Genetic and molecular control of seed tissue and nutrient partitioning : the role of the nucellus

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Chapter I - *General introduction*

The seed

The seed habit allows the dispersal of the progeny and represents a remarkable evolutionary advance in plant sexual reproduction that contributed to the successful colonization of land by gymnosperms and angiosperms (seed plants). Compared to spores, a more primitive mechanism of progeny dispersal used by ferns and mosses, seeds rely less on water, carry more nutrients, are better protected and can disperse in different ways.

Seed development

Seeds develop from ovules after fertilization. A typical ovule initiates as primordium in the ovary. The ovule primordium consists of three tissues: the proximal funiculus, the medial chalaza and the distal nucellus (Fig.1). The funiculus is connected to the placenta, which provides all the nutrients, the chalaza forms one or two protective integuments, and the nucellus undergoes meiosis to produce the megaspore (Schneitz et al., 1995a) (Fig. 2). Compared to mosses and ferns, which release the spores, the ovule retains the megaspore in the nucellus (Fig. 3). As a consequence, the megaspore develops into the female gametophyte (embryo sac) and grows inside the ovule at the expense of the nucellus (Magnani, 2018) (Fig. 3 and 4).

Seed development starts with the single (in gymnosperms) or double (in angiosperms) fertilization of the female gametophyte, which generates the embryo, the next plant generation, and, in the case of angiosperms, also the endosperm, a nourishing tissue (Figs. 5 and 6) (Skinner et al., 2004). Whereas the maternal tissues do not actively participate in the fertilization process, they undergo drastic changes in response to it. The integuments develop into the seed coat which surrounds embryo and endosperm and protects them from biotic and abiotic stresses (Coen and Magnani, 2018) (Figs. 6 and 7). The funiculus transports nutrients from the placental tissue and unload them into the chalaza from where are distributed to the rest of the seed. Finally, the nucellus is either eliminated to allow the growth for the fertilization products (for example in Arabidopsis) (Figs. 6 and 7) or it grows to become a nutrient storage tissue (for example in amaranth).

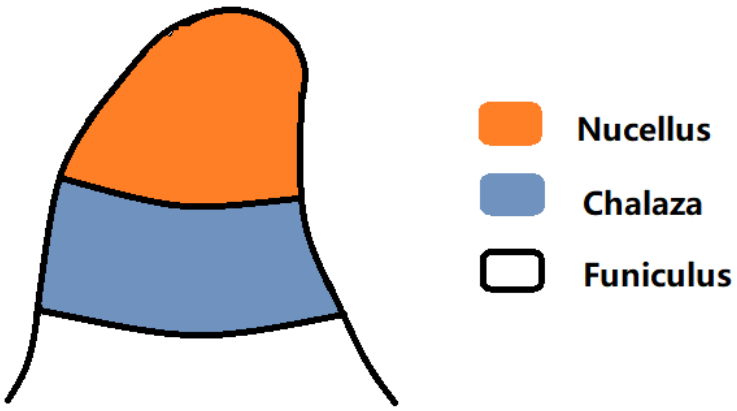

Figure 1. Arabidopsis ovule primordium.

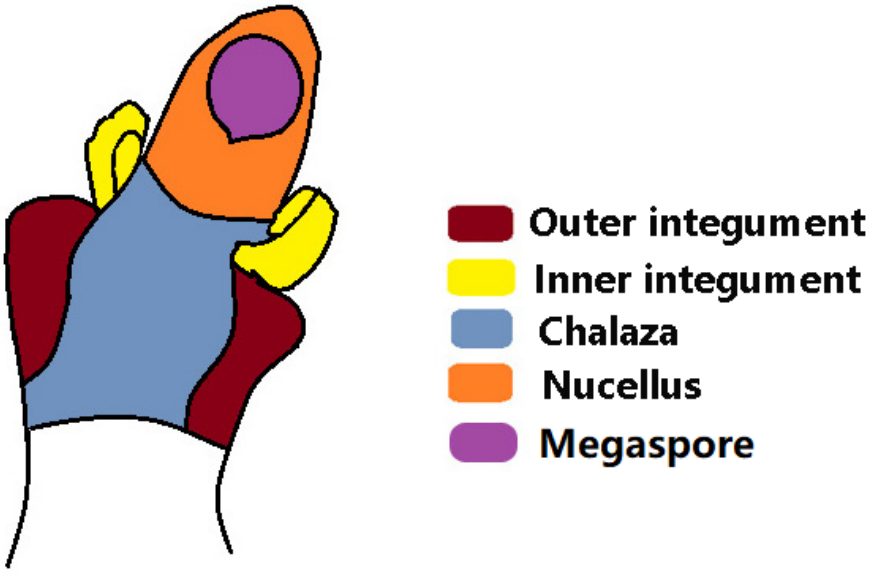

Figure 2. Arabidopsis ovule at stage 2-III (Schneitz et al., 1995a).

