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Comparative studies of live tapetum cells in sterile garlic (*Allium sativum*) and fertile leek (*Allium ampeloprasum*) using the fluorescence lifetime imaging analytical method

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ABSTRACT

Commercially cultivated *Allium sativum* has lost the ability to reproduce sexually due to various developmental abnormalities mainly in the male line associated with a phenomenon called male sterility. Numerous studies focused on discovering the cause of garlic male sterility at the tissue and cellular level have not yet fully resolved this issue. Here, we have focused our attention on the tapetum, i.e. a nutritive tissue regarded in plants as a critical factor for formation of viable pollen. We have applied a novel method, Fluorescence Lifetime Imaging (FLIM), which represents one of the most sensitive biophysical tools for assessment of changes in fluorophore lifetime, depending on its metabolic state in a live cell. Contrary to traditional methods based on cell fixation, FLIM analysis provides live tapetal cell imaging, which facilitates monitoring metabolic changes in the cell. We have shown qualitative metabolic discrepancies in live cells of the tapetum in sterile garlic and fertile leek and proposed a correlative metabolic model of microspore and tapetum development in garlic cultivars, indicating that the disturbances in the temporal coordination of programmed cell death within the anther might be regarded as the main cause of male sterility in the analysed *A. sativum* cultivars.

1. Introduction

In spermatophytes, formation of male gametophytes (pollen grains) takes place via microsporogenesis followed by gametogenesis in the anther loculus. Meiotically dividing sporogenous tissue cells (pollen mother cells – PMC) and mitotically dividing pollen grains in the loculus are surrounded by a nutritive tissue, i.e. the tapetum, which undergoes morphological, cytological, and structural changes during microsporogenesis and gametogenesis. These changes involve degeneration of the tapetum, which results from programmed cell death, called developmental PCD – dPCD (Beers, 1997; Olvera-Carrillo et al., 2015). The dPCD of the tapetum is strictly programmed in the ontogenetic plan of anther development, and the gradual degeneration of the cells of this tissue is aimed at providing compounds that are indispensable for normal development, formation, and function of the male gametophyte. The tapetum exhibits specific cytological characteristics, which distinguish it from other somatic cells. These traits include a larger nucleus and nucleolus, binuclearity and polyploidy of cells, the presence of numerous mitochondria and plastids in the cytoplasm, a large number of ribosomes, and a highly developed ER network. The traits indicate a high level of tapetal cell metabolism, and the architecture of all cellular structures suggests high activity associated with the transport of metabolic products from these organelles to the anther loculus (Steer, 1977). Besides their metabolic products, tapetal cells deliver nutrients from anther wall cells, mainly from the intermediate layer, to the anther loculus (Clément and Pacini, 2001) and receive substances from vascular bundles in the connective tissue to re-elaborate and release them by exocytosis or membrane permeability into locular fluid (Pacini, 2010). Such an efficient linkage system within the tapetum determines coordination of processes occurring in the anther, and changes in tapetal cells closely correlate with the phases of male gametophyte development and occur at a species-specific time point (Parkinson and Pacini, 1995). Proper functioning of the tapetum throughout microsporogenesis and gametogenesis is vital for formation of viable pollen capable of pollination. Disturbances in the development and functioning of this tissue during the formation and maturation of pollen grains have been reported as the main cause of male sterility in many plant species (Mariani et al., 1990; Engelke et al., 2002; Chauhan, 2005; Ma, 2005; Xie et al., 2005). This is supported by a number of male-sterile mutants having defects in the tapetum degradation and disintegration processes (Kawanabe et al., 2006; Li et al., 2006; Yang et al., 2007; Parish and Li, 2010). It can therefore be concluded that the process of formation of the male gametophyte, which is important for plant development, is inextricably linked with the tapetum.

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Allium sativum L. (garlic), which is currently widely cultivated, is a vegetatively propagated species, because garlic has lost the generative reproduction capability due to a number of developmental abnormalities occurring in both the male and female lines (Shemesh-Mayer et al., 2013). Garlic sterility is thought to have many determinants (Novak, 1972; Konvicka, 1973; Etoh, 1986; Tchórzewska et al., 2015; Tchórzewska et al., 2017) that are interrelated, but the cultivars persist due to intense vegetative propagation on all continents for hundreds of years. For instance, the complexity of the male sterility phenomenon is demonstrated by analyses of cytoplasmic male sterility (CMS) in species from the genus *Allium*. It has been proposed that oxidative stress might induce male sterility in garlic, showing that increased production of reactive oxygen species in deregulated mitochondria stimulates aberrant PCD in the tapetum (Aberrant PCD Model), resulting in pollen abortion (Shemesh-Mayer et al., 2015b). However, in another species of the genus *Allium* – onion (*A. cepa* L.), a different CMS mechanism results from an erroneous flow of information between cytoplasmic genes and nuclear genes (Yuan et al., 2018).

