Exogenous abscisic acid impacts the development of isolated immature endosperm in bread wheat

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Abstract

Endosperm in cereals such as wheat, is a part of the mature seeds and a valuable source of key substances for humans and animals. For this reason, the development of immature endosperm tissues *in planta* was the focus of this research. However, it is commonly known that tissue culture conditions can alter the developmental pathway of plant cells and can expose their potency. There is scarce information about research on isolated endosperm in wheat. The development of isolated immature endosperm in the winter bread wheat variety ‘Kobra’, depending on the media composition, is presented in this study. Abscissic acid (ABA) is a key plant growth regulator for proper seed development. The addition of exogenous ABA had a positive impact on the size and ultrastructural features in isolated endosperm, especially of the outer aleurone-like cells. Furthermore, the content of starch in the endosperm cultured on a medium with ABA did not significantly differ from that of caryopsis at the same age, in contrast to soluble carbohydrates. Fluorescein diacetate (FDA) staining and confocal microscopy observation confirmed the viability of the cells from the outer layers. The analysis of internucleosomal fragmentation of DNA in the explants suggests the induction of programmed cell death (PCD) and DNA degradation typical of necrosis. We concluded that the development of isolated immature endosperm in bread wheat depends on the composition of the media. Thus, it could be a model for *in vitro* studies of this specific storage tissue and its response to culture conditions in bread wheat.

Key message

Exogenous abscisic acid positively influences the size and viability of cells, as well as the ability to accumulate carbohydrates in isolated immature endosperm in wheat.

Keywords DNA fragmentation · *In vitro* culture · Soluble carbohydrates · Starch · *Triticum aestivum* · Ultrastructure

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Introduction

A storage tissue called endosperm plays a crucial role in embryo development. In some plants, like cereals, the endosperm is a major source of carbohydrates for human food and animal feed. Endosperm starts to form after the second fertilization during double fertilization. Fusion of the sperm nucleus and the central cell results in primary endosperm nucleus, the divisions of which result in multinuclear syncytium (Mares et al. 1975). The cellular stage of this tissue occurs at approximately 4 days post anthesis (DPA) for bread wheat (Evers 1970). In cereals the outermost endosperm cell layer differentiates as aleurone cells, while the cells inside develop into starch- and protein-storing cells. According to Kalinga et al. (2014), the

pre-physiological maturity stage (about 28 DPA) is distinguished from physiological maturity (35 DPA).

In cereals, PCD is a process strongly associated with endosperm development (Young and Gallie 1999; 2000; Dominguez and Cejudo 2014). During caryopsis maturation, most of the endosperm cells loaded with storage materials die, except the aleurone layer (Wei et al. 2002; Young and Gallie 2000; Sabelli and Larkins 2009).

Starch is considered to be the most universal form of carbon storage in plants, for energy and substrates (Martin and Smith 1995; Geigenberger 2011). Cereal crops are the main source of starch used for food (Agama-Acevedo et al. 2019). The starchy endosperm accounts for about 83% of the wheat grain's dry weight (Barron et al. 2007) and is stored in amyloplasts in starch granules. A plant's response to environmental stress factors such as drought, waterlogging, or excess metal also results in starch accumulation in the endosperm (Fábíán et al. 2011; Kurdziel et al. 2015; Skórka et al. 2020).

It has long been known that abscisic acid (ABA) plays a crucial role in seed dormancy (King 1976) – especially correct embryo dormancy in cereal seed (Gao and Ayele 2014) – but also for proper deposition of storage materials (Kermode 2005). An increase of both starch content and accumulation was reported by Yang et al. (2014) in studies of on post-anthesis of winter wheat.

In contrary to the endosperm of *Zea mays*, which has been examined under in vitro conditions for many years (Gruis et al. 2006; Reyes et al. 2010; 2011; Blehová et al. 2018; Olsen 2020, 2021), studies on isolated endosperm in *Triticum aestivum* are scarce. It has been shown that the isolated immature endosperm of selected cultivars of *Triticum* and *Triticosecale* are able to continue the development and that the efficiency of the response is genotype-dependent (Popielarska-Konieczna et al. 2013). Observations were conducted on explants for two weeks of culturing after their isolation from young caryopses. Cultures on the endosperm of *Z. mays* were maintained for a few days in order to study *Agrobacterium* transformation (Reyes et al. 2010), as well as prolamin deposition and the system of protein storage vesicles (Reyes et al. 2011). Interestingly, an in vitro culture of miniendosperm in *Z. mays* with the formation of tracheary elements was even maintained for two years (Blehová et al. 2018).

The objectives of the experiments were to investigate the changes in the development of isolated endosperm under different tissue culture conditions and to compare them with endosperm in caryopsis. Special attention was paid to ultrastructural features, viability, and starch accumulation. We could assume that the lack of embryo and maternal tissue like a pericarp certainly have significant and severe consequences on the development of immature endosperm (Gruis et al. 2006). Otherwise, in a controlled tissue culture we had

the opportunity to detect an appearance, which cannot be observed under natural conditions. To our knowledge this is the first observation of the development of isolated immature endosperm wheat for a period of several weeks under tissue culture conditions. These results can provide the basis for further study of the response of isolated endosperm in bread wheat at the molecular level.