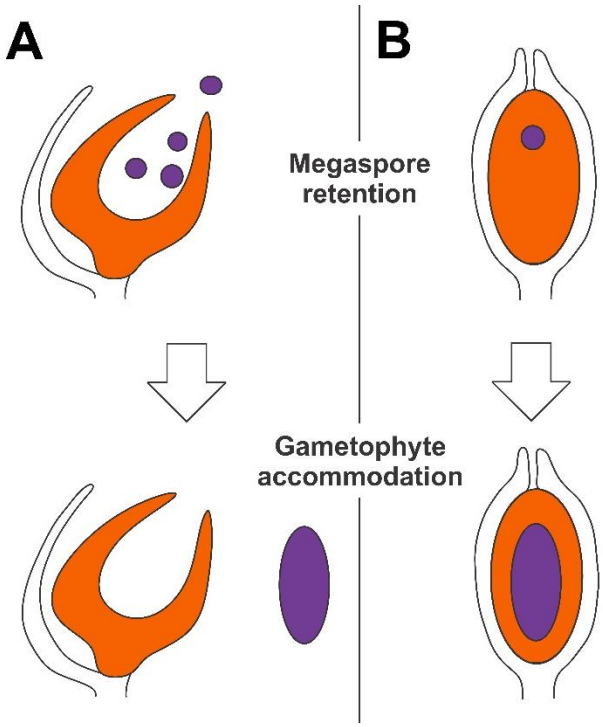

Figure 3. Megaspore retention and female gametophyte accommodation (Magnani, 2018). Female reproductive organ of a heterosporous non-seed vascular plant (A) and an ovule of a seed plant (B). The nucellus is highlighted in orange whereas megaspores and female gametophyte in purple.

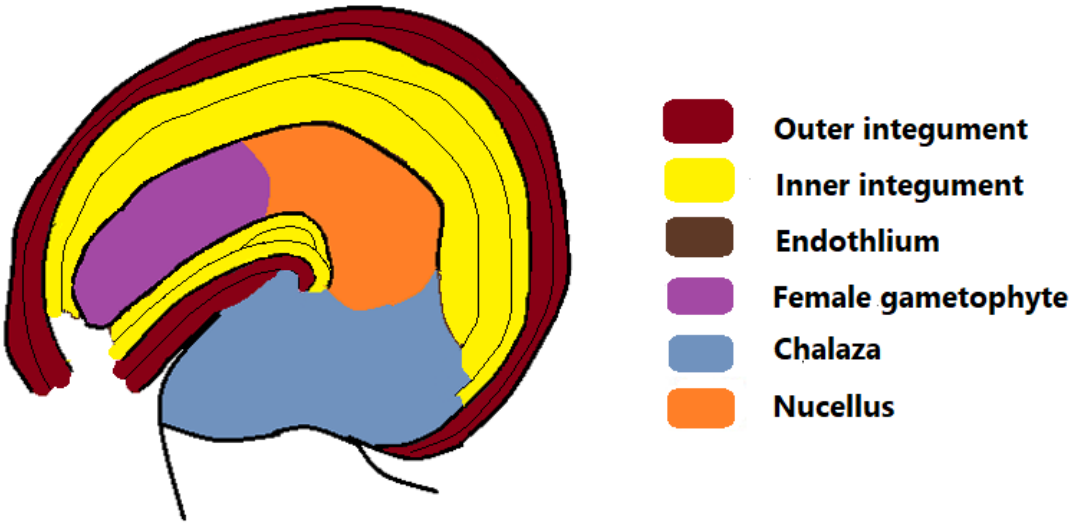

Figure 4. Arabidopsis mature ovule.

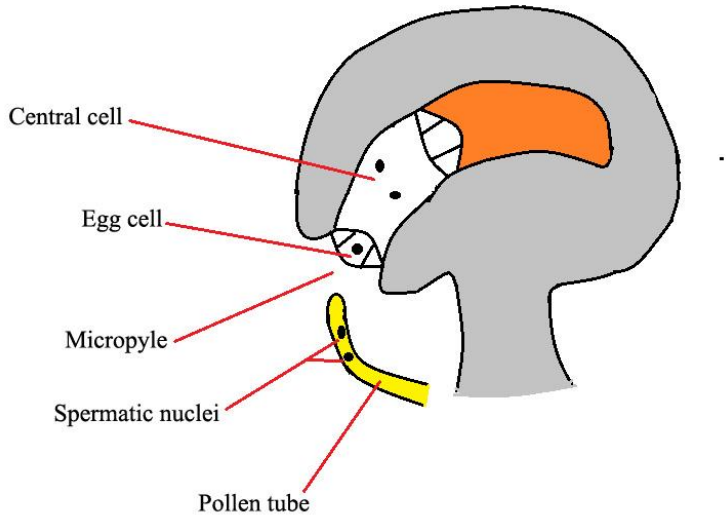

Figure 5. Double fertilization in Arabidopsis.

