Hordeins are expressed in microspore-derived embryos and also during male gametophytic and very early stages of seed development

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Abstract

Microspore-derived embryos induced by anther or isolated-microspore culture display certain characteristics of zygotic embryos. Furthermore, the expression of certain endosperm genes has been described in these non-zygotic embryos. The expression of hordein genes encoding the main barley endosperm proteins has been studied using a wide range of methods (RT-PCR, in situ hybridization, ELISA sandwich, western blotting immunocytochemistry, and cytochemistry) to ascertain their presence or absence during the induction and first stages of microspore embryogenesis. Due to the very sensitive techniques used it was possible to detect for the first time hordein expression during microspore embryogenesis. Surprisingly, these hordeins were also detected at different stages of male gametophytic development as well as during the very early stages of seed development, when they have not hitherto been detected. The expression and localization of these storage proteins and their corresponding transcripts provide new information about barley microspore embryogenesis and its relationship to zygotic embryogenesis. Although only small quantities of hordeins are accumulated during microspore embryogenesis they seem to be necessary for the initial development of the microspore-derived embryo. This idea is supported by the changes detected in their concentration throughout this process and is in accordance with previously published data concerning the importance of endosperm proteins for embryo development in both microspore culture and in planta.

Key words: Barley, hordeins, microspore embryogenesis, pollen development, protein bodies, storage proteins.

Introduction

Microspores programmed to differentiate and give rise to pollen tubes and gametes may be deviated to an alternate developmental pathway leading to the formation of haploid embryos (Reynolds, 1997). This dramatic shift from the gametophytic to the sporophytic pathway is usually induced as a consequence of stress treatment followed by anther or microspore culture under the appropriate conditions. Since this process, known as microspore embryogenesis, could have many advantages for breeding (Kasha et al., 1990) considerable efforts have been made to induce microspore embryogenesis in a wide variety of species and thus obtain higher induction frequencies (Ragavan, 1986; Reynolds, 1997; Touraev et al., 1997). These almost completely empirical approaches have afforded good results for cereals and, in particular, for barley, in which very efficient anther and isolated-microspore culture methods have been reported when using pretreatment with mannitol (Kasha et al., 1990; Hoekstra et al., 1992). Nevertheless, cellular and...
molecular studies of microspore embryogenesis are scarce and an understanding of the fundamental mechanisms of the induction of this process is still limited. One of the general characteristics of this embryogenic process is that the microspore-derived embryos develop following a pattern similar to that of zygotic embryos (Reynolds, 1997; Touraev et al., 1997; Pulido et al., 2001). The resemblances between the development of zygotic and microspore-derived embryos suggest that both pathways may have a partially common pattern of gene expression. Consequently, one of the strategies to characterize microspore embryogenesis and to find molecular markers is to study, during microspore embryogenesis, the expression of genes that are normally expressed during zygotic embryogenesis (Reynolds, 1997). Among the zygotic genes studied, those encoding the storage proteins of zygotic embryos, napins and cruciferins, have been reported as being expressed during microspore embryogenesis in Brassica (Crouch, 1982; Boutliier et al., 1994). In addition, it appears that endosperm genes (ZmAe1, ZmAe2, and ZmESR2) are expressed during the early stages of microspore embryogenesis in maize (Magnard et al., 2000; Massonneau et al., 2005). These authors have suggested that some endosperm products are needed for embryo development in microspore culture and have also pointed out the presence of endosperm-like domains showing a coenocytic organization in maize embryogenic microspores similar to that of endosperm initials. In barley microspore embryogenesis, the presence of two different kinds of multicellular pollen grain has recently been reported: homogeneous grains formed by a single type of cell and heterogeneous grains made up of cells with different cytological characteristics (Pulido et al., 2001, 2002, 2005; Maraschin et al., 2005b).

