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Endosperm variability: from endoreduplication within a seed to higher ploidy across species, and its competence†

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Review Paper

†This article is dedicated to the great academician Nikolay Ivanovich Vavilov (25 November 1887 to 26 January 1943), who had envisioned a hunger-free world but, ironically, died of hunger and malnutrition.

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Abstract

Endosperm tissue that nourishes the embryo during seed development, upon maturity, nourishes the global population with special reference to cereal crops like maize, wheat and rice. In about 70% of the angiosperms, endosperm genome content is '3n' with 2:1 (maternal:paternal) contribution, as a result of the second fertilization event. However, angiosperms evolution also documents diversity in endosperm genome content from '2n' to '15n', in scale with the corresponding maternal genome dosage variability ('1n' to '14n'), whereas paternal contribution is invariable. In apomicts, due to lack of fertilization, or pseudogamy (fertilization of the central cell for endosperm formation), endosperm genome dosage (m:p) has been reported to range between 1:1 and 8:3. Exceptionally, the central cell with one unreduced nucleus and fused with a reduced sperm cell, with 2:1 normal genome dosage, has been reported in *Panicum*. Altered genome dosage levels are reportedly correlative with eccentricities among maternal and paternal contribution to seed resource allocation. Besides endosperm ploidy variability between species of angiosperms, the present review gives an overview of the ploidy variability in endosperm cells within a seed, up to '690n'. In addition to genome-scale variability in the endosperm, some taxa of angiosperms exhibit chlorophyllous endosperms and some chlorophyllous embryos. Also, endosperm cell number during seed development is reported to have a strong association with grain weight at maturity. Genes underlying these traits of variability are unknown, and the present review underscores the variability and highlights the potential of the single-cell sequencing techniques towards understanding the genetic mechanisms associated with these variable traits.

Introduction

Endosperm is not just an embryo-nourishing tissue; being the major component of the cereal grain, it nourishes the global population. From an evolutionary perspective, the origin of endosperm (resultant of the second fertilization event) in angiosperms is predicted to have evolved from multiple fertilization events in gymnosperms, leading to polyembryony (Friedman, 1992). In 70% of the angiosperms genome dosage level of the embryo is '2n' with equal genetic contribution from the female (m, maternal) and male (p, paternal) parent, while in the endosperm, it is '3n' (2m:1p) (Raghavan, 2006). Diversity in endosperm ploidy level of angiosperms broadly ranges from 2n to 15n, especially due to the variability in the genetic contribution of the female parent from 1n to 14n (Friedman et al., 2008). Key genes regulating the cell cycle, mega-sporogenesis or -gametogenesis or endosperm developmental process, including ontogeny and cell numbers are well documented (Motamayor et al., 2000; Berger et al., 2006; Huh et al., 2007; Fiume and Fletcher, 2012; Batista et al., 2019; Huang et al., 2019; Kirkbride et al., 2019). However, the variability underlying these traits between species and its regulatory mechanism that flexibly accommodates and accepts the variable endosperm dosage level from 2n to 15n, and endosperm cell number in angiosperms is not yet fully understood. The present review highlights the endosperm variability patterns in angiosperms, from variable genome dosage to chlorophyllous endosperm involving carbon fixation; and the potentialities of the genomics tools and techniques to underscore the genetic mechanism for better scientific understanding.

Endosperm variability and development

Endosperm is a tissue in transition across dicot families, while it is the major source of reserves in monocots. However, early endosperm development is remarkably conserved across monocots and dicots (Becraft, 2001). Endosperm development is a dynamic and intricate process involving genome imprinting, dosage balance, positional cues, cell-cycle modifications and programmed cell death (PCD) for a normal ontogeny (Wangenheim, 1957; Becraft, 2001;

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Gehring and Satyaki, 2017; Satyaki and Gehring, 2019). In monocots, where the endosperm is persistent, it is comprised of three cell types, namely starchy endosperm, aleurone layer and transfer cell layers; among these, the aleurone layer retains its viability at seed maturity (Lopes and Larkins, 1993; Becraft, 2001; Becraft and Gutierrez-Marcos, 2012). In addition to cell-type variability, the endosperm exhibits variability of *inter alia* ploidy (both across species and within the seed, as well), chlorophylly and polyendospermy.

Endosperm ploidy variability in sexual and apomictic species

Around 70% of the angiosperms exhibit an endosperm ploidy level of '3n' with two-thirds of the genome dosage contribution from the maternal parent, and the remaining one-third through the paternal parent due to the second fertilization event (Raghavan, 2006). The maternal to paternal genome ratio of 2:1 is the most balanced dosage levels among parental genomes during sexual reproduction and is postulated as endosperm balance number (EBN) for normal endosperm and seed development (Johnston et al., 1980). However, in several angiosperms, endosperm ploidy deviates from '3n'. A central cell with 1 to 14 nuclei is the source of variability for the endosperm ploidy level, resulting in a genome dosage level of 2n to 15n upon the second fertilization event, reflecting altered maternal dosage levels, ranging from 1m:1p to 14m:1p (Friedman et al., 2008). Genera exhibiting variable endosperm ploidy level due to altered genome dosage levels of maternal parents include *Oenothera* (Von Wangenheim, 1962; Haig and Westoby, 1991), *Nuphar* (Williams and Friedman, 2002), *Manekia* (Arias and Williams, 2008), *Gagea* (Greilhuber et al., 2000), *Penea*, *Plumbago*, *Fritillaria*, *Plumbagella* and *Peperomia* (Friedman et al., 2008). Invariably, in all these species with seed development through a fertilization event, the paternal genome dosage contribution is uniformly '1n' only.

