RESEARCH PAPER

Time-lapse imaging of the initiation of pollen embryogenesis in barley (Hordeum vulgare L.)

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Abstract

Pollen embryogenesis provides exciting opportunities in the areas of breeding and biotechnology as well as representing a convenient model for studying the process of plant cell proliferation in general and embryogenesis in particular. A cell culture system was devised in which immature barley pollen could be cultured as a monolayer trapped between the bottom glass-cover slip of a live-cell chamber and a diaphanous PTFE membrane within a liquid medium over a period of up to 28 d, allowing the process of embryogenesis to be tracked in individual pollen. Z-stacks of images were automatically captured every 3 min, starting from the unicellular pollen stage up to the development of multicellular, embryogenic structures. The method should prove useful for the elucidation of ultrastructural features and molecular processes associated with pollen embryogenesis.

Key words: Barley, chamber cover slip, live-cell microscopy, pollen embryogenesis.

Introduction

The normal development of the male gametophyte of some plant species can be redirected into embryogenic development, resulting in the formation of embryos able to differentiate into haploid plants (Reynolds, 1997). Pollen embryogenesis is of importance in the breeding of certain crop species as it simplifies the fixation of meiotic recombination events via the doubling of the chromosome complement of haploid regenerants. barley is particularly amenable to pollen embryogenesis, a feature which has helped achieve the crop’s status as the leading model temperate cereal (Maraschin et al., 2005a). Defining reliable structural markers for pollen embryogenesis, which has proved to be difficult to date (Pechan and Smykal, 2001), requires the in vivo microscopy of cultured immature pollen (Krens et al., 1998). Previous efforts to track the fate of individual pollen (Bolik and Koop, 1991; Kumlehn and Lörz, 1999; Indrianto et al., 2001; Maraschin et al., 2005b, 2008) suffered from the inadequate delivery of signalling molecules produced by co-cultivated embryogenic pollen, a process which is inevitably compromised when the target pollen grains have to be immobilized in a gel matrix. Signalling has been shown to be essential for the definition of cell identity and proliferation in plant cell cultures (Kumlehn and Lörz, 1999; Kumlehn et al., 1999). In addition, these experiments have had to rely on the acquisition of a series of images, in which the time-interval between their capture has been of the order of several hours; such a long separation can be expected to fail to record a number of important cellular events.

A method whereby immature barley pollen, arranged in a monolayer within a liquid medium, develops normally and its growth can be followed over a period of several weeks is demonstrated here. The automated capture of Z-stacks of images allowed for an unprecedented level of temporal resolution to be achieved. The method should prove valuable for elucidating the ultrastructural features and the molecular machinery important for the initiation of pollen embryogenesis and other cellular processes occurring in developing pollen.
Materials and methods

Plant material and the induction of pollen embryogenesis

The protocol described by Coronado et al. (2005) was applied to raise barley (*Hordeum vulgare* L.) cv. ‘Igri’ (Saatzucht Ackermann, Irlbach, Germany) plants as a source of immature pollen amenable to pollen embryogenesis.

Construction and use of a live-cell chamber cover slip

All procedures were performed under aseptic conditions. Construction of a live-cell chamber was based on a two-well chambered coverglass (Thermo Scientific-Cat. No. 155380) in combination with an 0.4 µm hydrophilic, diaphanous PTFE membrane of a 12 mm Millicell Cell Culture Insert (Millipore-Cat. No. PICM01250) (hereafter referred to as a ‘PTFE membrane’) and a plastic mask fabricated from a Countess® Cell Counting Chamber Slide (Invitrogen, Cat. No. C10315) (Fig. 1).

The PTFE membranes were removed (Fig. 1B, 1C), and the plastic masks (Fig. 1E) adjusted in size to fit precisely within the well of the chambered coverglass (Fig. 1G). A square hole of size approximately 7 × 7 mm was cut in its centre using a heated scalpel blade (Fig. 1E). One chambered coverglass (Fig. 1G) was placed above the agarose layer (Fig. 2E). After a second plastic mask was placed above the agarose layer (Fig. 2F, 2G), 1 ml of embryogenic pollen culture was pipetted into the chamber to ensure a sufficient supply of signalling molecules for the test pollen (Fig. 2I). The combination of agarose and the second mask ensured that the optical focal plane was free of contamination from the feeder cells (Fig. 2J).

