



Review article

Live-cell imaging reveals the cellular dynamics in seed development

Ji Min Shin^{a,b}, Ling Yuan^{a,b}, Tomokazu Kawashima^{a,*}^a Department of Plant and Soil Sciences, University of Kentucky, KY, USA^b Kentucky Tobacco Research and Development Center, University of Kentucky, KY, USA

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ABSTRACT

Seed development in flowering plants is highly complex and governed by three genetically distinct tissues: the fertilization products, the diploid embryo and triploid endosperm, as well as the seed coat that has maternal origin. There are diverse cellular dynamics such as nuclear movement in gamete cells for fertilization, cell polarity establishment for embryo development, and multinuclear endosperm formation. These tissues also coordinate and synchronize the developmental timing for proper seed formation through cell-to-cell communications. Live-cell imaging using advanced microscopy techniques enables us to decipher the dynamics of these events. Especially, the establishment of a less-invasive semi-*in vivo* live-cell imaging approach has allowed us to perform time-lapse analyses for long period observation of *Arabidopsis thaliana* intact seed development dynamics. Here we highlight the recent trends of live-cell imaging for seed development and discuss where we are heading.

1. Introduction

Initiation of the next generation in sexual reproduction begins with fertilization; the female and male gametes fuse with each other through gamete membrane fusion (plasmogamy) and the gamete (pro)nuclei migrate toward each other, fuse (karyogamy), and blend their genomes to start embryogenesis. In flowering plants, unlike animals, two sperm cells are delivered via an elongated pollen tube into the embryo sac, including two female gametes: an egg cell and a central cell. Each sperm cell simultaneously fertilizes with the egg cell (haploid) and central cell (diploid in most flowering plants), producing a diploid zygote and a triploid primary endosperm, respectively, and this unique process is called double fertilization (Fig. 1a) (Doll and Ingram, 2022). The zygote first undergoes asymmetrical division into the two daughter cells with distinct cell fates in the process of embryo development. On the other hand, the primary endosperm (the fertilized central cell before division) proliferates as a coenocyte performing multiple mitotic nuclear divisions without cytokinesis until cellularization occurs (Fig. 1b-d) (Ali et al., 2022; Kimata et al., 2019, 2016; Brown et al., 2003; Boissard-Lorig et al., 2001). The endosperm plays important roles as a nourishing tissue for the developing embryo in eudicots such as *Arabidopsis thaliana* and as a persistent storage tissue in monocots such as rice and maize to support germination (Olsen, 2004; Olsen et al., 1999; Brown et al., 1996). The development of the embryo, endosperm, and seed coat enclosing them, is complex, yet well-orchestrated for successful seed

development (Doll and Ingram, 2022; Wang et al., 2022; Phillips and Evans, 2020). Understanding of morphological and physiological features has been advanced by the progress in microscopy techniques used with genetic and molecular tools, which can visualize subcellular components and manipulate their functions. However, unlike other developmental procedures in plants, the complex sexual reproduction processes including pollen tube guidance, double fertilization, and embryo/endosperm development, occur inside the maternal tissue (Shin et al., 2021; Sharma et al., 2021); demanding further development of methods to dissect out the mechanisms of individual processes (Kurihara et al., 2013).

Recently, semi-*in vivo* systems have been established, allowing us to investigate the fertilization events as well as embryo and endosperm development from intact ovules and seeds. This semi-*in vivo* system has provided a new avenue for discovering not only the snapshot of cellular processes, but also the temporal dynamics of sexual reproduction in plants. Here, we focus on updates in the knowledge of seed developmental dynamics accomplished specifically by time-lapse live-cell imaging and discuss the challenges and direction for future research. Dynamics in early events of sexual reproduction such as gametophyte development and pollen tube-female gametophyte interactions have been intensively reviewed (Hafidh and Honys, 2021; Hater et al., 2020; Sprunck, 2020). Different microscopy techniques for high-resolution live-cell imaging in plants are also well described elsewhere (Mizuta, 2021; Grossmann et al., 2018).