However, in aposporous apomicts, the paternal genome dosage level could be higher than '1n', at instances where the paternal ploidy level is higher than the maternal one (Haig and Westoby, 1991; Quarin, 1999; Alves et al., 2001; Šarhanová et al., 2012). In diplosporous apomicts, the maternal dosage alone is inherited with 4n (4m:0p) genome content without the fertilization event (Kollmann et al., 2000). In certain diplosporous apomictic grass species, such as *Elymus*, *Poa*, *Eragrostis* and *Tripsacum*, pollination is a necessary event for successful endosperm development – one of the three components of apomictic seed development (Bashaw and Hanna, 1990; Kaushal et al., 2019). For example, *Tripsacum* with diploids and tetraploids reported to exhibit genome dosage (maternal to paternal) of 8:1 and 8:2 ratios in apomictic plants, but 2:1, 4:1 and 4:2 ratios observed in sexual plants (Grimanelli et al., 1997). *Paspalum* with di-, tri-, tetra-, penta-, hexa- and octaploid species, the endosperm genome dosage level varies widely from 1:1, 2:1, 3:1, 4:1, 4:3, 8:1 to 8:3, with 4:1 genome dosage exhibiting the maximum reproductive efficiency (Burton, 1948; Quarin et al., 1984; Quarin, 1999; Ortiz et al., 2013; Felitti et al., 2015). An AFLP Marker associated with apomictic trait loci in *Paspalum* has been identified (Labombarda et al., 2002), its cross-transferability in other related species might be of advantage in differentiating the apomictic from sexual plants. Exceptionally, the occurrence of pseudogamy resulting in the normal EBN genome dosage level of 2:1 is possible only when the reduced sperm cell is fused with the central cell containing a single unreduced polar nucleus as reported in *Panicum maximum* (Warmke, 1954). However, in general, due to pseudogamy, 4:1 is formed when the two unreduced polar nuclei are fused with one reduced sperm nucleus (Grimanelli et al., 1997; Quarin, 1999; Felitti et al., 2015). Polyploidy and apomixis are reported to be strongly associated, although gametophytic apomixis is reported in diploids (Sharbel et al., 2009; Ortiz et al., 2013).

The occurrence of apomixis (gametophytic: apospory and diplospory; sporophytic: adventitious embryony type) is documented in at least 300 species, predominantly from four families, namely Gramineae, Compositae, Rosaceae and Rutaceae, but for more than 35 families in total (Koltunow, 1993; Khush, 1994). As a rule of thumb, gametophytic apomicts (apospory – *Cenchrus*, *Dicanthium*, *Panicum* and *Heracium*; diplospory – *Taraxacum*, *Ixeris* and *Antennaria*) are polyploid in nature, while the sporophytic ones (adventitious embryony – *Citrus*) exhibit diploid behaviour (Knox, 1967; Young et al., 1979; Asker and Jerling, 1992).

From an evolutionary perspective, polyploidization and hybridization events are the drivers of genetic modifications at the genome level for apomixis development (Barke et al., 2018). Photoperiod (natural) induced apomixis in *Dichanthium* was the first report documenting the role of the environment, in addition to genetic factors, leading to apomixis (Knox, 1967; Rodrigo et al., 2017). Moreover, experimentally induced apomixis through gamma-ray irradiation has also been documented for maize (Yudin, 1966). Chemically induced apomixis has employed *inter alia* nitrous oxide, dimethyl sulfoxide, gibberellic acid, 6-benzyl aminopurine, 2,4-dichlorophenoxy acetic acid and zeatin in *Datura*, *Solanum*, *Ficus*, *Gossypium* and *Zea* (Montezuma-de-Carvalho, 1967; Arendt, 1970; Zhou, 1980; Hu et al., 1991).

Facultative apomixis – coexistence of apomixis and sexual reproduction – have been demonstrated in *Hieracium* and *Sorghum* (Tang, 1977; Bicknell and Koltunow, 2004; Carman et al., 2011). Probably due to the apomictic nature of *Hieracium*, Gregor Mendel in 1869 obtained contrasting results as compared to *Pisum*, which he noted as 'almost opposed behaviour' at a time when the phenomenon of apomixis was unknown (Savidan, 2000; Bicknell and Koltunow, 2004). Inheritance of aposporous apomixis has been studied in *Pennisetum*, *Panicum* and *Brachiaria* and reported to be determined by a single dominant locus (Sherwood et al., 1994; Valle et al., 1994; Savidan, 2000). Genes (protein coding and lncRNA) and epigenetic mechanisms regulating apomixis are well documented (Guerin et al., 2000; Albertini et al., 2005; Laspina et al., 2008; Garcia-Aguilar et al., 2010; Polegri et al., 2010; Ortiz et al., 2013; Hand and Koltunow, 2014; Podio et al., 2014a,b; Felitti et al., 2015; Ortiz et al., 2017; Selva et al., 2017; Tang et al., 2017; Bocchini et al., 2018; Ochogavía et al., 2018; Tang et al., 2019; de Oliveira et al., 2020). However, complete molecular regulatory mechanisms are yet to be revealed and would help fertilize agricultural crops innovatively through fixing the hybrid heterotic vigour in order to propagate indefinitely with enhanced productivity (Dujardin and Hanna, 1983; Khush, 1994; Ramulu et al., 1999; Spillane et al., 2001, 2004; Ortiz et al., 2013; Brukhin, 2017).

Besides, in an inter-specific cross combination, when the ploidy level of the maternal plant (e.g. diploid) is less than that of the paternal plant (e.g. tetraploid); the genome proportion of the paternal plant in the resultant endosperm tissues is greater than '1n', and as per the example, it is '2n' (Quarin, 1999; Felitti et al., 2015; Batista et al., 2019). Evolutionarily, the occurrence of the character – endosperm development in angiosperms – resembles poly-embryonic events (more than one fertilization

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event leading to embryo formation) in gymnosperms, and later evolved to form endosperm in angiosperms. The genome dosage level variability in the endosperm, from 2n to 15n, might be termed 'radicals' or 'variables' for the said character, as defined by Vavilov (1922).