Cereal prolamsins (known as gliadins in wheat, hordeins in barley, secalins in rye, avenins in oats, and zeins in maize) are the main endosperm storage proteins in all cereal grains, with the exception of oats and rice (Shewry and Halford, 2002). Hordeins represent 35–50% of the total protein in barley seeds (Jaradat, 1991). They are classified into four groups, A, B, C, and D, in order of decreasing mobility (Field et al., 1982). The B hordeins are the main protein fraction, differing from C hordeins in their sulphur content (Kreis and Shewry, 1989). B hordeins account for 70–80% of the total and C hordeins for 10–20% (Davies et al., 1993). The A hordeins are not generally considered to be a storage fraction whereas D hordeins are homologous to the high-molecular-weight glutenins. Hordeins, along with the rest of the related cereal prolamsins, are not expressed in the zygotic embryo itself, unlike other storage proteins such as napins; they are believed to be expressed exclusively in the starchy endosperm during the middle-to-late stages of seed development (Davies et al., 1993; Shewry and Halford, 2002).

To gain further insight into the possible resemblances and differences between microspore and zygotic embryogenesis, the expression of hordeins was studied during microspore embryogenesis in barley. As a control, these proteins and their corresponding transcripts were also studied in developing seeds. Moreover, hordeins were examined at different pollen developmental stages: before the deviation to the embryogenic pathway and in the late stages of gametogenesis. To this end, very sensitive methods were used for the detection of prolamsins (Valdés et al., 2003).

Materials and methods

Plant material

Barley seeds of the winter cultivar ‘Igri’ were sown in pots of soil and germinated in a growth chamber with a photoperiod of 16/8 h (day/night) and a daytime temperature of 13 °C and night-time temperature of 11 °C. Germinated seedlings were vernalized at 4 °C for 1 month and grown in a climate chamber until tillering under the conditions described for seed germination. Tillers containing microspores at the different stages of male gametophytic development were excised and microspores were isolated from the rest of the sporophytic tissues for the mRNA and protein extractions by blending and maltose-gradient centrifugation according to Mordhorst and Lorz (1993). To study zygotic embryogenesis, developing seeds were collected at 3, 6, 8, 10, 12, and 35 d post-anthesis (DPA) (35 DPA seeds correspond to mature seeds). Finally, 14-d-old zygotic embryos were dissected from the rest of the caryopsis tissues prior to processing for RT-PCR, western blot, and ELISA tests.

Microspore culture

Tillers containing microspores at the late vacuolated stage were surface-sterilized by spraying with 70% ethanol and spikes were removed from the unsheathing leaves. Essentially Olsen’s protocol (Olsen, 1991) was followed with minor modifications. Aseptically dissected anthers were pretreated with 0.3 M mannitol in the dark for 3 d at 26 °C. Microspores were isolated by blending; maltose-gradient centrifugation was then applied according to Mordhorst and Lorz (1993). The upper microspore band was collected and cultured in MMS3 medium (Hu and Kasha, 1997) composed of MS medium containing 60 g l−1 maltose.

RT-PCR analysis

Total RNA was extracted from zygotic-embryogenesis samples (10 DPA complete caryopses and 14 DPA dissected zygotic embryos) and microspore pellets from in vivo pollen development and microspore cultures using the TRI REAGENT® LS (Molecular Research Center, Inc.) according to the manufacturer’s protocol. For RT-PCR, 2.5 μg of DNase I-treated total RNA was used for first-strand synthesis of cDNA by oligo(dT) primer reverse transcription using Moloney Murine Leucemia Virus Reverse Transcriptase as described by the manufacturer’s protocol (First Strand RT-PCR kit, ProSTAR; Stratagene). PCR was performed using the following primers, designed on the basis of database sequence information for the B1 Hordein gene (GenBank accession number X87232): B1 Hordein forward primer 5’-AACCATTTCACACGAAACCACCA-3’, and reverse primer 5’-ATTGTCGACTTGTCTCTCCTGC-3’. The B1 Hordein gene was amplified with an initial denaturation at 94 °C for 10 min followed by 40 denaturation amplification cycles (94 °C, 1 min), annealing (60 °C, 1 min), and extension (72 °C, 1 min), and a final elongation step at 72 °C for 10 min for all the stages studied. Saturation for S10 was reached at 36 cycles (data not shown) but 40 cycles were applied to have a positive control.
The amplification products were separated on a 1.5% agarose gel. Elongation factor 1α gene (EF1α) expression was assumed to be constitutive and equivalent in all the samples and was used as a loading control to normalize the amount of cDNA used in the amplification reaction. The primers used were: EF1α forward primer 5′-GGCTGTCAGGATCTCAGG-3′ and reverse primer 5′-AAC-TATGCGCACCACCA-3′, and the amount of cDNA used in the amplification reaction was normalized so that the EF1α amplification product showed a similar intensity in each of the samples assayed. 32 cycles for the EF1α gene were used because it had previously been established that this number of cycles did not lead to saturation in the amplification of the EF1α product (data not shown).