In studies focused on identification of the cause of garlic male sterility, tissues and cells were visualised mainly with traditional light, fluorescence, or electron microscopy, which provided only general insight into cytological and anatomical changes taking place within the anther loculus. The present study is a continuation of the research on sterility in representatives of the genus *Allium*, with the tapetum and its metabolic changes as the main research object. The investigations were conducted with the use of spectroscopic analyses and an analytical method that is novel in such research, i.e. Fluorescence Lifetime Imaging (FLIM), which is a sensitive tool for assessment of changes in the fluorophore lifetime depending on its metabolic state in the live cell. FLIM is gaining momentum as a very effective approach for analysis of metabolic changes in live cells of plant and animal tissues. Specifically, FLIM provides a useful approach for analysis of animal tissues, facilitating discrimination between e.g. healthy and carious dental tissue (Lin et al., 2010) or atherosclerotic plaques (Phipps et al., 2011) and identification of sub-structures within skin cells (Seidenari et al., 2012). The FLIM analysis, especially using chlorophyll fluorescence as the main probe, had already been successfully used to study lifetime distribution in the chloroplasts of moss leaves (Eckert et al., 2006), dehydration effects in *Arabidopsis thaliana* leaves (Holub et al., 2000), the states of photosystem reaction centres in *A. thaliana* and *Alocasia wentii* leaves (Broess et al., 2009), and metabolic fluctuations in avocado leaves upon light exposure (Matsubara et al., 2011). Importantly, it was used to distinguish between bundle sheath and mesophyll cells in maize leaves, which have differentially distributed photosystems 1 and 2 (Holub et al., 2000). FLIM was also applied to study algae (Iwai et al., 2010; Krumova et al., 2010), aggregates (Barzda et al., 2001; Janik et al., 2013), and crystals of photosynthetic complexes (van Oort et al., 2008). That chlorophyll fluorescence is intimately related to the physiology of the plant photosynthetic apparatus opens an avenue to the FLIM technique to be applied to monitor metabolic processes in plants (Janik et al., 2017). Here, we focus on plant cells, especially on the tapetum tissue during the microsporogenesis and gametogenesis process in male-sterile *A. sativum* and fertile *A. ampeloprasum* L., using the FLIM approach for live tapetal cell imaging. Combining our previous autofluorescence spectral imaging (ASI) (Tchórzewska et al., 2017) and the current FLIM analyses, we can propose a metabolic model of microspore and tapetum development in garlic species to discover the cause of male sterility in *A. sativum*.

2. Materials and methods

2.1. Plant material

The *A. sativum* (garlic, cv. Harnas and cv. Arkus) and *A. ampeloprasum* (leek) investigated in this study were cultivated in the Botanical Garden of Maria Curie-Skłodowska University (Lublin, 51° 16′ N, 22° 30′ E). Both cultivars of *A. sativum* were propagated from bulbs (cloves). *Allium ampeloprasum* was propagated from seeds and used as the control. Plants were grown on a universal, slightly acidic pH 5.5–6.5 soil, under a normal photoperiod depending on the season. No herbicide, fungicide, or any chemical inputs was incorporated, and manual weeding methods were employed in each case before and throughout the plant cultivation periods. Anthers from buds of spathe-covered inflorescences were sampled for analysis of microsporogenesis. Pollen grains from cv. Arkus and *A. ampeloprasum* were collected at the anthesis stage after spathe opening. The material was sampled randomly from 50 plants of each cultivar throughout the period of microsporogenesis taking place in the anthers (ca. 1 month). Each microsporogenesis stage was subjected to analysis in a minimum of 30 meiotically dividing cells.

2.2. Light microscopy (LM)

Squashed anthers of the analysed species were stained with acetocarmine (Gerlach, 1977). Observations were carried out with the use of a Nikon Eclipse *Ni* light microscope with Nomarsky contrast. Photographic documentation was made with a digital camera and NIS-Elements BP software.

2.3. Transmission electron microscopy (TEM)

For TEM, *A. sativum* and *A. ampeloprasum* anthers were fixed in 2.5% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 6.9) for 24 h at room temperature. Specimens were washed three times in phosphate buffer, post-fixed in 2% osmium tetroxide, dehydrated in a graded ethanol series and embedded in London Resin White Medium (Sigma). Ultrathin sections (60 nm) were stained with uranyl acetate (5 min) and lead citrate (10 min). The sections were observed under a JEM 100B TEM.

2.4. Scanning electron microscopy (SEM)

For SEM, freshly collected samples were fixed overnight in 2.5% glutaraldehyde in 0.2 M sodium phosphate buffer (pH 7.4), washed in distilled water, and dehydrated in increasing concentrations of ethanol (Hayat, 1981). The dehydrated specimens were then dried in a Critical Point Dryer (Denton Vacuum, Moorestown, NJ, USA) using liquid CO_2. The dried samples were mounted on aluminium stubs and sputter coated with gold (Hummer 6.2 Sputter Coater, Anatech USA, Union City, CA, USA). The samples were analysed under a scanning microscope (LEO1430VP) with an accelerating voltage of 15 kV equipped with a Bruker Ouantax 200XFlash EDX Spectrometer System attached to Zeiss EVO 50 Variable Pressure SEM at 15 kV, using INCA-Mapping software (Billerica, MA, USA).

2.5. Fluorescence lifetime imaging microscopy (FLIM)

Squashed anthers at different developmental stages of the two *A. sativum* cultivars and *A. ampeloprasum* were placed in distilled water and immediately analysed using fluorescence lifetime imaging microscopy. The FLIM analysis of plant cells was carried out according to a previously developed procedure (Janik et al., 2017). Lifetime images were acquired with a MicroTime 200 system from PicoQuant (Germany, GmbH). The instrument was based on an inverted microscope OLYMPUS IX71 coupled to the main optical unit in a confocal mode using a 150-μm pinhole. Scanning was performed with a 60 × 1.2 NA water-immersed objective and a 405-nm laser excitation wavelength to excite different kinds of fluorophores. The intensity and fluorescence lifetime data were gathered using an Avalanche Photodiode detector through ZT405RDC dichroic and 430 long wavelength pass filters (both from Analysentechnik). The time-correlated single photon counting mode was achieved and synchronised by HydraHarp 400. The photon arrival resolution was set at 16 ps. The images were collected with desirable frames in a 500 × 500 pixel format and 0.6 ms