Polyendospermy and chlorophyllous endosperm

Besides endosperm ploidy level variability (2n to 15n), *Arceuthobium americanum* Nutt. ex Engelm. has been reported to exhibit polyendospermy – formation of multiple '3n' endosperms (Friedman and Sumner, 2009). Non-endospermic species have also been reported, for example in Podostemaceae due to the lack of a central cell (Battaglia, 1971; Baroux et al., 2002) and in Trapaceae and Orchidaceae, due to either suppression of primary endosperm nucleus' cell division or disintegration of nuclei after few cell divisions (Johri et al., 1992). Very few taxa have been reported to exhibit chlorophyllous endosperm; Amaryllidaceae members are the notable ones (Meerow and Snijman, 2001). However, taxa from more than 70 families exhibit chlorophyllous embryos (Johri et al., 1992) during seed development. However, at maturity, the chlorophyllous nature disappears. The nature or type of photosynthesis, the quantity of carbon being fixed and its relative contribution with respect to the carbon translocated from leaves are open for further scientific research to assess the importance of chlorophyllous seed tissues. Irrespective of the statistical significance of the quantum of carbon fixed through endosperm or embryo, in comparison with translocated carbon, it will provide a greater understanding from a biological and evolutionary viewpoint, if the nature of photosynthesis is different from leaves. Answers from such studies will form a niche for further research questions, especially when the nature of photosynthesis in endosperm and/or embryo is different from leaves, as reported in wheat grain pericarp (Rangan et al., 2016). C_4 photosynthesis in wheat grain pericarp is yet an unsettled issue with arguments for and against (Henry et al., 2017). Species exhibiting variability in photosynthesis type, between leaves and endosperm/embryo parts of a plant might be of much help to throw light on this issue. The importance of non-foliar photosynthesis for yield improvement in crop plants is an untapped potential and is a current prioritized issue globally, on implementing C_4 photosynthesis in rice for improved productivity (Normile, 2008; Ermakova et al., 2020; Simkin et al., 2020). Carbon fixation in non-foliar (reproductive or sink) tissues of crop plants might, potentially, help accelerate grain filling to keep pace with the increase in cell size due to ploidy effects in endosperm tissues and its variability with respect to the cell position in the seed as detailed in the following section.

Endosperm ploidy variability within a seed

The variability in ploidy level of endosperm across species or hybrids derived between parents at different ploidy levels is prominent in crossable species wherein fertile seeds are produced, involving parents differing in their ploidy (Tomaszewska and Kosina, 2018). Remarkably, the variability of endosperm ploidy within a seed was reported well before the DNA-double helix structure (Duncan and Ross, 1950; Swift, 1950). First, report to point the variability in the size of cells and nuclei of the young endosperm cells present in the central portion of the endosperm, was made as early as 1931 in maize (Lampe, 1931). However, it was only in 1950 that increased cell and nuclei size were associated with the increased DNA content (Swift, 1950) or ploidy levels for those specific cells of enlarged size (Duncan and Ross, 1950) through quantified DNA content, and identified to be due to the endomitotic process. Genes (like *Rhl1*) associated with cell size enlargement and higher ploidy were reported much later (Sugimoto-Shirasu et al., 2005). An upsurge of around 64 or 125 and even up to 1000 times the volume of nuclei from the central portion of endosperm, when compared to its peripheral nuclei, was documented in maize (Duncan and Ross, 1950); the ploidy correlation with DNA content has been reported recently (Santeramo et al., 2020). The ploidy level variation of endosperm nucleus within a maize seed, when compared in the aleurone and central regions of the endosperm, was reported to be up to '6n' and '24n', respectively (Swift, 1950). Also, the variability in endosperm ploidy among the centrally located endosperm cells was as high as '690n' in maize (Kowles and Phillips, 1985). In addition to central region cells of the endosperm, suspensor cells too were reported to undergo endoreduplication events (Lee et al., 2009).

Apparently, the cells present in the central region of the endosperm undergo cell cycle only between S and G phases (endoreduplication) interrupted by gap periods and a doubling time of 24 h (Kowles et al., 1990, 1992b; Schweizer et al., 1995). The Cyclin A gene (*CYCA2;3*) is one of the key genes regulating the endoreduplication event (Imai et al., 2006), and the importance of this functionality has been viewed from a broader perspective on overall physiology and development (De Veylder et al., 2011). Besides increased mRNA and protein formation through endoreduplication, it has also been suggested that storing nucleotides by this mechanism might anticipate its use during embryogenesis and germination (Lee et al., 2009). It has been estimated that roughly 3% of the total endosperm cells (positioned in the central region) in maize seed exhibit the variability in ploidy from the normal triploid endosperm cells, and a few cells (approximately 1%) contained around 90 chromosomes (Lin, 1977). Ploidy variability, like in maize, has been reported in oats, as well (Tomaszewska and Kosina, 2018). Later, endoreduplication in plants was found in cotyledons, roots, cell suspensions, anthers, developing fruits (tomato) and leaves, as well (Joubès and Chevalier, 2000).

Defective kernel (*Dek*) mutants in maize exhibit reduced mitotic activity and, in turn, the endoreduplication, especially in the central regions of endosperm cell, was found to be controlled by a recessive gene (Kowles et al., 1992a) and also lacking an aleurone layer (Becraft and Yi, 2010). Water deficit (Artlip et al., 1995), abscisic acid (Mambelli and Setter, 1998), high temperature (Engelen-Eigles et al., 2000), parental dosage effect (Kowles et al., 1997; Leblanc et al., 2002; Tomaszewska and Kosina, 2018) and post-translational modification (Zhao and Grafi, 2000) may alter endoreduplication through regulating mitotic cycles in the endosperm, thereby suggesting multiple checkpoints, besides some recessive genes. Key molecular mechanisms involved in endoreduplication and increased ploidy level within the seed were identified as primarily due to the loss of activity of M-phase cyclin-dependent kinase and alterations in S-phase cyclin-dependent kinase (Larkins et al., 2001). Post-translational modifications such as hypophosphorylation on high mobility group I/Y protein by CDC2 kinase have been suggested to be associated with endoreduplication events in maize endosperm by alleviating the transcriptional repression by Histone H1 (Zhao and Grafi, 2000). Cyclin-dependent kinase inhibitors KRP1 and KRP2 inhibit the cell cycle, leading to the onset of endoreduplication events in the maize endosperm (Coelho et al., 2005).