To detect any possible amplification from contaminating genomic DNA, control PCR reactions were performed in which RNA was not retrotranscribed, but no contamination was found during these experiments. RT-PCR products were cloned in pGEM-T-Easy vector (pGEM-T-Easy Vector System I, Promega) and sequenced. Both strands were sequenced using the dideoxy chain-termination method (Prism Read Reaction DyeDeoxy Terminator Cycle Sequence Kit, Applied Biosystems) with an automated DNA sequencer (3100 Genetic Analyser, Applied Biosystems).

**Processing for microscopy**

Anthers containing microspores at different gametophytic developmental stages, isolated microspores at various stages of the embryogenic process and 3, 6, 8, 10, 12, and 35 DPA developing seeds were fixed in 4% paraformaldehyde and 0.25% glutaraldehyde in PBS (PBS: 137 mM NaCl, 3 mM KCl, 16 mM Na₂HPO₄, and 2 mM K₂HPO₄ at pH 7.3) for 4 h at 4°C. The isolated and fixed microspores were then embedded in 1% agarose solution in PBS and after solidification washed in PBS. The anthers and caryopses were not embedded in agarose but washed straight away in PBS after fixation. Isolated microspores as well as anthers and developing seeds were then dehydrated in an ethanol series with a progressive lowering of temperature, embedded in Unicryl (BioCell) at −20°C, and polymerized under ultraviolet irradiation.

**In situ hybridization**

RT-PCR product (426 bp) was cloned in pGEM-T-Easy vector and digoxigenin-labelled sense and antisense RNA probes were synthesized using SP6 and T7 RNA polymerases using digoxigenin-UTP (digRNA labelling mix ×10, Roche) according to the manufacturer’s instructions.

2 μm thick sections were attached to 3-aminopropyl triethoxy silane-coated slides. The slides were pretreated with 1 μg ml⁻¹ proteinase K in 100 mM TRIS–HCl, 50 mM EDTA, pH 7.5, for 30 min at 37°C, followed by 100 mM triethanolamine, pH 8.0, for 10 min and 0.25% acetic anhydride in 100 mM triethanolamine, pH 8.0, for 10 min, both at room temperature. The sections were then dehydrated in an ethanol series with a progressive lowering of temperature, embedded in Unicryl (BioCell) at −20°C, and polymerized under ultraviolet irradiation.

In situ hybridization was carried out in accordance with the manufacturer’s instructions.

The isolated and fixed microspores, and the amount of cDNA used in the amplification reaction was normalized so that the EF1α amplification product showed a similar intensity in each of the samples assayed. 32 cycles for the EF1α gene were used because it had previously been established that this number of cycles did not lead to saturation in the amplification of the EF1α product (data not shown).

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**Hordein extraction**

Hordeins were extracted from 3, 6, 8, 10, 12, and 35 DPA developing seeds and from microspore pellets from both in vivo pollen development and microspore culture according to Hernando et al. (2003) with minor modifications. First, homogenization was performed in 60% v/v aqueous ethanol by using a pestle vigorously (10 min) and using a rotary shaker at room temperature for 60 min. The samples were then centrifuged at 3220 g and the supernatant collected. The ethanol extracts were used for ELISA and western blot analysis.