To avoid interference from condensation on the inner surface of the chamber lid, a smooth edged 15 × 15 mm gap was cut into the lid of the chamber and covered with a sterile cover slip, which was replaced every day (Fig. 2H). The gap also facilitated the exchange of medium to compensate for evaporation. The set-up ensured that the test pollen remained immersed in liquid culture medium, thereby providing suitable culture conditions. After 2 d, the starvation medium was withdrawn using a 30 µm mesh-coated 1 ml pipette tip (Fig. 1H), and replaced by 1 ml KBP nutrient medium (Coronado et al., 2005). This step had to be done with care to avoid mechanical disturbance of the test pollen.

In a series of preliminary trials, in which non-immobilized cultured pollen were used as a control, it was established that neither 5 min nor 3 min interval recordings had any detrimental effect on pollen vitality and development. Reducing the time interval to only 2 min, however, caused the cultures to die between 3–5 d after the start of the recordings. Therefore the 3 min time-interval was selected as the standard to perform live-cell imaging of pollen embryogenesis. Z-stacks of nine images with a total scanning time of 40 s were acquired every 3 min. Pollen development was monitored for a period of up to 28 d using a HeNe 633 nm laser line. The optical interval of the Z-stacks was gradually increased from 2 µm to 4 µm over the course of the monitoring period to take account of the expansion of the test pollen. In vivo microscopy was performed using a Zeiss LSM 510 META (Carl Zeiss, Jena, Germany) equipped with a ×20 objective (NA 0.75). The microscope was installed in a dark room kept at a constant 25 °C to avoid focus drift. From every individual Z-stack a single 2D image was selected. Finally, 5069 images were used to compile a time-lapse movie depicting the course of pollen development (see Supplementary Video S1 at *JXB* online).

Results

The movie shown here is representative of a total of eight individual trials lasting between 14 d and 28 d that were performed to prove the usefulness of the system (see Supplementary Video S1 at *JXB* online).
Every trial comprised between 4 and 15 observed pollen and showed similar patterns of pollen development and similar pollen types were identified. Without the possibility of agitating the culture medium, the rate of development was significantly delayed to the extent that it took as long as 28 d for embryogenic microcalli to emerge from the exine. In conventionally grown embryogenic pollen, exine breakage typically occurs in the third week of cultivation. Nevertheless, the cultures appeared to retain a high level of viability which was not compromised by the recordings. Tracking of four pre-mitotic pollen over the course of 28 d resulted in a set of 91 242 images. The first mitotic division occurred in pollen #1 after 2 d (Fig. 3A).

Despite this mitotic activity, the dimensions of this pollen remained constant over the first 14 d (Fig. 3C–H), but expanded considerably thereafter. In pollen #2, the first pollen mitosis occurred after 3 d (Fig. 3B), following this the large vacuole remained for approximately 10 d (Fig. 3C, 3D). Thereafter, the pair of nuclei migrated towards the centre, so that the cytoplasm assumed a star-like pattern. Finally, the volume of the vacuole gradually diminished (Fig. 3E, 3F). No further mitoses were observed, and this pollen retained a constant size over the entire period of observation. Starting at day 15, detectable starch granules began to accumulate within the cells (Fig. 3H, 3I).

Pollen #3 did not develop at all and collapsed on day seven (Fig. 3F). Pollen #4 initially swelled (Fig. 3A–E) before the first pollen mitosis took place at the cell periphery on day seven, resulting in the generation of a pair of similar-sized nuclei (Fig. 3F). Several further synchronized series of mitoses subsequently converted this pollen grain into an expanding multicellular structure (Fig. 3G–I). None of the four test pollen described above had its exine ruptured by day 28, although rupture was observed in some pollen grains of other trials that were run under the same conditions. For comparison, conventionally cultured embryogenic pollen (non-immobilized, incubated in 35 mm Petri dishes) was analysed at representative time points after induction of pollen embryogenesis. The results revealed that there are no significant differences between non-immobilized and immobilized pollen with respect to viability, developmental pattern, and ratio of identified cell types (dead pollen, starch accumulated pollen, multicellular structures).
The entire series of images from which those shown in Fig. 3 have been selected is available online in the form of a time-lapse video (see Supplementary Video S1 at JXB online).