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Tolerance to altered genome dosage levels is evident in angiosperms, albeit in a few species only, and in different cells of a tissue, depending on the position (Kowles and Phillips, 1985; Joubès and Chevalier, 2000; Friedman et al., 2008). Understanding the linkage between the genetic nature of embryosac variability and endosperm ploidy might help shed light on the genetic regulatory mechanism underlying the ploidy syndrome. The following section associates endosperm ploidy with embryosac variability, using the common genetic factor. However, how far they could be influenced by environmental factors on ploidy variability with respect to the different cell positions within a tissue is yet to be revealed.

Are endosperm ploidy and embryosac variability genetically linked?

Cells destined for endosperm formation are determined during embryosac formation. Later, with the onset of fertilization, the determined central cell, upon second fertilization, gets differentiated into endosperm tissue. To understand the variability in endosperm ploidy level, primarily, the megaspore mother cell (MMC) formation process – its root, needs to be understood. Identification of the diversity in embryosac development might shed some light on the endosperm variability. Normal meiosis of MMC leads to monosporic functional megaspores. The absence of cytokinesis during the second meiotic division or both meiotic divisions yields bi- (two haploid nuclei) or tetra-sporic (four haploid nuclei) functional megaspores. Evolutionarily, bi- and tetrasporic trait types are derived from the monosporic trait (Arias and Williams, 2008). The single functional megaspore undergoes three mitotic division to yield an eight-nucleate (seven cellulars) mature embryosac (ES), classified as *Polygonum* type – the most common one across angiosperms (Friedman, 1998). However, mega-gametogenesis devoid of the second or third mitosis, in combination with mono-, bi- or tetra-sporic megaspore, potentially generates 4 or 16 nucleate ES instead of the most frequently found eight-nucleate ES. Structured arrangement of 4 or 8 or 16 nuclei within ES in different species exhibits diverse forms of ES. Structurally (and the number of nuclei as well) differing ES were named upon the taxa from which they were described and reported at first. Documented ES types are *Polygonum*- (most common), *Oenothera*-, *Allium*-, *Peperomia*-, *Penaea*-, *Drusa*-, *Fritillaria*-, *Plumbagella*-, *Plumbago*-, *Adoxa*-, *Butomopsis*-, *Acalypha indica*-, *Peperomia hispidula*-, *Apinagia*- and *Dicraea* types (Maheshwari, 1950; Battaglia, 1971; Friedman et al., 2008). Due to the variability in the number of nuclei of the central cell of ES, accordingly the endosperm ploidy level gets modulated from the normal '3n' endosperm during the second fertilization event.

To obtain an overall understanding on such variable ES for number of nuclei, and, in turn, endosperm genome dosage levels, ES classified on genetic basis can be grouped broadly into seven types (Friedman et al., 2008), namely monosporic 2n (1m:1p), monosporic 3n (2m:1p), bi-sporic 3n (2m:1p), tetra-sporic 3n (2m:1p), tetra-sporic 5n (4m:1p), tetra-sporic 9n (8m:1p) and tetra-sporic 15n (14m:1p). Taxonomically, the order *Piperales* alone accommodates six of these seven genetic types (Arias and Williams, 2008) excluding the monosporic 2n type, suggesting the possibility for better understanding of the genetic variability of embryosac or endosperm and its development. The law of homologous variation, described by Vavilov (1922), might help foresee the endosperm variability in allied taxa as well for the genera reported with variable endosperm ploidy levels. Most of the understanding of the process of organogenesis of MMC and flower or seed development has been reported only in few plant models like *Arabidopsis*, *Medicago*, *Zea* and *Nicotiana* (Table 1). With the present understanding from these model plants, the genetic mechanism underlying endosperm or ES variability may be revealed.

Tolerance of altered genome dosage

Several reports have highlighted the importance of balanced genome dosage levels, positional cues, PCD and cell-cycle modifications primarily involved in normal endosperm development and, in turn, seed development and viability (Wangenheim, 1957; Becraft, 2001; Satyaki and Gehring, 2019). Whenever there is a deviation from the 2m:1p genome dosage ratio during endosperm formation, either premature cellularization (increased maternal dosage) or lack of cellularization (increased paternal dosage), seed abortion is the most probable result (Scott et al., 1998). However, the evolution (on parsimony basis) of 2m:1p from 1m:1p further from 1m:0p (Cailleau et al., 2010), and the existence of species accommodating the altered genome dosage, indicates nature's tolerance of genome dosage levels deviating from 2m:1p (Satyaki and Gehring, 2019). Likely, this tolerance mechanism is responsible for the 30% of angiosperms exhibiting altered genome dosage levels, from 1m:1p up to 14m:1p (Raghavan, 2006; Friedman et al., 2008). It is essential to underpin the genetic regulatory mechanisms of the tolerance of the deviation from the most common 2m:1p, especially so because understanding the genetic regulation and genes involved in endosperm development have been reported mostly in the model plant *Arabidopsis* (Table 1). However, in well-studied cereal crops like rice, wheat and maize, the initial phase of endosperm development (cell determination phase) is largely unknown (Li and Song, 2020; Olsen, 2020). Information gained from model plant species may help understand the endosperm development and the genome dosage balance in the species deviating from 2m:1p ratio. Also, methylation patterns involved in parental imprinting to functionalize parent-of-origin effects, when neutralized in both the parents, the impact of the nature of imprinting no longer hinders or alters normal endosperm development (Adams et al., 2000). Hence, this potentially gives clues on the possible tolerance for the nature's acceptance of deviation from the 2m:1p dosage level.