**Hordein quantification by ELISA**

To quantify hordeins during male gametophytic development and zygotic and microspore embryogenesis, a home-made ELISA based on an R5 monoclonal antibody was used according to Valdés et al. (2003). Polystyrene EIA/RIA flat-bottomed plates (Corning Incorporated) were coated overnight at 4°C with 2.2 μg ml⁻¹ R5 antibody in 50 mM carbonate/bicarbonate buffer, pH 9.6. After washing three times with 0.05% Tween 20 in PBS and blocking with 1% BSA, 0.05% Tween 20 in PBS for 1 h, the plates were incubated for 1 h with 100 μl of sample extract dilutions. Three independent experiments were made for each stage studied and three dilutions of every sample were done in 0.05% Tween 20 in PBS before being added to the ELISA plate wells. Due to the different quantities of hordein present at the various stages studied, different dilutions were made. The European gliadin standard IRMM-480 (Institute for Reference Materials and Measurements of the European Commission in Geel, Belgium) was used in the ELISA system as a gliadin reference, as previously described in Valdés et al. (2003). The calculations in this study were based on a gliadin protein content of 86.4% on the basis of dry matter (van Eckert, 2002). After incubation with the sample extracts the plates were washed and incubated with 100 μl of a peroxidase R5 conjugated antibody (R5-HRP) diluted in 0.05% Tween 20 in PBS for 1 h at room temperature. Finally, after washing the plates six times in 0.05% Tween 20 in PBS, 100 μl of the substrate solution, K-blue (Neogen) was added and the reaction was stopped 10 min later with 50 μl of 2.5M H₂SO₄. Absorbances at 450 nm were measured in a microplate reader. Multiple Range Tests was applied for statistical analysis of the results.

**Western blot analysis of hordeins**

R5 western blotting was performed according to Valdés et al. (2003). Briefly, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was done under the same conditions as those described by Rocher et al. (1996). The proteins were then electrotransferred onto polyvinylidene difluoride (PVDF) membranes, incubated directly with HRP-R5, a peroxidase R5 conjugated antibody, and developed using the ECL Western Blotting Analysis System for immunodetection (Amersham Biosciences) according to the manufacturer’s instructions.

**Spatial localization of hordeins by immunofluorescence**

1 μm thick sections were attached to 3-aminopropyl triethoxy-silane-coated slides and incubated in 5% BSA, 0.1% Tween 20 in PBS for 20 min in PBS for 2 h at room temperature. Silver enhancement was then carried out in accordance with the manufacturer’s instructions (BioCell); 1 volume of initiator was mixed with 1 volume of enhancer; the mixture was applied and the reaction monitored under LM for 10–15 min. The reaction was stopped by washing the slides in distilled water, after which the sections were air-dried, stained with safranin and mounted.

**Hordeins in microspore embryogenesis**

Hordeins in microspore embryogenesis
RNA from the developing seeds [caryopses 10 DPA (S10) and dissected embryos 14 DPA (E14, zygotic embryogenesis)] were also analysed to be used as controls. Equal loading of the samples was ensured by the comparable intensity of bands resulting from RT-PCR amplification of the EF1α gene (GenBank accession number L11740) in equivalent samples because EF1α mRNAs display steady-state levels (Liboz et al., 1990).

The RT-PCR analysis for this gene showed no signal when RNA from dissected zygotic embryos (E14) was used (negative control). A band of 426 bp was observed, however, in 10 DPA complete caryopses (S10) (positive control). With regard to the male gametophytic process, bands of the same size (426 bp) were observed at all the stages studied (A0, P9, and P12). In embryogenetic development induced by isolated-microspore culture, bands were detected after mannitol pretreatment (A3) and after 3 d (C3) and 13 d (C13) culture.