Materials and methods

Plant material and culture conditions

The plant material was chosen according to previous studies (Popielarska-Konieczna et al. 2013), which showed that isolated immature endosperms from the 'Kobra' cultivar of winter bread wheat were one of the best developing explants under tissue culture. Bread wheat (*Triticum aestivum* L.) 'Kobra', after nine weeks of vernalization at 5 °C, was grown in pots in the greenhouse of the Institute of Plant Physiology of the Polish Academy of Sciences (Krakow, Poland). Each pot contained three wheat plants growing to the anthesis in a 3:2:1 potting mix of soil, peat, and sand, respectively, under controlled conditions: 20/17 °C (day/night), 800 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR, and a long day (16/8 h light/dark). The photoperiod and light intensity were maintained using high-pressure sodium lamps (400 W; Philips SON-T AGRO, Belgium) between 6 and 8 a.m., 6 to 10 p.m., and on cloudy days. The plants were watered twice a week with a soluble fertilizer (N:P:K = 15:15:18).

For the plant culture, the immature caryopsis were collected from the middle part of main spikes at 8 DPA, then were sterilized and prepared as previously described (Popielarska-Konieczna et al. 2013). In brief, the green chaff was removed from the cut-off flowers and the ovaries were then sterilized. The micropylar part of the ovule containing the embryo was cut off and removed together with the whole integument of the ovule (Fig. 1a and b). Explants, i.e. part of the endosperm (Fig. 1c) were cultured on basal Murashige and Skoog media (1962) solidified with 6% agar (Plant Agar, Duchefa). A solution of macro- and microelements and vitamins (Duchefa) was supplemented with 10% sucrose, amino acid (glutamine 2 mg L⁻¹, Sigma), and plant growth regulators: 0.5 mg L⁻¹ of thidiazuron (TDZ) or 2.0 mg L⁻¹ of ABA. Three kinds of media were tested: Medium 1 with TDZ, Medium 2 with glutamine and ABA, and Medium 3 with glutamine and TDZ. Solutions with glutamine, ABA, and TDZ were filter-sterilised with 0.22- μm Millex Syringe Filter Units (Millipore, Merck), and then were added to the rest of the autoclaved medium. Before sterilization, the pH was adjusted to 5.7–5.8. The explants were cultured at 25 $\pm$ 1 °C in the dark.

Fig. 1 Source of explants (**a, b**) and isolated endosperm (**c–f1**) of *Triticum aestivum* ‘Kobra’. **a** Immature caryopsis at 8 DPA. **b** Ovule after removing the ovary wall; dotted line and star indicated the micropylar part of the ovule with the embryo, which was cut and removed. **c** Isolated endosperm after removing the integument, with a visible cut area (star). **d–f** Explants at 8 DPA at the beginning of the culture on Medium 2. **d1–f1** Corresponding explants at 34 days old (8 DPA + 26 DIV); open arrows indicate the developing part of the explant, in contrast to the non-growing part (asterisks). Scale bar: 500 μ m

Collection of samples

Endosperm tissues for samples were taken from fresh-cut caryopses and from tissue culture plates. The age of the explants from culture conditions was described as a number of days: 8 DPA (age of caryopsis as a source of isolated endosperm) and number of days under in vitro (DIV) culture, e.g. an 18-day-old explant had the endosperm isolated at 8 DPA and was cultured 10 DIV. The endosperm explants were collected for analysis at 8 DPA + 4 DIV, 8 DPA + 10 DIV, 8 DPA + 16 DIV, and 8 DPA + 26 DIV. For a comparative analysis, endosperms developed in caryopsis *in planta* were taken at 12, 18, 24, and 34 DPA. Endosperm tissue from caryopsis at 8 DPA was also collected as a primary explant.

During the three years of the experiment (2014–2016), a total of more than 3,000 explants were isolated and cultured. The observations and images were taken using a dissecting binocular microscope (Zeiss, Germany, Stemi SV 11) equipped with a digital camera (Canon Power Shot G6).

Histological analysis and measurement of cells

Samples were fixed overnight at 4 °C in 5% (v/v) glutaraldehyde in 0.1 M phosphate buffered saline solution (PBS, pH 7.2) for 24 h at room temperature. The samples were then washed four times in phosphate buffer, dehydrated gradually in an ethanol series, embedded in Technovit® 7100

synthetic resin, cut into 5- μ m thick sections, stained with 0.1% (w/v) toluidine blue (TBO) and finally mounted in Entellan (Merck, Darmstadt, Germany). Semi-thin sections were also stained with Aniline-Blue Black for protein identification and Sudan Black B for lipid identification. Observations and documentation were performed using the visible light system in a Nikon Eclipse E400 microscope equipped with a Zeiss AxioCam MRe digital camera with Zeiss Axio-Vision 3.0 software and a Nikon DS-Fi2 with NIS-Elements 4.0 software. The images were processed with CorelDRAW Graphics Suite 2020 software.

The perimeter of cells was measured using ImageJ software version 1.51j8. At least 100 cells (classified as aleurone and starchy cells, as well as aleurone-like and starchy-like cells) were acquired for the measurements from semi-thin sections randomly chosen for 8-, 18-, and 34-DPA-old caryopsis stage and 8DPA + 10DIV, and 8 DPA + 26DIV explants of each culture treatment were scored.

Transmission electron microscopy

The samples were fixed in a mixture of 2.5% (w/v) formaldehyde (prepared from paraformaldehyde) and 2.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.0) for 2 h at room temperature. Next, they were rinsed in the same buffer with four changes (15 min each) and post-fixed in buffered 1% OsO₄ at 4 °C overnight. After being rinsed in distilled water, the samples were treated with 1% uranyl acetate in

distilled water for 1 h, dehydrated in a graded acetone series, and embedded in Spurr's resin. Ultrathin sections were cut on a Leica EM UC7 ultramicrotome, stained with uranyl acetate and lead citrate, and examined with an FEI Tecnai G2 Spirit TWIN/BioTWIN transmission electron microscope at the Laboratory of Electron Microscopy, Faculty of Biology, University of Gdańsk.