* Corresponding author.

E-mail address: tomo.k@uky.edu (T. Kawashima).<https://doi.org/10.1016/j.plantsci.2022.111485>

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2. Application of time-lapse live-cell imaging to the study of seed development

Fertilization in most flowering plants occurs deep-inside the maternal tissue where the female gametophyte is embedded under layers of ovule coat cells, making clear observation and investigation of the process extremely hard. To overcome this difficulty, the *in vitro*

fertilization system, where the isolated gametes are electrically fused, has been established in monocots and allowed the direct observation of gamete fusion events, accelerating numerous discoveries in the dynamics of plant fertilization such as gamete nuclear migration, karyogamy, and polyspermy block (Peng et al., 2017; Toda et al., 2016; Ohnishi et al., 2014; Kranz et al., 1995). With the optimization of the growth medium, the resultant zygote can develop into an embryo, which can

The cellular dynamics revealed by live-cell imaging techniques

Two synergid cells • Sperm cell release [1] • Cytoskeleton dynamics [2] • Persistent synergid cell – endosperm fusion [3,4,5,6]		
Egg cell • Polarity [7,8,9] • Organelle dynamics [7,9,10] • Cytoskeleton dynamics [8,11] • Gamete nuclear migration [11] • Karyogamy [12]	Zygote to 1-celled pro-embryo • Polarity & asymmetric division [7,8,9] • Organelle dynamics [7,9] • Cytoskeleton dynamics [8] • Zygotic genome activation [13,14]	Late globular embryo (64-cell) • Cell lineage from 1-celled pro-embryo [15] • Organelle dynamics from 1-celled pro-embryo [16] • Cytoskeleton dynamics from 1-celled pro-embryo [16,17]
Central cell • Organelle dynamics [18,19] • Cytoskeleton dynamics [11,18,19,20] • Gamete nuclear migration [11,18]	Primary to coenocytic endosperm • Vacuole dynamics [21] • Cytoskeleton dynamics [21] • Nuclear dynamics & chalazal cyst formation [21,22]	Cellularized endosperm • Cellularization [21]
Seed coat • Microtubule dynamics during coenocytic endosperm development [23]		
Cell-to-cell communications • Female & male gametes/gametophytes interaction [reviewed in 24,25,26] • Seed coat – early embryo communication mediated by auxin [27] • Coenocytic endosperm – seed coat communication mediated by auxin [28] • Coenocytic endosperm – early embryo communication mediated by peptide [29]		
Double fertilization in ovule	Developing seed during coenocytic phase	Developing seed after cellularization

Fig. 1. The cellular dynamics during double fertilization and post-fertilization in *Arabidopsis* revealed by both snapshot and time-lapse live-cell imaging. Depending on the developmental stages (color-boxed), the cellular dynamics found by live-cell imaging analyses (references: [1] Hamamura et al., 2011; [2] Susaki et al., 2022; [3] Maruyama et al., 2015; [4] Heydlauff et al., 2022; [5] Motomura et al., 2016; [6] Motomura et al., 2017; [7] Kimata et al., 2019; [8] Kimata et al., 2016; [9] Kimata et al., 2020; [10] Yu et al., 2021; [11] Kawashima et al., 2014; [12] Maruyama et al., 2020; [13] Kao and Nodine, 2019; [14] Zhao et al., 2019; [15] Gooch et al., 2015; [16] Liao and Weijers, 2018; [17] Vaddepalli et al., 2021; [18] Ali et al., 2020; [19] Kawashima and Berger, 2015; [20] Ali and Kawashima, 2021; [21] Ali et al., 2022; [22] Boissard-Lorig et al., 2001; [23] Creff et al., 2015; [24] Hafidh and Honys, 2021; [25] Hater et al., 2020; [26] Sprunck, 2020; [27] Robert et al., 2018; [28] Figueiredo et al., 2016; [29] Costa et al., 2014) are summarized in the table. The ovule/seed scheme representing each stage is shown at the bottom of the table. From left to right, the first scheme (a) depicts double fertilization in the ovule (orange circles indicate nuclei of the central cell, egg cell and synergid cell). The second (b) and third (c) schemes indicate the developing seed consisting of the 1-celled pro-embryo and coenocytic endosperm after the 4th division and the developing seed at the globular-shaped embryo (64-cell) stage with enlarged coenocytic endosperm, respectively. The different color nuclei in the coenocytic endosperm represent the compartmentalized endosperm subregions. The last scheme (d) shows the developing seed at the heart-shaped embryo stage with cellularized endosperm. MCE; micropylar endosperm, PEN; peripheral endosperm, CZE; chalazal endosperm generating chalazal cyst.

