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Development of the endosperm of *Sorghum bicolor* during the endoreduplication-associated growth phase

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Abstract

Spatial occurrence of endoreduplication, a variant of the cell cycle resulting in endopolyploidy, was investigated in the developing sorghum caryopsis between 5 and 16 DAP (days after pollination). This was a period of intense mitotic and endoreduplication-associated endosperm growth. Endopolyploidy was quantitatively analyzed on median caryopsis sections using image densitometry that provided in situ cytometrical data. In the endosperm, the first endopolyploid nuclei with a nuclear DNA content of 12C (where 1C represents the nuclear DNA content of a non-replicated haploid genome) were detected at 5 DAP. In subsequent days progressively higher levels of endopolyploidy occurred, and nuclei with the highest amount of DNA (96C) were first observed at 10 DAP. The highly endopolyploid nuclei were located only in the central region of the endosperm and their occurrence coincided with the onset of starch deposition in the endosperm. Cells with non-endopolyploid nuclei (3C and 6C) were found exclusively in the peripheral layers of the endosperm. No starch was observed in the basal part of the endosperm where the highest level of endopolyploidy was 24C. The volume of endosperm nucleai and cells showed a positive correlation with the level of endopolyploidy. Endoreduplication was also prominent in the pericarp, where the highest level of endopolyploidy was 16C.

Keywords: Endoreduplication; Endosperm; Grain; Pericarp; Sorghum bicolor

1. Introduction

Sorghum (*Sorghum bicolor* (L.) Moench), is a major cereal crop plant and is evolutionarily closely related to maize (Swigoňová et al., 2004). As in all cereal species, the caryopsis is composed of three major parts: the pericarp (seed coat), the endosperm and the embryo.

The pericarp is derived from the ovary wall and adheres strongly to the seed coat of the ovule (Evers and Millar, 2002). In sorghum and rice, the pericarp is the major site of starch deposition during first days after pollination (DAP) and before starch deposition in the endosperm (Earp et al., 2004; Sato, 1984). By maturity all starch disappears and the cells in which it was present are squashed or broken down. Exceptions to this are some varieties of sorghum where cells and starch granules persist until maturity (Evers et al., 1999).

In flowering plants, the result of double fertilization is a diploid zygote and a triploid primary endosperm cell. The latter subsequently develops into a storage tissue, the endosperm, which is structurally adapted to ensure efficient translocation of nutrients from the sporophyte to the developing embryo. At maturity, the cereal endosperm is composed of five specialized cell types: the central starchy endosperm, the subaleurone layer, the aleurone layer, the basal endosperm transfer layer and the embryo-surrounding region (Olsen et al., 1999). Endosperm development is determinate and includes syncytial and cellular phases (Berger, 2003; Costa et al., 2004; Olsen et al., 1999). In sorghum in the syncytial phase, as many as 28 nuclei were observed 8 h after pollination (Paulson, 1969). About 48 h after pollination, the syncytial endosperm is cellularised, and this is followed by a period of intense mitotic activity (Berger, 2003; Costa et al., 2004; Olsen et al., 1999; Paulson, 1969). The outer cells of developing cereal endosperm give rise to the aleurone layer (Olsen et al., 1999). In sorghum, the aleurone layer consists of roughly rectangular cells that are much smaller than in the rest of the

Abbreviations: DAP, days after pollination; IOD, integrated optical density.

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endosperm. Small, elongated cells also occur at the base of the sorghum endosperm (Artschwager and McGuire, 1949).

In maize the endosperm cells undergo endoreduplication, resulting in highly endopolyploid nuclei in the starchy endosperm cells (Kowles and Phillips, 1985; Kowles et al., 1990; 1997; Larkins et al., 2001; Vilhar et al., 2002) and aleurone cells in barley (Keown et al., 1977). Endoreduplication is a variant of the cell cycle, in which chromosomal DNA is replicated without intervening mitotic division, leading to endopolyploid nuclei with polytene chromosomes (Joubès and Chevalier, 2000). The resulting larger, endopolyploid nuclei are often, but not always, associated with an increase in cell size (Leiva-Neto et al., 2004; Sugimoto-Shirasu and Roberts, 2003; Vilhar et al., 2002). Nevertheless, in maize, cells with highly endopolyploid nuclei occupy a major part of the volume of the starchy endosperm (Vilhar et al., 2002). Central endosperm cells in sorghum caryopsis have very large nuclei with diameters often measuring 15 μm (Artschwager and McGuire, 1949).

