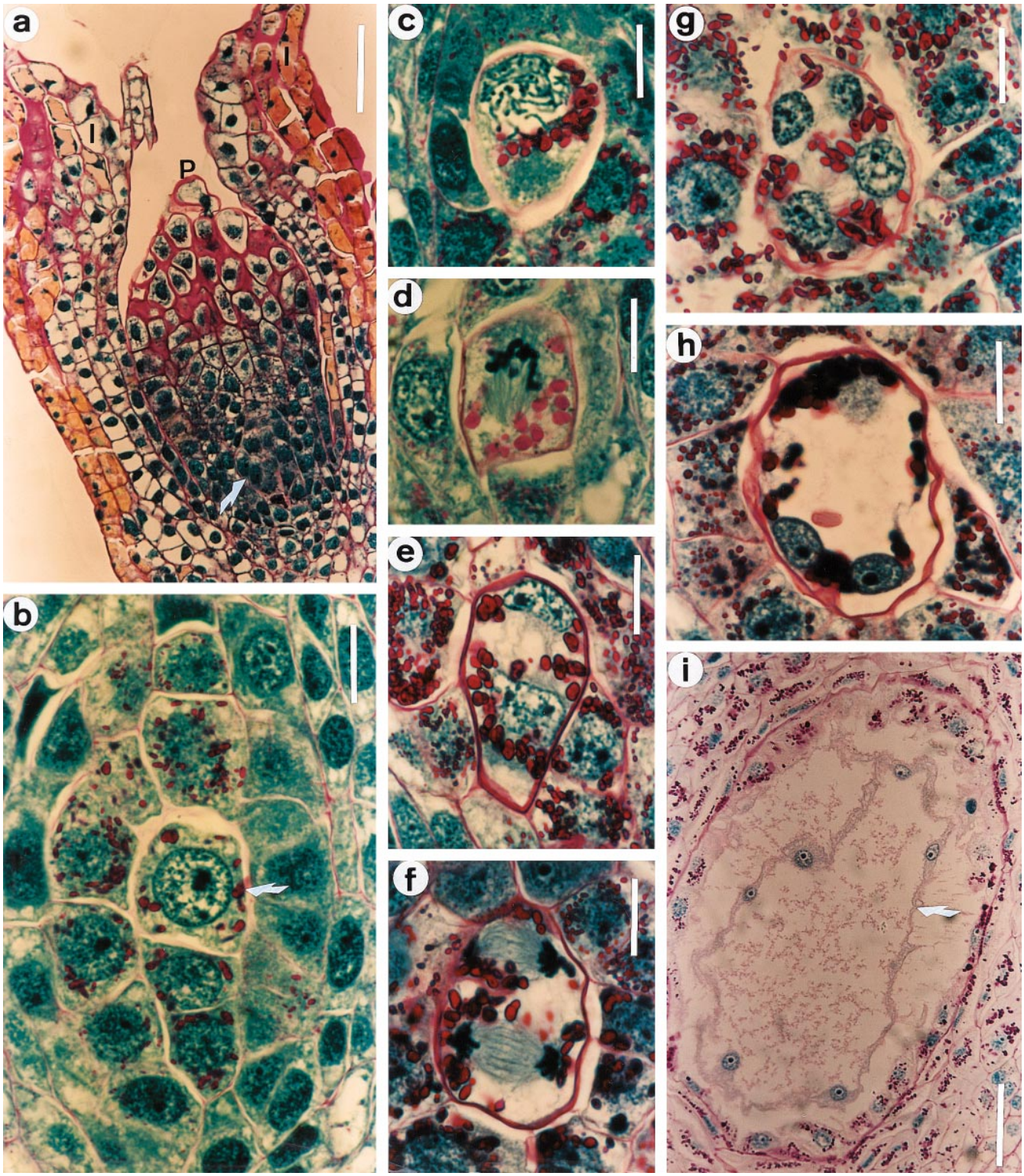

Fig. 1a–i Main steps of megasporogenesis and megagametogenesis in *Cupressus sempervirens*. **a** Longitudinal section of a post-pollination ovule showing integuments (*I*) and germinating pollen grain (*P*). Sporogenous cells (*arrow*) differentiate at the base of nucellus (*bar*: 23 μm). **b** High magnification of the chalazal region of the nucellus where only one megaspore mother cell (*arrow*) develops (*bar*: 5 μm). **c–g** Different stages of meiosis and megasporogenesis: **c** prophase I, **d** anaphase I, **e** telophase I (dyad), **f** telophase II and **g** four nucleate cœnomegaspore (tetrad). Note the absence of cell-wall formation and the persistence of the four meiotic nuclei (all *bars*: 5 μm). **h–i** Early stages of megagametogenesis: **h** enlargement of the cœnomegaspore (*bar*: 5 μm), **i** free nuclear stage (*arrow*) (*bar*: 20 μm)

high levels of DNA content in the megagametophyte nuclei (Pichot and El Maâtaoui 1997).

Futhermore, this tetrasporic origin of the megagametophyte raises some important problems in genetic analyses of Cypress. In fact, the megagametophyte of gymnosperms is frequently used for genetics studies because the genotype of this usually haploid tissue represents exactly the maternal contribution to the embryo. The diploid phenotype observed in the *C. sempervirens* zymograms (Raddi et al. 1990; Papageorgiou et al. 1993) led us to hypothesize the cellular origins of *C. sempervirens* megagametophyte: two (or more) megaspores (1C) from one or multiple MMC, or a mixture of one (or more) unreduced MMC (maternal 2C tissue) and one (or more) megaspore (1C) (Pichot and El Maâtaoui 1997). The second hypothesis can now be dismissed : the diploid maternal tissue does not directly contribute to allelic variability of megagametophyte because the whole nucellus is digested by the megagametophyte development. When our findings are taken into consideration, the first hypothesis is valid and can be clarified in the way that only one meiosis occurs, producing four persisting haploid nuclei.

Other deviations from the expected haploid phenotype were reported, at low frequencies, in *Pinus attenuata* Lemm. (O'Malley et al. 1988) and *Ginkgo biloba* L (O'Malley and Kelly 1988) megagametophytes. In *P. attenuata* a multisporic origin was hypothesized when non-haploid zymograms were observed. For dimeric enzymes, no heterodimer band was noticed, and thus, *P. attenuata* megagametophyte was a mosaic tissue composed of genetically different haploid cells. In *C. sempervirens*, the heterodimer bands observed by Papageorgiou (1995) can not be explained without the coexistence of the two alleles in the same cell. This requirement is in agreement with our hypothesis of nuclei fusions leading to the observed multiple ploidy levels (Pichot and El Maâtaoui 1997).

In *C. sempervirens*, the four persisting haploid nuclei represent different genotypes determined by the first meiotic division. For maternal heterozygous loci, the presence of the two maternal alleles in equal quantities at the very first stage of the megagametophyte development (four nuclei) lets us suppose that the mature megagametophyte will still contain in more or less equal quantities these different alleles, except if the rhythm of nuclei division is influenced by specific alleles at some loci. Any variation in the proportion of the alleles probably does not significantly affect the mature megagametophyte "genotype". Thus, molecular genetic studies using megagametophyte or diploid maternal tissue should produce identical results. This feature was observed for isozyme analyses of *C. sempervirens* megagametophytes and needles (Papageorgiou 1995).

These results show that the control of ploidy level(s) and of the origin of the megagametophyte must be a prerequisite for genetic studies in gymnosperms.

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