Confocal microscope

Endosperm explants were transferred into sterile units of a Lab-Tek® Chambered Borosilicate Coverglass System (Nalge Nunc Int., Rochester, NY) and filled with a sterile solution of 8% (w/v) sucrose (pH 5.7–5.8). FDA (Sigma) was dissolved in acetone to a stock concentration of 1 g/ml. A working solution (5 µl/ml) was obtained by mixing 5 µl of the stock with 1 ml of sterile sucrose solution in the chambers with explants. After 5 min incubation in the dark, explants were observed with a Zeiss LSM 510 Meta laser scanning confocal microscope (Carl Zeiss GmbH, Jena, Germany). The fluorescein was excited using a 488-nm and 405-nm laser, and emissions were collected between 505 and 530 nm. The images were processed using Zeiss LSM Image Browser software (version 4.2.0.121), with Color Depth Coding (pseudo-color presentation of height information).

DNA laddering detection

The samples were collected in Eppendorf tubes, with the weight being determined as 100 mg of the fresh weight. They were frozen in liquid nitrogen and stored at -80 °C until the analysis. The DNA was extracted using a GeneMATRIX Food-Extract DNA Purification Kit (EURx) according to the manufacturer's protocol; they were then precipitated. DNA was resolved on 1.8% agarose gel and stained with SimplySafe (EURx) for visualization.

Analysis of soluble carbohydrates and starch

The samples were collected in Eppendorf tubes—the weight was determined as 50 mg of the fresh weight—then frozen in liquid nitrogen and stored at -80 °C until the analysis. The total sugar content (starch and soluble sugar) was determined according to the anthrone method by spectrophotometry (Perkin Elmer UV VIS) at 620 nm according to McCready et al. (1950) with the modifications of Janeczko et al. (2010).

Statistical analysis

All data were pooled means from the replicates and were statistically evaluated using Duncan's test and one-way analysis

of variance (ANOVA) using the statistical package STATISTICA 13.0 (Stat-Soft, Inc.).

Results

Response of explant under tissue culture

In comparison with the day of the inoculation (Figs. 1d–f), whole or parts of the explants were visibly enlarged during the next 4–10 DIV (Fig. 1d1–f1). The developing (active) part of the explants was bulgy and harder to compare with the non-developing (non-active) part, which retained a gel-like consistency. The appearance of explants was similar under all tested media, and no morphological differences were observed until 26 DIV. Additionally, ANOVA revealed that the efficiency of the explants' response was not medium-dependent ($F = 0.07$, $P \leq 0.786$).

Histology, ultrastructure, and size of aleurone- and starchy-like cells

Cross-sections of the controls, that is, the endosperm of 8 DPA and 34 DPA caryopsis, were analyzed. Observations of sections of the 8 DPA caryopsis revealed differences between cells from the surface and from the inside of the endosperm (Fig. 2a). Smaller and similarly sized cells corresponded to the aleurone layer. On the other hand, the starchy cells inside the endosperm were larger and high vacuolated (Fig. 2b). Thin cell walls and large nuclei were noticed for both kind of cells. Endosperm from caryopsis at 34 DPA showed an abundance of storage materials: starch granules in starchy cells and proteins and lipid bodies in aleurone cells (Fig. 2c, d). The cell walls within the aleurone layer were thick, with numerous plasmodesmata (Fig. 2d).

Sections of cultured endosperm after 10 DIV showed clearly visible differences between cells from the outer and deeper parts of the explants (Fig. 3, Suppl. Figure 1). Smaller aleurone-like cells at the surface of the explants and larger starch-like cells filled with starch granules were observed on all types of media. Aleurone-like cells possessed dense cytoplasm on Media 2 and 3. Cells from the surface of explants cultured on Medium 1 were highly vacuolated (Suppl. Figure 1). During next 16 days of culturing, the aleurone-like cells continued to develop and increased in size only on Medium 2 (Fig. 4a). It is worth noting that the irregular shape of these cells was not typical of an aleurone layer. Large aleurone-like cells had abundant organelles, e.g. mitochondria and profiles of endoplasmic reticula, amyloplasts with starch granules, protein bodies, and many lipid bodies (Fig. 4b–d). Sections of explants from Media 1 and 3 at the same age (8 DPA + 26 DIV) revealed small, empty aleurone-like cells (Suppl. Figure 1). The microscope slides

Fig. 2 Cross sections of the outer part of the endosperm of *Triticum aestivum* ‘Kobra’ from caryopses at 8 DPA (**a, b**) and 34 DPA (**c, d**). **a, c** Histological sections stained with TBO; notice the aleurone (arrows) and subaleurone cells (open arrows) and starchy cells (arrows). **a** Aleurone cells with large nuclei. **c** Visible dark-stained aleurone cells with thick cell walls; a starchy cell filled with starch granules; the remnants of a husk (star). **b, d** Ultrastructure of aleurone (arrows) and subaleurone cells. **b** Vacuole (v). **d** Magnification of part of an aleurone cell with numerous lipid bodies (lb), electron-dense protein deposits (pt), and plasmodesmata (pd) in the cell wall (cw). Scale bar: 20 μ m (**c**), 10 μ m (**a**), 5 μ m (**b**), 1 μ m (**d**)

showed clear differences between active and non-active endosperm tissue (Suppl. Figure 1), which corresponded with the developing and non-developing parts of explants visible under macroscopic observation.