give rise to a fertile plant in several monocots such as rice, maize, and wheat (Toda et al., 2016; Maryenti et al., 2019; Uchiumi et al., 2007; Kranz and Lorz, 1993). This optimization further enabled the investigation of the effect of polyspermy on embryo development; multiple sperm cells were electro-fused into the egg cell and the subsequent embryo development was monitored in detail (Toda et al., 2016). However, the embryo from *in vitro* fertilization does not follow the same morphological pattern as *in planta* embryo development; the *in vitro*-fertilized embryo at a stage equivalent to the globular-shaped embryo stage *in planta* develops into a cell mass and the fertile plant is regenerated from the cell mass in rice (Uchiumi et al., 2007). The embryo, endosperm, and seed coat, grow together as a seed *in planta* and the cell-to-cell communication among these tissue types plays important roles in their coordinated development, especially during early embryogenesis (Doll and Ingram, 2022; Wang et al., 2022; Kurihara et al., 2013; Kawashima and Goldberg, 2010). Therefore, an *in planta* or semi-*in vivo* system where the dissected, yet-intact seed could be observed using time-lapse live-cell imaging was awaited to complement the *in vitro* system.

With significant advances in the signal detection sensitivity in confocal microscopy, time-lapse live-cell investigation of the fertilization event as well as the initial stage of embryo and endosperm development from the intact ovule/seed dissected from the plant became available in *Arabidopsis* (Fig. 1) and *Torenia fournieri*. *Torenia* produces a unique ovule from which the female gametophyte cells protrude, exposing the egg cell and synergid cells out from the ovule coat. This unique structure allows direct observation of gamete cell dynamics during fertilization, and the function of the synergid cell (Fig. 1a) to attract pollen tubes toward an unfertilized ovule (pollen tube guidance) as well as the chemical attractants secreted from the synergid cell for pollen tube guidance were first identified in *Torenia* (Kurihara et al., 2013; Higashiyama et al., 1998). In *Arabidopsis*, events immediately after plasmogamy such as karyogamy and the degeneration of the persistent synergid cell have been monitored directly from ovules dissected from pollinated pistils (Ali et al., 2020; Maruyama et al., 2020, 2015; Heydlauff et al., 2022). In the ovule, there are two synergid cells and one is immediately degenerated when it receives the pollen tube contents containing the two sperm cells, while the other remains intact (persistent synergid cell). The persistent synergid cell then becomes degenerated after successful fertilization when the primary endosperm initiates the first nuclear division. Live-cell imaging enabled to identify the timing of persistent synergid degeneration as well as the degeneration mechanism, which turned out to be the cell-cell fusion of the persistent synergid and developing endosperm (Maruyama et al., 2015; Heydlauff et al., 2022).