Roadmap for understanding the endosperm genome variability

Gametophytic mutations (from completion of meiosis until fertilization and maternal control of seed development) affecting mega-gametogenesis follows non-Mendelian segregation (Yadegari and Drews, 2004). However, sporophytic mutations affecting mega-gametogenesis (from MMC till completion of meiosis) are yet to be studied. Hence, to sufficiently address this, two approaches may be followed. Firstly, working with the existing knowledge on the reproductive ontogeny in model flowering plants, potential candidate genes could be identified, and further, its validation in the taxa of interest, either through forward or reverse genetics approaches (Jankowicz-Cieslak and Till, 2015). Alternatively, differential gene expression (RNA-seq) between the two nearest taxa variable for endosperm and embryosac formation may be used to identify and validate the genes or genetic mechanism underlying variability. Irrespective of the

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Table 1. Potential candidate genes involved in regulating plant reproductive development (based on reports of genes/metabolites associated with cell cycle)

Gene/ metabolite/ pathway	Name	Species	Function	Reference
Auxin	–	*Arabidopsis*	Prevents endosperm cellularization and leads to seed arrest	Batista et al. (2019)
CYCD	D-type cyclin	*Arabidopsis Medicago*	Cell cycle entry	den Boer and Murray (2000) and citations therein
CDC2A	Cyclin-dependent kinase (*cdk*)	*Arabidopsis Zea*	Cell cycle regulation	
CAK	CDK-activating kinase	*Arabidopsis*	Phosphorylation of CDK to regulate cell cycle	
RML	Root meristemless	*Arabidopsis Nicotiana*	Encodes for Glutathione (GSH) biosynthesis linking cell cycle	
CDC26	Cell division cycle protein 26 (uORF)	*Arabidopsis*	Regulates cell cycle at anaphase	Lorenzo-Orts et al. (2019)
TOR pathway	Target of Rapamycin pathway	*Arabidopsis*	Integrates cytoplasm growth, cell expansion and cell cycle. Also, cell size checkpoint	Sablowski and Carnier Dornelas (2014) and citations therein
ARP6, E2FA, E2FB and *E2FC*	Actin-related protein 6, elongation factor complex	*Arabidopsis*	Distinguishing MMC identity to take meiosis; while other cells to take mitosis	Pinto et al. (2019) and citations therein
KNU	Knuckles	*Arabidopsis*	Drives expression specifically in MMC (even at tetrad) – the primary germline cell	
THO/TREX complex	Includes *Tex1*, *Hpr1* and *Tho6*	*Arabidopsis*	Leads to multiple MMC-like cells through involvement of tasiRNA that represses ARF3	Lora et al. (2019) and citations therein
Auxin cytokinin ratio	–	*Arabidopsis*	Positional information with auxin at distal and cytokinin at proximal polarity in ovule primordia development. Possibly, the absence of auxin leads to multiple MMC-like cells?	
ASF1	Anti-silencing function 1	*Arabidopsis*	Involved in gametophyte (both male and female) development and acquiring fertilization competency	Min et al. (2019)
F-box, E3 ligases	–	*Boechera*	Distinguishes sexual and apomictic germline	Zühl et al. (2019)
FRK3	Fertilization-related kinase3	*Solanum chacoense*	Gametophyte (male and female) development	Daigle et al. (2019)

approaches taken to identify the candidate genes, validating the identified genes is an important part of the studies for understanding the genome dosage variability. A schematic overview of the roadmap towards understanding the genetic mechanism underlying endosperm or embryosac variability is provided in Fig. 1 and detailed in the following section.

Regulators of the cell cycle as potential candidates

With the brief highlights of the genetic or genomic variation in endosperm and embryosac formation, as explained in previous sections, first a framework with identified potential target taxonomical groups for better understanding of the underlying genetic mechanism needs to be made. Secondly, potential candidate genes regulating the cell cycle (mitotic and meiotic) and cytokinetic checkpoints across species are to be identified and charted out (den Boer and Murray, 2000; Sablowski and Carnier Dornelas, 2014; Keçeli et al., 2017; Barrada et al., 2019; Daigle et al., 2019; Eekhout and De Veylder, 2019; Lora et al., 2019; Lorenzo-Orts et al., 2019; Min et al., 2019; Pinto et al., 2019; Zühl et al., 2019). For easy reference and as a starting point, a list of potential candidate genes (including miRNA and other non-coding RNAs) regulating reproductive development in angiosperms is provided in Table 1. A brief comparison between the identified taxa with its nearest taxa with normal (3n) endosperm, at structural and functional levels for those genes might throw some light on the existing variability and its underlying genetic mechanism.

Epigenetic regulation of genomic imprinting

In addition to genetic mechanisms, the importance of epigenetic mechanisms, especially during ontogeny of endosperm and seed development, is well documented with a role for non-coding small RNAs (Gehring and Satyaki, 2017; Yu et al., 2020). Methylation patterns of DNA and various modifications (acetylation and methylation) of histone protein (Song and Chen, 2015), in addition to small RNAs (Ng et al., 2012; Yakovlev et al., 2020; Yu et al., 2020), are the epigenetic regulators playing key roles in developmental processes. The coordinated act of the Polycomb group (PcG) of proteins and the DNA methylation essentially regulates the epigenetic systems for endosperm development, including the central cell stage of mega-gametogenesis. Gene products of *MEDEA* (*Mea*) *FERTILIZATION INDEPENDENT SEED DEVELOPMENT* (*FIS2*) and *FIS3* (a.k.a:

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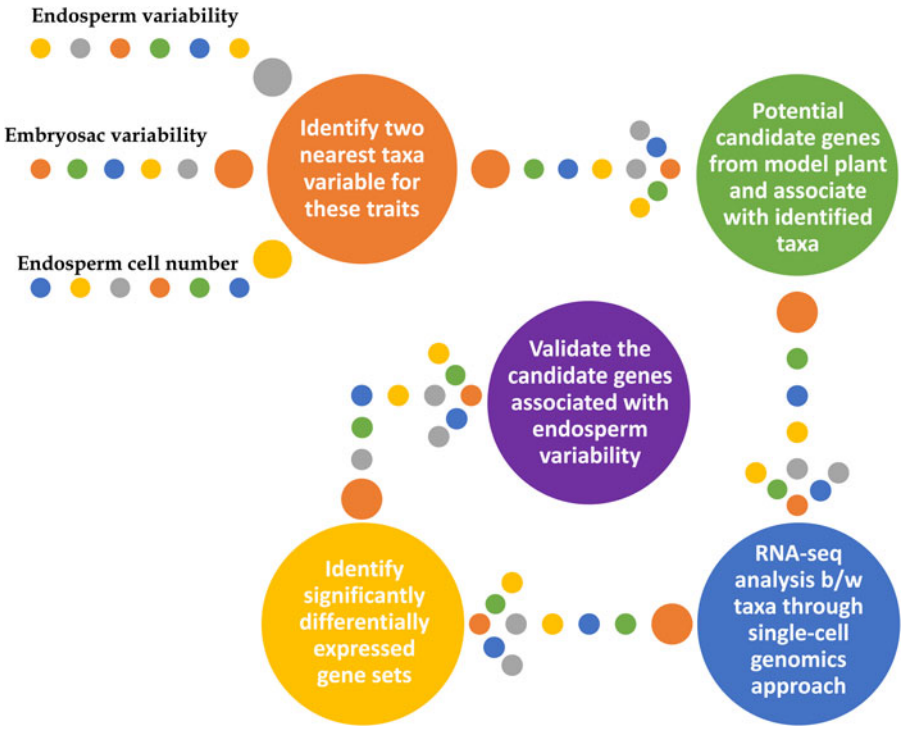


Fig. 1. An overview of the genetic mechanism regulating endosperm and embryosac variability.

FIE, *FERTILIZATION INDEPENDENT ENDOSPERM*) classified as PcG type are well-known regulators of the embryo and endosperm development (Köhler et al., 2003). PcG proteins are well-known transcriptional regulators through histone modifications mediated gene silencing mechanisms, with its homologues present across the animal and plant kingdoms in establishing cell identity and memory (Hsieh et al., 2003; Di Croce and Helin, 2013). The activity of most of the imprinted genes of the endosperm are regulated through epigenetic mechanisms and its complex nature poses difficulties in association with the corresponding phenotype (Pignatta et al., 2018). Besides, the complexities involved in understanding the embryo or endosperm development *per se*, crosstalk between embryo and endosperm also plays a crucial role in reproductive isolation and seed development. During wide hybridization, such crosstalk induced barriers for genetic exchange lead to hybrid incompatibilities (Fishman and Sweigart, 2018; Roth et al., 2019). Importantly, crosstalk between embryo and endosperm in mature seed during germination is also crucial for successful germination through the supply of seed reserves for the germination processes (Yan et al., 2014; Doll et al., 2020).

Although methylation patterns generally occur in heterochromatic (gene-poor) regions, in transposable element regions, the genic regions appear more methylated than the flanking regions. However, when compared between embryo and endosperm for genome-wide methylation patterns, it was reported that the *DEMETER* (*DME*) gene product demethylates the genes of transposable elements in the central cell nuclei of maternal origin before fertilization (Gehring et al., 2009). This highlights the role of epigenetic regulation in enforcing the parent-of-origin effect on the endosperm development. In maize endosperm, through genome-wide methylation pattern analysis, it has been reported that there is a reduction of 13–34% methylation when compared to embryo or leaf tissues (Lauria et al., 2004; Wang et al., 2015). DNA glycosylases (DME) and RNA-directed DNA methylation (RdDM) through siRNA pathways are the two major epigenetic mechanisms regulating plant development with the former for imprinting female gametogenesis and the latter for vegetative tissues (Law and Jacobsen, 2010). The importance of Histone H1.2 in regulating DME for its downstream epigenetic regulation to impart an imprinting effect has been identified (Rea et al., 2012). Also, histones modified by PcG proteins are well-documented regulators at the transcriptional level involved in the normal embryo and endosperm development (Schubert et al., 2005; Moreno-Romero et al., 2019). Possibly, this corroborates the reports on endoreduplication in endosperm cells of the central region to have a reduced H1/DNA ratio and thereby enhancing the transcription and translation towards grain filling in endosperm cells (Zhao and Grafi, 2000; Larkins et al., 2001). This highlights the importance of epigenetic regulation through silencing as well as expression (through negative repression) for normal endosperm and embryo development.

There are very few reports that underscore the association between epigenetic regulation and ploidy level at the species level but none to the author's knowledge with reference to endosperm or seed development. Primarily, a non-linear relationship between the DNA methylation pattern and ploidy level was documented (Li et al., 2011), when compared between species with different ploidy (di-, tri- and tetra-). Altered ploidy might potentially alter the epigenetic silencing mechanism and thereby altering the normal developmental processes (Scheid et al., 1996).

Role of genomics on understanding the endosperm variability

The candidate gene approach is the most traditional, long-standing and widely used methodologies in identifying genes associated with the trait of interest, particularly when the trait is of a quantitative or complex nature (Pflieger et al., 2001; Tabor et al., 2002; Zhu and Zhao, 2007). However, preliminary

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knowledge of the trait is required for the identification of the genes underlying it. Also, contrasting genotypes for the trait of interest may be identified at first for generating a QTL map (Wayne and McIntyre, 2002; Zhu and Zhao, 2007). With the availability of computational facilities and robust sequencing technologies, the importance of genomics for the identification of genes and pathways is undisputed. Notably, genome-wide scanning approaches and digital candidate gene identification approaches, such as QTL, LD mapping, GWAS, GBS, and functional annotation, either alone or in combination with traditional candidate gene approaches are now frequently used (Mackay and Powell, 2007; Götz et al., 2008; McCarthy et al., 2008; Chen et al., 2009; Zhang et al., 2010, 2020; Bush and Moore, 2012; Glaubitz et al., 2014; Torkamaneh et al., 2020). The following three subsections provide an overview of the strength of genomics, with special reference towards understanding endosperm variability (both across species and within the seed).