The measurement of perimeter cells did not show the real size of the cells, but it could suggest differences between aleurone- and starchy-like cells. The results showed that the endosperm cells differed in size between *in planta* and *in vitro* conditions—in case of the aleurone and aleurone-like cells. The cells from the surface of explants were almost four times larger under culture conditions. The development of immature isolated wheat endosperm was supported, especially in the presence of glutamine and ABA (Table 1). The size of starchy-like cells was similar to that of the endosperm developing in caryopsis.

Viability of cells

The analysis with FDA staining and confocal-laser scanning microscope of endosperm after 26 DIV cultured on Medium 2 revealed a few layers of cells with fluorescence on the surface of the explants (Fig. 5). The shape and composition of cells corresponded with cells observed on the histological sections. The area inside the explants did not display a positive signal (Fig. 5f), which suggested that

there were no living cells in this area. Similarly, no fluorescence was detected in the case of explants cultured on Media 1 and 3 after 26 DIV, either inside or on the surface of the explants (data not shown).

DNA specific fragmentation is a typical characteristic of PCD. The degradation of nuclear DNA produces fragments with 180–200 bp or multimers, which show DNA laddering on gel electrophoresis. The results of DNA electrophoresis revealed that fragmentation of DNA in the endosperm of caryopsis occurred at 18 DPA (Fig. 6a) and progressed in the following days of grain maturation. At 30 and 34 DPA, laddering was clearly visible. The results of DNA electrophoresis of samples from cultured explants revealed that laddering was detected even after 4 DIV, and progressed in the following days of culturing (Fig. 6b). Interestingly, the fragmentation of DNA was clearly visible in the non-active parts of the explants. In the active part, fragmentation was not observed or the laddering was blurred (Suppl. Figure 2). However, in following days of culturing, until 26 DIV, the amount of nuclear DNA decreased distinctly (Fig. 6b). It is worth noting that the degradation of all nuclear DNA was observed after 26 DIV for the explants cultured on Medium 1, in contrast with the other treatments (Suppl. Figure 2c).

Fig. 3 Cross-sections of the outer part of isolated endosperm of *Triticum aestivum* 'Kobra' at 18 days old (8 DPA + 10 DIV) cultured on Medium 2. **a** Histological sections stained with TBO; notice the aleurone-like (*open arrows*) and starchy-like cells (*arrows*). **b–d** Ultrastructure of aleurone-like cells. **b** Profiles of endoplasmic reticulum (*er*) and protein bodies (*pb*). **c** Amyloplast (*am*) with starch grain (*sg*), lipid bodies (*lb*), protein bodies (*pb*), mitochondria (*mt*), and vacuoles (*v*). **d** Mitochondria (*mt*), lipid bodies (*lb*), plasmalemma (*pl*) next to cell wall (*cw*) and protein bodies (*pb*) next to crenelated vacuole tonoplast (*vt*). Scale bar: 50 μ m (**a**), 1 μ m (**b–d**)

Fig. 4 Cross-sections of the outer part of isolated endosperm of *Triticum aestivum* 'Kobra' at 34 days old (8 DPA + 26 DIV) cultured on Medium 2. **a** Histological sections stained with TBO; notice the aleurone-like (*open arrows*) and starchy-like cells (*arrows*). **b–d** Ultrastructure of aleurone-like cells. **b** Lipid bodies (*lb*) visible next to the outer cell wall (*cw*) and inside the cytoplasm; protein bodies (*pb*) in vacuoles (*v*), myelin-like body (*ml*); amyloplast (*am*) with starch grain (*sg*). **c** Profiles of endoplasmic reticulum (*er*). **d** Mitochondria (*mt*). Scale bar: 50 μ m (**a**), 1 μ m (**b–d**)

Table 1 Perimeter (μm) of endosperm cells of *Triticum aestivum* ‘Kobra’ from caryopses at 8, 18, and 34 DPA and explants at 18 and 34 days old (8 DPA + 10 DIV and 8 DPA + 26 DIV, respectively) cultured on three kind of Media

Aleurone cells			
	8 DPA	18 DPA	34 DPA
Caryopsis	45.34 ± 2.33 a	117.53 ± 5.23 b	110.25 ± 4.68 b
Aleurone-like cells			
		8 DPA + 10 DIV	8 DPA + 26 DIV
Medium 1	–	57.56 ± 5.67 a	48.36 ± 4.11 a
Medium 2	–	254.44 ± 13.67 c	397.89 ± 15.61 c
Medium 3	–	105.34 ± 7.21 a	100.31 ± 10.52 b
Starchy cells			
	8 DPA	18 DPA	34 DPA
Caryopsis	105.45 ± 14.47 a	1115.72 ± 30.21 b	1838.56 ± 28.67 b
Starchy-like cells			
		8 DPA + 10 DIV	8 DPA + 26 DIV
Medium 1	–	1154.47 ± 40.24 b	1244.89 ± 45.66 b
Medium 2	–	1688.67 ± 42.21 b	1424.52 ± 26.94 b
Medium 3	–	1347.21 ± 50.20 b	1144.76 ± 32.12 b

Mean and standard error ($\pm$) values are presented. Different letters at mean values indicate statistical difference between means ($p < 0.05$ by the post hoc Tukey test)