In contrast to post fertilization processes, events prior to or during fertilization are very short and it is extremely hard to capture the right moment using *in planta* fertilized ovules mentioned above. To overcome this problem, semi-*in planta* fertilization system has been coupled to control the timing of fertilization. In *in planta*, pollen tubes germinated from pollen grains attached on the stigma grow through the pistil and reach to individual ovules. In the semi-*in planta* fertilization system, the pollinated stigma is dissected and placed together with the dissected ovules on an agar medium plate. The pollen tubes coming out from the dissected stigma elongate on the agar plate and are guided to the ovules by sensing the pollen tube attractants secreted from the synergid cell (Higashiyama et al., 1998; Hamamura et al., 2011; Palanivelu and Preuss, 2006; Susaki et al., 2017). This system enables to monitor individual short fertilization processes with time-lapse live-cell imaging and the dynamics of sperm cell release from the pollen tube (Hamamura et al., 2011) and sperm nuclear migration (Kawashima et al., 2014) have been revealed (Fig. 1).

The duration of seed development (approximately 8 days until the embryo becomes mature in *Arabidopsis*) is much longer than the fertilization processes (total about 8–10 h from pollination in *Arabidopsis* (Maruyama et al., 2020; Faure et al., 2002); therefore, a further

optimization of the growth medium was necessary to perform time-lapse live-cell imaging of later seed development. Gooh et al. (2015) tested several plant growth media with different sugars and identified that Nitsch medium with 5% (w/v) trehalose significantly increases seed survival rate and the frequency of normal embryo development (Sauer and Friml, 2004; Gooh et al., 2015). This optimized growth liquid medium has enabled long-term culturing of the *Arabidopsis* intact seed, allowing live-cell imaging of not only the behavior of the zygote after fertilization (Kimata et al., 2019, 2016, 2020; Kao and Nodine, 2019; Zhao et al., 2019), but also embryo development (Gooh et al., 2015; Liao and Weijers, 2018; Vaddepalli et al., 2021) as well as unique early stage endosperm development (Fig. 1) (Ali et al., 2022; Boissnard-Lorig et al., 2001). The primary endosperm initiates the first nuclear division within 2 h after fertilization and continues mitotic divisions without cytokinesis, generating a multinuclear large endosperm cell (coenocytic endosperm; Fig. 1b, c). The nuclear division continues up to ten times, followed by cellularization (Ali et al., 2022; Boissnard-Lorig et al., 2001; Brown et al., 1999; Berger, 1999). The advancement of live-cell imaging finally made it possible to track timing intervals of the entire nuclear divisions as well as to dissect cellular dynamics of the endosperm subregions: micropylar, peripheral, and chalazal endosperm subregions (MCE, PEN and CZE; Fig. 1b, c) (Ali et al., 2022; Boissnard-Lorig et al., 2001). Each subregion shows a different timing of nuclear division and the PEN nuclei dynamically move toward the chalazal pole, generating a nuclei aggregate called the chalazal cyst with unknown functions (Fig. 1c) (Ali et al., 2022; Boissnard-Lorig et al., 2001). Gene expressions among these three subregions are also distinct (Picard et al., 2021; Belmonte et al., 2013), indicating that they have different roles in seed development. More advanced live-cell imaging with genetics and genomics will reveal this unique endosperm development and function.

To image deeply the embedded embryo for a longer time (over 3 days) or capture high resolution cytoskeleton and organelle dynamics during zygote asymmetric division in *Arabidopsis*, two-photon excitation microscopy (2PEM) has been used (Fig. 1) (Kimata et al., 2019, 2016; Gooh et al., 2015; Kimata et al., 2020). 2PEM enables the excitation of target fluorescent protein with longer, near-infrared wavelengths which can penetrate deeply embedded tissue with less light scattering (Mizuta, 2021). Still, late embryogenesis, from the late-heart embryo stage, has not been observed clearly because the embryo resides further inside the seed as the seed develops and expands (Ali et al., 2022; Gooh et al., 2015). During coenocytic endosperm development, the protoplasm is all pushed toward the plasma membrane by the large central vacuole (Ali et al., 2022; Brown et al., 1999). Thus, unlike the embryo, the endosperm protoplasm is a single layer just beneath a few layers of the seed coat, allowing relatively easy visualization of cellular dynamics compared to the embryo. However, strong autofluorescence especially from later stage seeds has been detected from the RFP channel (excitation; 559 nm, emission; 560–620 nm), and sensitivity to photobleaching and phototoxicity causing cell damage under long time-lapse live-cell imaging also becomes more apparent when tracking the endosperm dynamics from cytoskeleton defective transgenic line (Ali et al., 2022). Nevertheless, the optimized liquid growth medium for dissected *Arabidopsis* seed time-lapse live-cell imaging allows the developmental characteristics of *in planta* seed development to be recreated both structurally and temporally (Ali et al., 2022; Boissnard-Lorig et al., 2001; Gooh et al., 2015); providing a great platform to further investigate the complex seed development dynamics.