Differential gene expression studies

Significant progress has been made on the genetic regulation of endosperm formation that enhances our understanding of endosperm ontogeny (Table 1). Most of the studies involved the model plant *Arabidopsis thaliana* to understand the basic genetic mechanism underlying endosperm ontogeny (Table 1). However, genetic regulation underlying the genome-scale variability in endosperm ontogeny between closely related taxa (within *Piperales*) are yet to be uncovered. Differential gene expression (RNA-seq) in combination with single-cell genomics (scRNA-seq) might be of huge potential to address this. Additionally, for apomixis developmental processes, candidate genes like *SERK* and *APOSTART* have been identified (Albertini et al., 2005; Podio et al., 2014b). Since apomixis is reported to be tightly associated with the polyploidy mechanism (Ortiz et al., 2013), species within a genus (or variable cytotypes within a species) exhibiting variable ploidy level with apomictic behaviour are ideal models to identify the molecular mechanisms underlying the apomictic phenomena (Ortiz et al., 2013; Felitti et al., 2015; Ochogavía et al., 2018).

Comparison between nearest taxa variables for apomictic behaviour helped identify a set of genes including *AGO9* (Olmedo-Monfil et al., 2010), *CASEIN KINASE* (Depetris et al., 2018), *LORELEI* (Felitti et al., 2011), a MAP3K coding gene *QUI-GON-JINN* (Mancini et al., 2018), *THAUMATIN-LIKE*, *COPIA*, *CCD*, *LEISHMANOLYSIN-LIKE PEPTIDASE* and *FAR1-RELATED* (Ortiz et al., 2017), *ORC3* (Siena et al., 2016), *DORN1* and eATP pathway (Choi et al., 2014; Felitti et al., 2015), *AMP SYNTHASE*, *EF-1α*, *COP9 SIGNALOSOME* and *ACETOLACTATE SYNTHASE* (Cervigni et al., 2008), long non-coding RNAs like *N13* (Ochogavía et al., 2018) and *QGJ* (Mancini et al., 2018), and small RNAs like *ATHILA*, *LINE* and *ATLANTYS* (Olmedo-Monfil et al., 2010), involved in the regulatory processes for apomictic expression. In addition, some recent reports have identified apomixis-related genes in *Paspalum* (de Oliveira et al., 2020) and *Boehmeria* (Tang et al., 2019), which provide insight in genetic control of apomeiosis and polyploidy events leading to apomixis behaviour (Savidan, 2000; Hand and Koltunow, 2014). Also, regulatory mechanisms modulated at the transcriptional, translational and post-translational levels dissecting the apomictic trait, including epigenetic regulation, signal transduction and evolutionary aspects have been covered in detail (Ortiz et al., 2013; Brukhin, 2017; Schmidt, 2020). Notably, most of the genetic or molecular mechanisms associated with apomictic traits have been reported for the genus *Paspalum* (Quarin, 1999; Labombarda et al., 2002; Laspina et al., 2008; Polegri et al., 2010; Ortiz et al., 2013, 2017; Podio et al., 2014a; Felitti et al., 2015; Siena et al., 2016; Bocchini et al., 2018; Depetris et al., 2018; Mancini et al., 2018; Ochogavía et al., 2018; de Oliveira et al., 2020). These details will be of significant help in mining the genes associated with apomictic behaviour in other species as well, based on homology, using sequences obtainable through next-generation sequencing (NGS) tools, followed by further functional validation.

Differential gene expression in combination with digital candidate gene identification approaches using functional annotation tools is a potential method to identify candidate genes underlying endosperm variability and apomixis. This is easily doable in qualitative traits and is feasible in quantitative traits when the quantum of expression results in a differential expression pattern associated with the contrasting phenotypic traits (Rangan et al., 2020a,b). On these advantages, differential gene expression or RNA-seq with functional annotation is a robust upcoming tool for candidate gene identification. While this approach could be used directly for endosperm ploidy variability across species, its utility for endosperm ploidy variability within a seed, and apomictic developmental processes will require additional single-cell sequencing methods (Wagner et al., 2016; Tanay and Regev, 2017).

Single-cell sequencing for ploidy and epigenetic identification

Single-cell sequencing to understand ploidy level (aneuploidy) and its effect on development are well documented in animal and cell models, and upcoming in plant systems. The techniques in the whole genome, epigenetic and transcriptome studies have been applied to identify differences underlying cancer cells and regular ones (Ferrarini et al., 2018). Gene regulation and genome dosage imbalance due to aneuploidy in humans for trisomy dependent regulation, studied using RNA from skin fibroblast cells through single-cell RNA-seq (scRNA-seq) approaches have been reported.

Utilization of single-cell genomics in generating a cell-type atlas, cell-type specificity, resolving molecular relations and functional genomics, and cell differentiation for tissue specificity underlying biological or analytical problems are well documented (Efroni and Birnbaum, 2016; Tanay and Regev, 2017; Ryu et al., 2019; Rich-Griffin et al., 2020). Such studies might potentially be of help as starting material to plan for combining RNA-seq with single-cell genomics – scRNA-seq (Ryu et al., 2019), to study ploidy variability of individual cells within a seed or endosperm tissues.

For this, NGS and single-cell sequencing methodologies (Nawy, 2014), with the current understanding of the endosperm ontogeny in model plants (Table 1) might be of great help in understanding genome-scale variability of the endosperm. This may provide deeper insights in sporogenesis, gametogenesis, endosperm ontogeny and its variability across species from evolutionary, breeding and crop improvement perspectives. Gene regulatory network relationships and molecular interaction could very well be revealed using the scRNA-seq approaches. The peculiarities of seed resource allocation between maternal and paternal parents are not well-founded on hypotheses explaining the variability (Cailleau et al., 2010). Such studies will help underscore the genetic mechanisms underlying the biology of dosage compensation, genome imprinting and maternal/paternal effect with special reference to seed development.