Starch and soluble carbohydrate content

It was found that the content of soluble carbohydrates in the endosperm tissue taken from caryopses increased between 8 and 12 days of age, and then slightly decreased (Fig. 7a). In all the samples from isolated tissue culture, the content of soluble carbohydrates was higher, and significantly so for most of them. The tissues cultured on Media 2 and 3 were especially rich in soluble sugars. In endosperm tissue taken from caryopses, increased starch deposition was noted from day 8 until day 18, and a slight decrease was found at the maturation stage (Fig. 7b). The content of starch in isolated cultured endosperm was lower at all time-points, except for the 12-day-old tissue (8 DPA + 4 DIV) from Medium 1. On this medium, the content of starch distinctly decreased during the following days of culture. A higher amount of starch in comparison to caryopsis was observed for tissue cultured on Medium 2. The content of starch for this culture was measured in 12-day-old tissue (8 DPA + 4 DIV) and did not change much until day 34 (8 DPA + 26 DIV). Medium 3 revealed a similar content of starch in 12-day-old tissue, but in the following days it significantly decreased. Generally, there were no significant differences between starch content detected in endosperm from caryopsis and the cultured explants – until day 24 (caryopsis: 24 DPA; explants: 8 DPA + 16 DIV). The soluble carbohydrate concentration decreased significantly in comparison with the

caryopsis. ANOVA revealed significant differences in the starch and carbohydrate content relative to the tissues’ age ($F = 10.23$, $P < 0.001$), but not significant differences regarding the source of endosperm (caryopsis and explant from different media). Medium 2 supplemented with ABA was significantly better than other media in the maintenance of starch ($F = 15.56$, $P < 0.01$), but not for carbohydrates.

Discussion

It is commonly known that tissue culture conditions can alter the developmental pathway of plant cells and can enhance their potency and plasticity (Feher 2019). The endosperm of cereals—considered the storage tissue of starch and protein—is an interesting object for studying the response to culture conditions. This is the first detailed biochemical, histological, and ultrastructural study on isolated cultured endosperm tissue in bread wheat to focus on the addition of exogenous ABA. Based on previous studies (Popielarska-Konieczna et al. 2013), the winter bread wheat variety ‘Kobra’, with a highly efficient response of explants isolated at 8 DPA, was chosen. The histological and ultrastructural analysis in this study confirmed that cells continued to develop. However, the response of starch- and aleurone-like cells in cultured endosperm differed. The size and histological features of starch-like cells were similar for

Fig. 5 Confocal images of isolated endosperm of *Triticum aestivum* ‘Kobra’ at 34 days old (8 DPA + 26 DIV) cultured on Medium 2. Light (a) and fluorescence (b) images of explant. c Magnification of aleurone-like cells. d–g Visualization with the depth coding processed from ten slices in stack, and pseudo-color (scale in left corner), which represent the z-depth; (d – f) selected slices (f as the deepest one), and the image merged from all ten slices (g). Scale bar: 200 μ m (a, b), 10 μ m (c), 50 μ m (d–g)

all treatments, whereas the size and appearance of aleurone-like cells depended on culture conditions, that is, the type of medium. Aleurone-like cells were greatly enlarged, especially in the presence of ABA. These cells did not show the isodiametric shape that is characteristic of aleurone layer cells, but a round or irregular shape, probably because of a lack of maternal tissue. The pericarp is responsible for the protection and promotion of embryo and endosperm development (Radchuk and Borisjuk 2014). The final size of the seed is determined by collaboration between the seed coat and the endosperm. It is obvious that the absence of an outer seed envelope impacts the shape of the developed endosperm. Aleurone-like cells are rich with protein and lipid bodies. Large, irregularly shaped cells with many lipid

droplets were observed in cultured endosperm in corn (Blehov et al. 2018). However, in case of corn, the lipids were secreted to the subcellular regions on the surface of explants. Fan et al. (2013) observed that under stress (waterlogging treatment) the endosperm tissue was deformed and the cell shape was irregular during *in planta* development. In aleurone-like cells in the presence of ABA, the circles of endoplasmic reticulum and myelin-like bodies were observed. These organelles separate and isolate cytoplasm zones and are considered to be the formation of a lytic compartment with a different contents (Belyavskaya 2004). On the other hand, a concentric arrangement of endoplasmic reticulum is considered to be a more stable configuration characteristic of conditions of low cell energy (Smith et al. 2002).

Fig. 6 DNA laddering detection in endosperm of *Triticum aestivum* 'Kobra'. Isolated endosperm from caryopsis (a) 1–6 from left to right: at 8, 12, 18, 26, 30, and 34 DPA. Explants cultured on Medium 2 (b) 1–7 from left to right: at 8 DPA + 4DIV, 8 DPA + 10 DIV active, 8

DPA + 10 DIV non-active, 8 DPA + 18 DIV active, 8 DPA + 18 DIV non-active, 8 DPA + 26 DIV active, and 8 DPA + 26 DIV non-active. The progression of PCD is noted for lanes 4–6 (a), 1–3, 5, and 7 (b)

Additionally, myelin-like bodies were detected in plant cells, which induced PCD after an attack by parasites (Matuszkie-wicz et al. 2018). The shape and ultrastructure of mitochondria changed during culturing. Globular mitochondria with visible cristae were noted after 10 DIV, but after 26 DIV elongated mitochondria without cristae were observed. The elongation of mitochondria and the degradation of cristae is connected with the aging of the cell and PCD (Fahn and Shimony 1998; Gladish et al. 2006; Adamakis and Eleftheriou 2019). Specific features related to adaptive and protective functions also against pathogens were shown by the aleurone layer under *in planta* conditions (Zheng and Wang 2015, and references therein). This may explain the variation and growth of aleurone-like cells under *in vitro* conditions which was observed in this study.