Understanding the development of cereal grains and particularly, the distribution of functionally- and nutritionally-important components, is important if the utilization of the grain is to be optimized. In this study we demonstrate a technique which allows analyses to be performed on tissue sections, combining information on spatial distribution of various cytological parameters at the light microscope level. Using image cytometry we demonstrate that caryopsis development in sorghum is similar to that of maize and other cereals. We show the spatial and temporal occurrence of endoreduplication in different parts of sorghum caryopsis between 5 and 16 DAP, the period of intense mitotic and endoreduplication-associated growth of the endosperm. Additionally, we demonstrate a relationship between starch accumulation and endoreduplication.

2. Experimental

2.1. Preparation of tissue sections

Sorghum (*Sorghum bicolor* (L.) Moench) samples were prepared using the male-sterile line A3T×7000 and pollination with B3T×7000. Flowering proceeds basipetally in sorghum and about four days are required for style emergence to the base of the panicle (Pring and Tang, 2004). The line A3T×7000 was pollinated when flowering was complete at the base of the panicle. The pollination results in 100% fertilization at the fourth day and thus is in synchrony when samples were collected over time. Developing caryopses were collected at different days after pollination (DAP) and immediately fixed in cold FAA fixative (3.7% formaldehyde, 5% acetic acid, 50% ethanol) for 24 h, followed by dehydration in series of ethanol and tertiary butyl alcohol and embedding in Paraplast Plus (Sherwood Medical Co., St Louis, MO, USA). Paraffin embedded caryopses were sectioned to 12–20 μm thick sections (depending on the development stage) on a rotary microtome (Microm 325, Carl Zeiss, Germany).

2.2. Staining for starch with I_2/KI

Tissue sections were dewaxed in xylene and rehydrated in an ethanol series to water, stained in aqueous solution of 2% iodine and 3% potassium iodide (I_2/KI) for 1 min, washed in water, dehydrated quickly through the ethanol series to xylene and mounted with DPX (Fisons Scientific Equipment, Loughborough, England).

2.3. Image analysis

The image analysis system consisted of the Axioskop 2 MOT microscope (Carl Zeiss, Jena, Germany) with the KS400 software package (Carl Zeiss Vision, Hallbergmoos, Germany) and either a video CCD camera (DXC-950P, Sony, Tokyo, Japan) for DNA measurements or a digital color camera (AxioCam MRc, Carl Zeiss Vision, Hallbergmoos, Germany) for measurements of cell size and pericarp thickness.

2.4. Measurement of pericarp thickness and number of cell layers

Pericarp thickness and numbers of cell layers were measured on median longitudinal tissue sections of sorghum caryopses imaged using UV excitation. Both parameters were measured on the dorsal side of the caryopsis, away from the embryo, in three different caryopses for each developmental stage. Pericarp thickness was measured perpendicularly to the pericarp surface at its thickest part and the number of cell layers was counted in the same region.

2.5. Measurement of nuclear DNA and nuclear volume

The nuclear DNA was measured by image densitometry using the interphase-peak method (Vilhar et al., 2001; Vilhar and Dermastia, 2002) adapted for use with tissue sections (Kladnik et al., 2004; Vilhar et al., 2002). Longitudinal sections of caryopses were dewaxed in xylene, rehydrated through an ethanol series to water, hydrolysed in 5 M HCl for 75 min at 20 °C, stained with Schiff's reagent for 120 min at 20 °C, washed for 45 min in six changes of 0.5% potassium metabisulphite in 50 mM HCl, dehydrated and mounted in DPX (Fisons Scientific Equipment, Loughborough, England). Integrated optical density (IOD) and coordinates of the nuclei were measured on a series of grayscale images of caryopses recorded with a 40× objective. IOD is linearly related to the amount of DNA and was used to estimate the relative amount of DNA in individual nuclei. The nuclear amount of DNA was expressed in C-value units, where 1C represented the nuclear DNA content of a non-replicated haploid genome. The volume of a nucleus was estimated as the volume of a sphere based on the area of projection of a nucleus in the *z*-axis of the tissue section.