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Single-cell genomics in combination with RNA-seq and functional annotation approaches might be a robust tool to study and elucidate the underlying genetic events affecting the sporophytic and gametophytic mega-gametogenesis and, in turn, fertilization and seed developmental processes. This will also help in identifying the key candidate genes or molecular events responsible for the variability in the genome/nuclear content of ES from 1n to 14n. Additionally, this may also provide a better understanding of the genetic mechanism that favours seed resource allocation that begins with the occurrence of meiosis and completes before fertilization in cycads and ginkgoes whereas the same begins only after fertilization in angiosperms, with conifers in between (Cailleau et al., 2010). It will also be of use in endoreduplication events occurring in the central region of endosperm tissue leading to higher ploidy in those specific cell types as compared to the ones at the periphery (Duncan and Ross, 1950; Swift, 1950; Kowles and Phillips, 1985; Larkins et al., 2001).

Merits and challenges of single-cell RNA-sequencing

Key merit is the availability of techniques and tools reported in model organisms or cells or tissue types, which can be applied with minor modifications to plants (Efroni and Birnbaum, 2016; Rich-Griffin et al., 2020). However, the presence of cell walls and various secondary metabolites in plants warrants for protocol standardization. Great advantage and strength are expected for studying cell-type or single-cell systems in plants, including the availability of laser-assisted microdissection tools to isolate what we actually see (Kehr, 2003; Nelson et al., 2006; Brandt et al., 2018; Sakai et al., 2018; Florez-Rueda et al., 2020). With these strategies, it is quite possible to achieve the isolation of genetic material from the cell lines or cell types of interest in plants, especially from endosperm during seed development. Once the genetic material is isolated; then, it is equal to any other system wherein common tools and techniques available for single-cell sequencing are directly applicable. Differential expression through RNA-seq might help identify the key candidate genes involved in endosperm ploidy variability.

In spite of these merits and potential possibilities of the applicability of scRNA-seq methods to understand endosperm ploidy variability, one should be cautious on the possible challenges that might be faced during the process: (1) epigenetic regulation including parent-of-origin and endoreduplication mechanisms are modulated through cell cycle regulators and isolating cell types for comparison might end up in different results; (2) sufficient quantity of homogenous cell types at the same cellular stage within the group is also an important criterion. It could potentially affect the results, and hence, uniformity of samples is imperative; (3) requirement of sufficient biological replicates of the same cellular stage and developmental level for higher confidence scores when the results are subjected to statistical analyses. Addressing these challenges might help save time and resources with proper planning towards understanding the genetic mechanism modulating endosperm variability within the seed, and across species, and their differences.

Conclusion

Understanding the overall seed development is important for seeds that are not just the fulcrum for the survival of many life forms but also are the carriers of genetic imprints across generations, which helps pass through the successful speciation events in the evolutionary timeline. Sporophytic (Mendelian segregation pattern) mutations and their molecular interactions affecting mega-gametogenesis are known to affect fertilization events and, at times, leads to ovule abortion yielding fewer seeds. Variability in endosperm ploidy within a seed (between the central region and the periphery) and at species level has been known for some 90 years. However, its underlying (epi)genetic mechanisms linking imprinting and parent-of-origin effects during its ontogeny are not completely understood. Present-day genomic tools like scRNA-seq might help gain a better picture of gametophyte development and fertilization in plants, especially of the ontogeny of embryo and endosperm, with special reference to variability within the seed and across species. Comparative studies between taxa exhibiting variability for ES structure and genome dosage level might shed light on the tolerance for altered genome dosage, imprinting and endosperm development under varied genetic environments, and its mechanism of action from an evolutionary perspective. The role of epigenetic mechanisms (both silencing and expression through negative repression) in combination with varied ploidy level (within the seed and species level) interactively regulating the embryo and endosperm development are yet to be elucidated. Additionally, genetic mechanisms underlying the other forms of variability like the disintegration of endosperm after a few divisions in certain taxa, cell identity for endoreduplication in specific cells from the central region of the endosperm, chlorophyllous endosperm and its importance in contributing to carbon fixation, apomictic endosperm dosage, composite endosperm and polyendospermy would also be revealed. This will potentially shed light on the variability in resource allocation during seed development processes and might help extend the duration of resource allocation in agricultural crop plants for enhanced productivity.

To exploit the triploid nature (3n) of endosperm tissues, endosperm culture (*in vitro*) is mostly used for generating triploid plants with superior traits like disease resistance, high yield, larger fruits, etc., in species where the seed is not an economic product (including propagation mode), or felt undesirable as in case of banana, grapes or berries (Thomas and Chaturvedi, 2008; Miyashita et al., 2009). Direct somatic embryogenesis or callus mediated *in vitro* propagation, as reported in many species (Trolinder and Goodin, 1987; Novak et al., 1989; Li et al., 1998; Rangan et al., 2011), might be useful in deriving triploid plantlets with increased vigour using endosperm culture techniques (Sita et al., 1980; Tulecke et al., 1988; Gmitter et al., 1990; Sun et al., 2011; Antoniazzi et al., 2018; Van Thang et al., 2018). In addition, endosperm cells are targeted for improved grain quality and nutrition, like quality protein maize (Gibbon and Larkins, 2005; Vivek et al., 2008) and golden rice (Beyer et al., 2002; Paine et al., 2005). Generation of soft, friable and off-white to creamy yellow callus from endosperm tissues of cereal crops, in combination with processing (dried and powdered forms), would gear up agriculture towards lab farming that might lead to culture edible endosperm directly on Petri dishes. At the verge of a climate change scenario, transformation and utilization of the understanding of variability towards an entirely new form (petri cultures of endosperm) may also help feed humanity in the future, contributing towards fulfilling the dreams of the great academician N.I. Vavilov: a hunger-free world.

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