Nuclear DNA specific fragmentation, typical of PCD, produces fragments with 180–200 bp or multimers, which show DNA laddering on gel electrophoresis (Fan et al. 2013). The starchy cells of endosperm start PCD from 16 DPA, according to Young et al. (1999). In this study, laddering in the samples from caryopsis was observed at about 18 DPA and progressed until 34 DPA, when the whole sample was fragmented. In the samples from the cultured endosperms, the DNA fragmentation started earlier; however, the intensity depended on the type of medium. This study indicated that tissue culture conditions could promote PCD progression in endosperm cells, especially in the non-active parts of explants. However, some of the nuclear DNA, especially from the active part of the explants, remained unfragmented and the sample migrated slowly through the agarose gel. On the other

hand, a blurred trail on the electrophoresis gel indicated rather random fragmentation of DNA and the degradation typical of necrosis (Mittler et al. 1997; Potten and Wilson 2004). It was reported in research on *Z. mays* mutants that ABA stopped the precocious PCD (Dominguez and Cejudo 2014, and references therein).

By interacting with other signaling molecules, including phytohormones, sugars are considered to be molecules that can regulate each stage of the plant life cycle (McNeill et al. 2017). The level of sugars in plant cells, as well as their distribution, use, and storage, are carefully controlled and conditioned by the physiological activity of the cells, the type of tissue, the environmental conditions, and the stage of plant development during ontogenesis (Lastdrager et al. 2014). The content of carbohydrates detected in caryopsis were in agreement with Kang et al. (2013), who reported a rapid accumulation of starch until 15 DPA which then gradually declined during grain maturation. The starch deposition in 34-day-old cultured endosperm was only at a similar level as 34 DPA caryopsis in the presence of ABA. For other treatments, the starch accumulation seemed to be lower compared with grains. However, the addition of exogenous ABA improved the accumulation stability of amyloplasts. Zhao et al. (2007) showed that ABA regulates the activity of enzymes involved in the regulation and accumulation of starch synthesis in grains. The accumulation of starch in cereal grains is both genetically and environmentally controlled (Peña et al. 2002), and among numerous factors the function of ABA in controlling seed maturation and proper storage material deposition is well known (Kermode 2005; Dominguez and Cejudo 2014; Liu et al. 2020; Ali et al.

Fig. 7 Carbohydrate (a) and starch (b) content in isolated endosperm of *Triticum aestivum* ‘Kobra’. Samples were taken from caryopsis at 8, 12, 18, 24, and 34 DPA, and from cultured tissues at 8 DPA + 4 DIV, 8 DPA + 10 DIV, 8 DPA + 16 DIV, and 8 DPA + 26 DIV on three types of media. Values are mean $\pm$ SE. Mean values followed by the same letter are not significantly different ($p=0.05$)

2021; Sano and Marion-Poll 2021). A recent experiment on transgenic barley (Staroske et al. 2016) with increased accumulation of ABA revealed great metabolic plasticity of grains, which matured earlier but with a weight and composition similar to the wildtype. Yang et al. (2014) showed that the external application of 10 mg L^{-1} ABA changed the endogenous hormone content in developing wheat grains. The addition of ABA resulted in significant increases of endogenous indole-3-acetic acid and ABA content from 7 to 21 DPA. IAA mediated grain filling and was involved in promoting starch synthesis. Compared to the other media used (Media 1 and 3), the ABA in Medium 2 increased the starch accumulation rate in the cultured wheat's immature endosperm. Based on our results, we may summarize that exogenous ABA also maintained a constant level of carbohydrate accumulation in the wheat's developing endosperm. Moreover, the enlargement of aleurone-like cells may suggest that the size of endosperm cells can be controlled and

enhanced by the exogenous application of plant growth regulators, like ABA. This phenomenon might be used for the modification of endosperm and further, for the crops improvement.

Conclusions

The medium with ABA was more appropriate for starch accumulation than the media without this plant growth regulator. Additionally, ABA supplementation improved the condition and viability of the isolated tissue. The ultrastructural observation revealed features that are characteristic of aleurone-like cells, but their size was significantly larger than aleurone cells developing in caryopsis. To our knowledge, this is the first observation of the development of isolated immature wheat endosperm over several weeks under tissue culture conditions, corresponding to maturation

in *in planta* caryopsis. Our study concluded that under tissue culture conditions it is possible to maintain the development of isolated immature endosperm and to modify the development through media composition. Isolated immature endosperm of cereals could be a model for *in vitro* studies on the response of this specific storage tissue. The ability to culture *in vitro* developing endosperms provides biochemical and molecular systems to analyze the modification of cereal endosperm cell fate.

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Author contribution MPK devised the research. IM, MPK, and ICh performed the tissue cultures. MKK and ICh performed the histological and TEM studies. MPK and ICh performed confocal observations. DK and ICh carried out the molecular analysis and evaluated the results. IM, AP, and ICh carried out the biochemical analysis and evaluated the results. MPK and ICh contributed to the writing of the manuscript. All of the authors read and approved the final manuscript.

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Data availability The data generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