In situ spatial expression of the B1 Hordein gene

To study the distribution of the B1 Hordein gene transcripts during in vivo pollen development and at the first stages of microspore embryogenesis, hybridization was carried out in situ with a digoxigenin-labelled riboprobe using semi-thin sections (Fig. 2). Labelling corresponding to gold particles intensified by silver enhancement was evident at every stage of male gametophytic development studied: vacuolated microspore (Fig. 2A), early bicellular (Fig. 2B) and tricellular (Fig. 2C) pollen. No labelling was found in the tapetum at any of these stages however (Fig. 2A). With regard to microspore embryogenesis, after 3 d mannitol pretreatment the microspores also presented labelling (Fig. 2D). The labelling was more abundant and homogeneously distributed later in the homogeneous and heterogeneous multicellular pollen grains obtained after 3, 6, 9, and 13 d culture. In heterogeneous multicellular pollen grains the labelling was distributed in both types of cell (Fig. 2E). Labelling was also observed in microspore-derived embryos released from the microspore wall (Fig. 2F). Labelling was very abundant in the 10 DPA seed endosperm cells (Fig. 2G). No labelling was obtained after hybridization in embryos of 10 DPA seeds (Fig. 2H). No signal was ever detected in the nucleolus, cell wall, vacuoles, or starch granules or when the corresponding B1 Hordein sense riboprobe was applied in parallel sections (data not shown).

Hordeins are present at very early stages of seed development and microspore embryogenesis

In view of the above results, to detect and quantify hordeins during zygotic and early microspore embryogenesis, the ELISA ‘sandwich’ method was used (Fig. 3), which has inter-assay (reproducibility) and intra-assay (repeatability) variabilities of 8.7% and 7.7%, respectively (Valdés et al.,...
In zygotic embryogenesis (Fig. 3A) it was possible to detect hordeins as early as 3 DPA, although they were very scarce at this moment. This quantity grew progressively from 3 DPA onwards, slowly at first but much faster later on in seed development. In microspore embryogenesis (Fig. 3B), hordeins could already be detected in the vacuolated microspores (A0) before pretreatment with mannitol and they were not affected by this treatment (A3). The quantity of hordeins changed throughout the embryogenic development of microspores, increasing at first after 3 d culture (C3), then decreasing until 9 d isolated-microspore culture, and finally increasing again after 13 d culture.

Hordein patterns during zygotic embryogenesis and early microspore embryogenesis were analysed by western blotting using HRP-R5 conjugated antibody to identify any differences between the hordein patterns shown in these two different embryogenic pathways (Fig. 4). Due to the considerable difference in the quantities of hordeins present in the samples analysed, the protein samples were loaded in such a way as to be able to visualize the protein patterns for all the samples. Hordeins were first detected in zygotic embryogenesis at 3 DPA (Fig. 4A). Bands ranging from 30 kDa to 70 kDa were obtained at all stages of seed development. The relative abundance of the different hordeins changed, with hordein B proteins increasing throughout seed development. With regard to microspore-derived embryogenic development (Fig. 4B), differences were found in the accumulation of hordeins at the various developmental stages. B hordeins were more abundant than C hordeins before induction, as also occurred in seed development, whilst after 13 d the relative
amount of C hordeins was higher than that of B hordeins (Fig. 4B).

**Tissue location of hordeins**

To study the precise location of hordeins during male gametophytic development and microspore and zygotic embryogenesis, immunocytochemical studies were made at light microscope level at different developmental stages of these processes (Fig. 5).

During endosperm cellularization (Fig. 5A), hordeins were first detected in the endosperm at 6 DPA (Fig. 5B, C). The labelling was concentrated in protein bodies that were also visible with the chloramine T-Schiff reagent and with toluidine blue staining (Fig. 5D, E). These protein bodies fused (Fig. 5C) and grew in size within the endosperm throughout seed development. In mature seeds the immunofluorescent protein matrix formed by the fusion of the protein bodies, together with starch granules, occupied the whole cytoplasm of the endosperm cells (data not shown). No labelling was observed in the zygotic embryo in any of the seed development stages studied (data not shown).