3. Concluding remarks

Histological and genetic research have elucidated that the complex interplay of these genetically distinct tissues is responsible for seed development including the control of seed size, early embryogenesis, endosperm cellularization, and seed dormancy, which are mediated by diverse factors such as translocation of sucrose, peptides, plant hormones, and mechanical stimulus (Doll and Ingram, 2022; Wang et al.,

2022; Orozco-Arroyo et al., 2015; Creff et al., 2015; Robert et al., 2018; Figueiredo et al., 2016; Costa et al., 2014). Studies identifying such cell-to-cell communications have heavily relied on observed phenotypes together with snapshots of particular stages of development (Fig. 1) (Robert et al., 2018; Figueiredo et al., 2016; Costa et al., 2014). The application of established time-lapse live-cell imaging systems should allow us to capture cellular behaviors and the dynamics of signaling molecules governing the cell-to-cell communications important for seed development in the coming years.

Imaging of reproductive processes in crop plants has been challenging (Kalinowska et al., 2020; Gutiérrez-Marcos et al., 2006) due to large size of reproductive tissues, lower tissue transparency, and that their positions are embedded more deeply inside multiple cell layers compared to *Arabidopsis*. Multiple photon microscopy such as 2PEM and 3PEM, well suited for deep tissue imaging, has advanced, in combination with different microscopy systems. For instance, with the utilization of an adaptive optics (apical optics) system, which allows correction of optical aberrations and wavefront distortions induced by system or sample, non-invasive *in-vivo* imaging of deep tissue such as mouse brain beyond 1 mm deep has been developed (Wang and Xu, 2020; Wang et al., 2018). 2PEM has also been applied to light sheet fluorescence microscopy (LSFM) and structured illumination microscopy (SIM) (Lin et al., 2021; Zheng et al., 2017). LSFM is capable of high acquisition speed and axial confinement of illumination by using two optical axes so that it causes less photobleaching and phototoxicity with deep tissue imaging (Höckendorf et al., 2012). SIM, a super-resolution microscopy system, mathematically deconvolves the interference signal of different orientations and phase-shifts acquired from a series of raw images, resulting in significant increase of the spatial resolution (Schermelleh et al., 2019; Gustafsson et al., 2008). Thus, the application of advanced microscopy techniques, allowing high-resolution imaging with less photodamaging, can help to observe not only the late embryogenesis of *Arabidopsis* but also the development of crop seeds which have much deeper tissue. Although the optimization of the growth medium must be performed for individual species, the semi-*in vivo* system for crop plants should be adjusted with such advanced microscopy systems to start investigating seed development in crop plants which directly link with our food security.

Microscopy systems can also be equipped with diverse accessories such as stage-top incubators and microplate stages. By manipulating environmental conditions such as temperature and carbon dioxide in the incubator, how the seed development is altered can be investigated in real time and in a high-throughput manner. Understanding not only the basis of seed development, but also how seeds respond to their environment and how their development is affected by the environment is becoming more critical so that we can come up with new strategies to mitigate unstable climates and stable food production.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

No data was used for the research described in the article.

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