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References

- Adamakis IDS, Eleftheriou EP (2019) Structural evidence of programmed cell death induction by tungsten in root tip cells of *Pisum sativum*. *Plants* 8:62. <https://doi.org/10.3390/plants8030062>
- Agama-Acevedo E, Flores-Silva PC, Bello-Perez LA (2019) Cereal starch production for food application. In: Silva Clerici MTP, Schmiege M (eds) *Starches for food application*. Chemical, technological and health properties. Elsevier Inc., pp 71–102. <https://doi.org/10.1016/B978-0-12-809440-2.00003-4>
- Ali F, Qammar G, Li F, Wang Z (2021) Updated role of ABA in seed maturation, dormancy, and germination. *J Adv Res*. <https://doi.org/10.1016/j.jare.2021.03.011>
- Barron C, Surget A, Rouau X (2007) Relative amounts of tissues in mature wheat (*Triticum aestivum* L.) grain and their carbohydrate and phenolic acid composition. *J Cereal Sci* 45:88–96. <https://doi.org/10.1016/j.jcs.2006.07.004>
- Belyavskaya NA (2004) Biological effects due to weak magnetic field on plants. *Adv Space Res* 34:1566–1574. <https://doi.org/10.1016/j.asr.2004.01.021>
- Bleňová A, Škorňková M, Šamajová O, Kaštier P, Matušiková I (2018) Maize miniendosperm proliferation *in vitro* is characterized by tracheary element formation. *Plant Cell Tiss Org Cult* 135:455–462. <https://doi.org/10.1007/s11240-018-1478-6>
- Dominguez F, Cejudo FJ (2014) Programmed cell death (PCD): an essential process of cereal seed development and germination. *Front Plant Sci* 5:366. <https://doi.org/10.3389/fpls.2014.00366>
- Evers AD (1970) Development of the endosperm of wheat. *Ann Bot* 34:547–555. <https://doi.org/10.1093/oxfordjournals.aob.a084390>
- Fábrián A, Jäger K, Rakszegi M, Barnabás B (2011) Embryo and endosperm development in wheat (*Triticum aestivum* L.) kernels subjected to drought stress. *Plant Cell Rep* 30:551–563. <https://doi.org/10.1007/s00299-010-0966-x>
- Fahn A, Shimony C (1998) Ultrastructure and secretion of secretory cells of two species of *Fagonia* L. (Zygophyllaceae). *Ann Bot* 81:557–565. <https://doi.org/10.1006/anbo.1998.0591>
- Fan HY, Zhou ZQ, Yang CN, Jiang Z, Li JT, Cheng XX, Guo YJ (2013) Effects of waterlogging on amyloplasts and programmed cell death in endosperm cells of *Triticum aestivum* L. *Protoplasma* 250:1091–1103. <https://doi.org/10.1007/s00709-013-0485-z>
- Feher A (2019) Callus, dedifferentiation, totipotency, somatic embryogenesis: what these terms mean in the era of molecular plant biology? *Front Plant Sci* 10:536. <https://doi.org/10.3389/fpls.2019.00536>
- Gao F, Ayele BT (2014) Functional genomics of seed dormancy in wheat: advances and prospects. *Front Plant Sci* 5:458. <https://doi.org/10.3389/fpls.2014.00458>
- Geigenberger P (2011) Regulation of starch biosynthesis in response to a fluctuating environment. *Plant Physiol* 155:1566–1577. <https://doi.org/10.1104/pp.110.170399>
- Gladish DK, Xu J, Niki T (2006) Apoptosis-like programmed cell death occurs in procambium and ground meristem of pea (*Pisum sativum*) root tips exposed to sudden flooding. *Ann Bot* 97:859–902. <https://doi.org/10.1093/aob/mcl040>
- Gruis DF, Guo H, Selinger D, Tian Q, Olsen OA (2006) Surface position, not signaling from surrounding maternal tissues, specifies aleurone epidermal cell fate in maize. *Plant Physiol* 141:898–909. <https://doi.org/10.1104/pp.106.080945>
- Janeczko A, Biesaga-Kościełniak J, Oklestkova J, Filek M, Dziurka M, Szarek-Lukaszewska G, Kościełniak J (2010) Role of 24-epibrassinolide in wheat production: physiological effects and uptake. *J Agron Crop Sci* 196:311–321. <https://doi.org/10.1111/j.1439-037X.2009.00413.x>
- Kalinga DN, Bertoft E, Tetlow I, Liu Q, Yada RY, Seetharaman K (2014) Evolution of amylopectin structure in developing wheat endosperm starch. *Carbohydr Polym* 112:316–324. <https://doi.org/10.1016/j.carbpol.2014.05.008>
- Kang GZ, Xu W, Liu GQ, Peng XQ, Guo TC (2013) Comprehensive analysis of the transcription of starch synthesis genes and the transcription factor RSR1 in wheat (*Triticum aestivum*) endosperm. *Genome* 56:115–122. <https://doi.org/10.1139/gen-2012-0146>