During male gametophytic development immunofluorescence was observed in some spots of the cytoplasm of the vacuolated microspores (Fig. 5F, G) and bicellular (Fig. 5H, I) and tricellular pollen (Fig. 5J, K), although the labelling was concentrated in a very few small spots which were never visible as protein bodies after toluidine blue staining.

As far as microspore-derived embryogenic development was concerned (Fig. 6), scarce labelling was observed after mannitol pretreatment (Fig. 6A, B). After 3 d (Fig. 6C, D), 6 d and 9 d (Fig. 6E, F) culture, immunofluorescence was stronger and concentrated in bigger spots present in homogeneous as well as heterogeneous multicellular pollen grains, the labelling being present in both types of cell in the latter. When dead microspores were present no labelling was found (Fig. 6C, D). After 13 d culture, fluorescent protein bodies were also present in microspore-derived embryos released from the microspore wall (Fig. 6G). Specific techniques for detecting proteins, such as Coomassie Blue staining (Fig. 6H) and chloramine T-Schiff reagent (Fig. 6I), revealed, after 13 d culture, the presence of protein bodies very similar to those observed inside the endosperm. The same occurred after 18 d (Fig. 6J, K), but in these embryo-like structures there were, in addition, some protein bodies that presented no immunofluorescence at all whilst others fluoresced only in certain spots within the protein bodies (insert Fig. 6J).
Discussion

To date, cereal prolamin genes have been reported as being expressed exclusively in the starchy endosperm during the middle-to-late developmental stages of seed development (for a review see Shewry and Halford, 2002). The results of this study show, however, that hordeins in barley are expressed in microspore-derived embryos and also during male gametophytic development. These results could be obtained due to the high sensitivity of the techniques employed, especially the ELISA sandwich (Valdés et al., 2003), thus finding them as early as 3 DPA in seed development. The presence of hordeins so early in seed development has not been described before. As far as is known, the earliest detection of hordeins was 5 DPA, made in Sundance barley by Davies et al. (1993) using immunocytochemistry.

In situ hybridization, which allowed the spatial localization of B1 Hordein gene transcripts, was used because, although hordein transcription was evident after RT-PCR, the microspore-derived embryogenic response is non-homogeneous and unsynchronized, as has been demonstrated elsewhere (Pulido et al., 2005).

Although these results show that the quantity of these proteins was very low in both male gametophytic and microspore-derived embryogenic development, the coincidence in the results obtained by the different techniques reinforces their value.

Hordeins in the microspore-derived embryogenic pathway

The presence of either storage proteins or their messengers in microspore-derived embryos of barley or any other monocots has not been described previously. Nevertheless, Crouch (1982) detected the presence of a 12S glycoprotein (cruciferin) during microspore embryogenesis in the dicot Brassica napus, and later Boutiplier et al. (1994) described the presence of napin transcripts in embryogenic microspores of the same species. Other storage proteins have also been reported in the somatic embryos of different species (Crouch, 1982; Shoemaker et al., 1987; Joy et al., 1991; Misra et al., 1993; Koltunow...
et al., 1996; Chatthai and Misra, 1998; Šunderlíková and Wilhelm, 2002). All the storage proteins found in microspore-derived and somatic embryos have been detected previously in the corresponding zygotic embryos. Hordeins, however, are not present in the zygotic embryo of barley but in the endosperm of the corresponding seeds, as has been described elsewhere (for a review see Shewry and Halford, 2002) and has been confirmed in this investigation. During monocot seed development the endosperm acts as a storage tissue, but this tissue is not present in the embryos originated from microspores. These results may suggest that, due to the lack of endosperm, the microspore-derived embryos themselves need to synthesize some of the substances necessary for giving rise to the future plantlet, hordeins among them. Comparing microspore-derived and zygotic embryo development by ELISA it was seen that there were fluctuations in the hordein content throughout microspore-derived embryogenic development, whereas in seed development they increased constantly. Whilst it is known that, during seed development, hordeins are stored for later use in germination and seedling development, these results suggest that in microspore-derived embryogenic development these proteins may be synthesized and consumed according to the requirements of the embryogenic microspores and early embryos.