Discussion

The experimental set-up described here provided appropriate growing conditions while simultaneously permitting high-quality imaging of cellular events. It also allowed for the culture medium to be completely exchanged, which was not possible in previous attempts to perform time-lapse imaging where the targets were immobilized within a solidified medium (Kumlehn and Lörz, 1999; Indrianto et al., 2001; Maraschin et al., 2005b) and were thus subject to sub-optimal conditions since they had limited access to the signalling molecules produced by freely growing feeder cells. The presence of the PTFE membrane ensured that the test pollen were trapped within the liquid medium, while at the same time allowing for the free flow of nutrients and signalling molecules. Since the test pollen were in close contact to the cover slip of the chamber coverglass, it was possible to use high numerical aperture objectives to acquire high quality images.

Fig. 3. Representative example of in vivo microscopy of cultured immature pollen over a 28 d period. (A–I) Individual pollen #1 and #4 underwent pollen embryogenesis and generated a multicellular structure. (E, F) After just one mitotic division, development was arrested in pollen #2, and starch granules began to accumulate. (F–I) Pollen #3 did not show any sign of development and died after 7 d. N, nucleus. Bar=10 μm.
It has been shown previously that well-spaced cells undergoing pollen embryogenesis can be successfully observed by light microscopy, since spacing avoids the problems of cell overgrowth and optical interference from non-target cells (Kumlehn and Lörz, 1999; Indrianto et al., 2001). However, successful embryogenesis does require a certain cell density. This requirement was satisfied by separating the monolayer of observed pollen from a highly concentrated (200 000 pollen ml$^{-1}$) feeder population, using a thin layer of solidified medium sandwiched between two permeable PTFE membranes. Previous observations of the in vitro development of wheat (Indrianto et al., 2001) and barley (Bolik and Koop, 1991; Kumlehn and Lörz, 1999; Maraschin et al., 2005a, b, 2008) embryogenic pollen invariably used time-intervals of one to several hours. Although this level of temporal resolution may be sufficient to identify major events, such long time-intervals are not well suited for the documentation of the full detail of events associated with pollen embryogenesis. The temporal resolution was increased here to 3 min and our set-up has proved to be able to maintain the test pollen in a viable state for at least 28 d.

Cultured immature pollen characteristically develops at a variable rate, as illustrated by the differential performance of the four individual pollen shown in Fig. 3. Three contrasting fates, as also outlined by Maraschin et al. (2005a, b), were observed: some cells underwent proliferation, and subsequently formed embryogenic microcalli; some were arrested in development after initial rounds of mitosis had occurred; and some failed to develop at all. Indrianto et al. (2001) and Maraschin et al. (2005a, b) reported that, prior to the first mitosis of pollen-embryogenesis-competent pollen of wheat and barley, the nucleus migrates to a central position, leading to the cytoplasm taking on a star-like appearance within the highly vacuolated pollen. This feature has been considered to be a predictive marker for the initiation of pollen embryogenesis. By contrast, in both pollen #1 and #4 (as well as in many other pollen not shown here), the first mitosis took place while the nucleus was still at the cell periphery. A star-like pattern did occur after the first pollen mitosis in pollen #2 (Fig. 3E, 3F), which went on to accumulate starch rather than switch to embryogenic development. In our view, therefore, the formation of the star-like cytoplasm is not a reliable morphological marker for embryogenic pollen development.

The goal of the 28 d study was a test-case to establish the technical limits of our set-up. In a typical experiment, such long running times are not necessary for detailed scientific observations focusing on the initiation of pollen embryogenesis but could be useful to study cell proliferation and embryo formation.

We suggest that the improvement in efficiency and temporal resolution achieved by the cell culture method described here will facilitate a more comprehensive analysis of the key events in pollen embryogenesis, leading potentially to the identification of molecular triggers. In particular, its application to transgenic material expressing fluorophore-labelled cellular structures such as nuclei, chromatin or components of the cytoskeleton will generate a wealth of detailed information concerning the process of pollen embryogenesis.

**Supplementary data**

Supplementary data can be found at JXB online.

Supplementary Video S1. A time-lapse movie (5069 images, 7 pictures per second) depicting the course of pollen development over 28 d.

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