- Kermode AR (2005) Role of abscisic acid in seed dormancy. *J Plant Growth Regul* 24:319–344. <https://doi.org/10.1007/s00344-005-0110-2>
- King RW (1976) Absciscic acid in developing wheat grains and its relationship to grain growth and maturation. *Planta* 132:43–51. <https://doi.org/10.1007/BF00390329>
- Kurczel M, Dłubacz A, Weselucha-Birczyńska A, Filek M, Łabanowska M (2015) Stable radicals and biochemical compounds in embryos and endosperm of wheat grains differentiating sensitive and tolerant genotypes—EPR and Raman studies. *J Plant Physiol* 183:95–107. <https://doi.org/10.1016/j.jplph.2015.05.018>
- Lastdrager J, Hanson J, Smeekens S (2014) Sugar signals and the control of plant growth and development. *J Exp Bot* 65:799–807. <https://doi.org/10.1093/jxb/ert474>
- Liu F, Zhang H, Ding L, Soppe WJJ, Xiang Y (2020) Reversal of RDO5 1, a homolog of rice Seed dormancy4, interacts with bHLH57 and controls ABA biosynthesis and seed dormancy in Arabidopsis. *Plant Cell* 32:1933–1948. <https://doi.org/10.1105/tpc.20.00026>
- Mares DJ, Norstog K, Stone BA (1975) Early stages in the development of wheat endosperm. I. The change from free nuclear to cellular endosperm. *Aust J Bot* 23:311–326
- Martin C, Smith AM (1995) Starch biosynthesis. *Plant Cell* 7:971–985. <https://doi.org/10.1105/tpc.7.7.971>
- Matuszkiewicz M, Sobczak M, Cabrera J, Escobar C, Karpiński S, Filipecki M (2018) The role of programmed cell death regulator LSD1 in nematode-induced syncytium formation. *Front Plant Sci* 9:314. <https://doi.org/10.3389/fpls.2018.00314>
- McCready RM, Guggolz J, Silveira V, Owens HS (1950) Determination of starch and amylase in vegetables. *Anal Chem* 22:1156–1158. <https://doi.org/10.1021/ac60045a016>
- McNeill GJ, Mehrpouyan S, Minow MAA, Patterson JA, Tetlow IJ, Emes MJ (2017) Starch as a source, starch as a sink: the bifunctional role of starch in carbon allocation. *J Exp Bot* 68:4433–4453. <https://doi.org/10.1093/jxb/erx291>
- Mittler R, Del Pozo O, Meisel L, Lam E (1997) Pathogen-induced programmed cell death in plants, a possible defense mechanism. *Dev Genet* 21:279–289
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol Plant* 15:437–497
- Olsen O-A (2020) The modular control of cereal endosperm development. *Trends Plant Sci* 25:3. <https://doi.org/10.1016/j.tplants.2019.12.003>
- Olsen O-A (2021) A first step towards *in vitro* cultured cereals. *J Cereal Sci*. <https://doi.org/10.1016/j.jcs.2021.103182>
- Peña RJ, Trethowan R, Pfeiffer WH, van Ginkel M (2002) Quality (end-use) improvement in wheat. Compositional, genetic, and environmental factors. *J Crop Prod* 5:1–37. https://doi.org/10.1300/J144v05n01_02
- Potten C, Wilson J (2004) Apoptosis—the life and death of cells. Cambridge University Press, Cambridge, pp 17–22, 37–57
- Popielarska-Konieczna M, Kozieradzka-Kiszkurno M, Tuleja M, Ślesak H, Kapusta P, Marcińska I, Bohdanowicz J (2013) Genotype-dependent efficiency of endosperm development in culture of selected cereals: histological and ultrastructural studies. *Protoplasma* 250:361–369. <https://doi.org/10.1007/s00709-012-0419-1>
- Radchuk V, Borisjuk L (2014) Physical, metabolic and developmental functions of the seed coat. *Front Plant Sci* 5:510. <https://doi.org/10.3389/fpls.2014.00510>
- Reyes FC, Sun B, Guo H, Gruis DF, Otegui MS (2010) *Agrobacterium tumefaciens*-mediated transformation of maize endosperm as a tool to study endosperm cell biology. *Plant Physiol* 153:624–631. <https://doi.org/10.1104/pp.110.154930>
- Reyes FC, Chung T, Holding D, Jung R, Vierstra R, Otegui MS (2011) Delivery of prolamins to the protein storage vacuole in maize aleurone cells. *Plant Cell* 23:769–784. <https://doi.org/10.1105/tpc.110.082156>
- Sabelli PA, Larkins BA (2009) The development of endosperm in grasses. *Plant Physiol* 149:14–26. <https://doi.org/10.1104/pp.108.129437>
- Sano N, Marion-Poll A (2021) ABA metabolism and homeostasis in seed dormancy and germination. *Int J Mol Sci* 22:5069. <https://doi.org/10.3390/ijms22105069>
- Skórka M, Sieprawska A, Bednarska-Kozakiewicz E, Gawrońska K, Kornaś A, Telk A (2020) Genotype-dependent differences between cereals in response to manganese excess in the environment. *Agronomy* 10:510. <https://doi.org/10.3390/agronomy10040510>
- Smith MB, Palmer RG, Horner HT (2002) Microscopy of cytoplasmic male-sterile soybean from an interspecific cross between *Glycine max* and *G. soja* (Leguminosae). *Am J Bot* 89:417–426. <https://doi.org/10.3732/ajb.89.3.417>
- Staroske N, Conrad U, Kumlehn J, Hensel G, Radchuk R, Erban A, Kopka J, Weschke W, Weber H (2016) Increasing abscisic acid levels by immunomodulation in barley grains induces precocious maturation without changing grain composition. *J Exp Bot* 67:2675–2687. <https://doi.org/10.1093/jxb/erw102>
- Wei C, Lan SY, Xu ZX (2002) Ultrastructural features of nucleus degradation during programmed cell death of starchy endosperm cells in rice. *Acta Bot Sin* 44:1396–1402
- Yang D, Luo Y, Ni Y, Yin Y, Yang W, Peng D, Cui Z, Wang Z (2014) Effects of exogenous ABA application on post-anthesis dry matter redistribution and grain starch accumulation of winter wheat with different staygreen characteristics. *Crop J* 2:144–152. <https://doi.org/10.1016/j.cj.2014.02.004>
- Young TE, Gallie DR (2000) Programmed cell death during endosperm development. *Plant Mol Biol* 44:283–301. <https://doi.org/10.1023/A:1026588408152>
- Young TE, Gallie DR (1999) Analysis of programmed cell death in wheat endosperm reveals differences in endosperm development between cereals. *Plant Mol Biol* 39:915–926. <https://doi.org/10.1023/A:1006134027834>
- Zhao BH, Kai LIU, Zhang HX, Zhu QS, Yang JC (2007) Causes of poor grain plumpness of two-line hybrids and their relationships to the contents of hormones in the rice grain. *Agric Sci China* 6:930–940. [https://doi.org/10.1016/S1671-2927\(07\)60131-X](https://doi.org/10.1016/S1671-2927(07)60131-X)
- Zheng Y, Wang Z (2015) The cereal starch endosperm development and its relationship with other endosperm tissues and embryo. *Protoplasma* 252:33–40. <https://doi.org/10.1007/s00709-014-0687-z>

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