Other endosperm genes, unrelated to storage proteins, have been described as being expressed in microspore-derived embryos, but not in the corresponding zygotic embryos (Magnard et al., 2000; Massonneau et al., 2005). The expression of hordein genes in microspore-derived embryos is in harmony with the recent suggestion of Maraschin et al. (2005a) and Massonneau et al. (2005) that the development of embryogenic microspores requires endosperm-like functions, which might be needed to establish interactions that probably exist in planta between embryo and endosperm. Massonneau et al. (2005) suggested
that certain proteins deriving from non-embryogenic structures are secreted into the medium to stimulate microspore-embryo development. In barley, hordeins are expressed in embryogenic structures, as has been shown, but they are not secreted proteins.

Magnard et al. (2000) showed the existence of endosperm-like domains in multicellular maize pollen grains. From these immunolocalization and in situ hybridization results, however, it is not possible to distinguish any domain which might be considered as being endosperm-like, either in the homogeneous or heterogeneous multicellular pollen grains.

Blumenthal et al. (1990) discovered the presence of several heat-shock elements upstream of the ‘TATA’ box in the nucleotide sequence of gliadin genes. These elements were conserved sequences associated with the switching on of all known heat-shock genes. Within this context it is worth noting that the expression of heat-shock proteins has been widely described during the induction and early stages of microspore embryogenesis induced by different kind of stresses, including starvation (Pechan et al., 1991; Zarsky et al., 1995). Thus it is possible that hordein expression increased in the cultured microspores used here as a consequence of the stress treatment to which they had been subjected. This hypothesis would need to be proved by further research.

The localization of hordeins by immunofluorescence showed that, in seed development, these proteins were included in protein bodies of different sizes that were also visualized by cytochemical techniques. During microspore-derived embryogenic development, protein bodies were not visible in young multicellular pollen grains by cytochemical techniques, but fluorescent spots revealed the presence of hordeins. In microspore cultures from 13 d culture onwards, protein bodies were clearly stained by cytochemical techniques but the distribution of hordeins was not the same in all of them, as was shown by immunofluorescence. These results suggest that either some protein bodies are not made up exclusively of hordeins or that hordein epitopes are only partly accessible to these antibodies. It has been proved that barley endosperm protein bodies are formed entirely of hordeins B and C, which are assembled in the centre of the body and surrounded by an external layer of γ1 and γ2 hordeins (Rechinger et al., 1993). In other cereals such as oats and wheat, however, endosperm protein bodies may be made up of different proteins: in oats, globulins and prolamins are co-located in the same protein bodies (Lending et al., 1989) and in wheat the endosperm protein bodies formed by gliadins may contain inclusions of a globulin known as triticin (Bechtel et al., 1991). Perhaps some of the protein bodies present during the embryogenic development induced in microspores in barley are composed of different storage proteins. Further studies are necessary to discover the exact composition and to unravel the accurate function of all the proteins contained in protein bodies present in embryogenic microspores.

**Hordeins in male gametophytic development**

Although it has been reported that the storage proteins synthesized during pollen maturation provide building blocks for rapid growth and pollen-tube germination (Mascarenhas, 1989; Herman and Larkins, 1999), storage proteins in pollen have not been extensively studied and, to date, cereal prolamsins had not been described during gametophytic development. With regard to these results, napins, which are zygotic-embryo storage proteins in *Brassica napus*, have been reported as being expressed during male gametophytic development as well as in embryogenic microspores (Boutilier et al., 1994). These results may represent a similar pattern of expression for storage proteins in both a dicot and a monocot species.

Most cereal prolamsins and glutelins belong to one class of related seed proteins, which have common evolutionary roots (Shewry and Tatham, 1990), except for rice glutelins and maize γ-zein (Müntz, 1998). In this context, these results concerning hordein expression during both embryogenic and male gametophytic development open new perspectives concerning the possible expression of other prolamsins, such as gliadins and secalins, in these developmental pathways in related cereals such as wheat and rye.

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