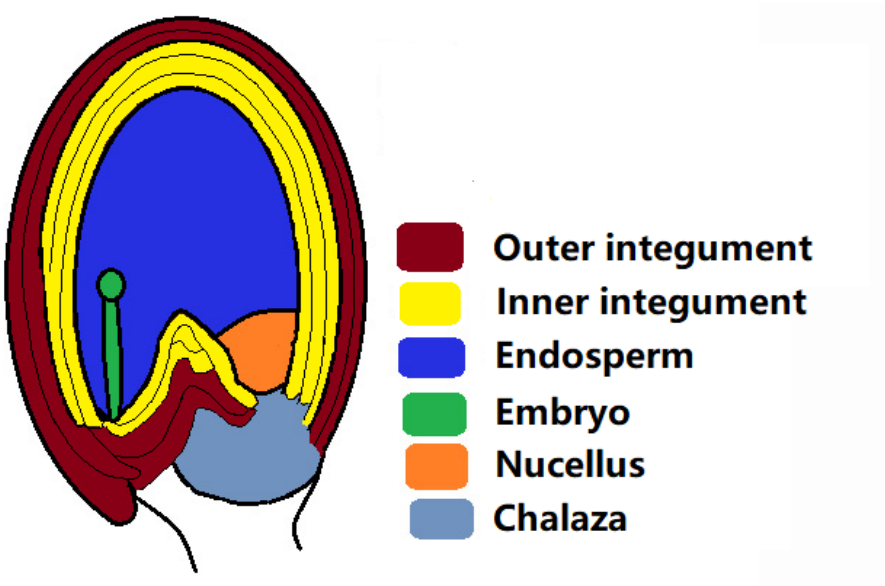

Figure 6. Arabidopsis globular embryo stage seed.

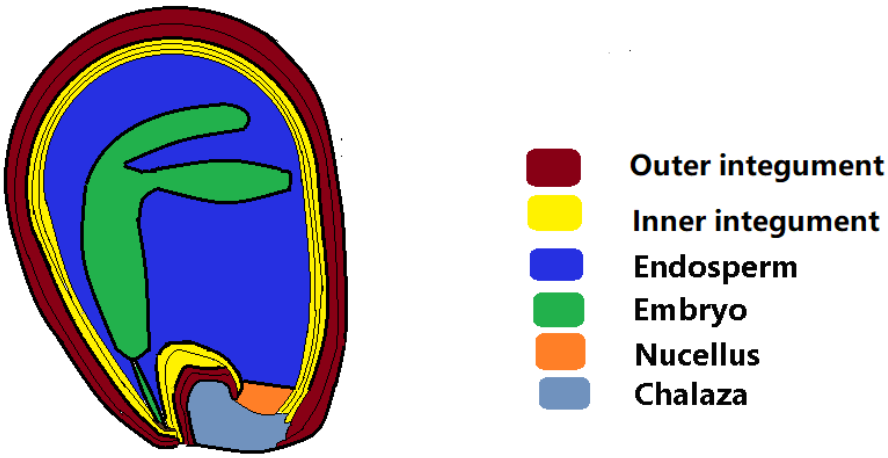

Figure 7. Arabidopsis walking stick embryo stage seed.

Seed tissue partitioning

Despite having a common function and same tissues, seeds evolved different architectures. In gymnosperms, the female gametophyte grows at the expense of the nucellus to become the main storage tissue alongside the embryo. Angiosperm seeds have been classified into three major architectures according to the relative volumes of the fertilization products, embryo and endosperm, and the nucellus (Fig. 8). In endospermic seeds (e.g., Arabidopsis and cereals), the endosperm surrounds the embryo and plays an important role in nutrient storing (Sreenivasulu and Wobus, 2013). By contrast, the endosperm of non-endospermic seeds (e.g., most legumes) is completely consumed by the embryo, which becomes the primary storage tissue (Weber et al., 2005). Finally,

perispermic seeds (e.g., pseudocereals such as amaranth and quinoa) develop a large perisperm, a tissue originating from the nucellus, alongside a minute endosperm (Burrieza et al., 2014). The ancestral condition of angiosperm seeds is still debated between endospermic and perispermic as basal angiosperms display either a large nucellus or endosperm as primary seed storage compartment (Friedman and Bachelier, 2013). Plants shifted several times between the endospermic and perispermic seed condition highlighting the antagonistic development of endosperm and nucellus as a defining mechanism for seed evolution (Lu and Magnani, 2018)

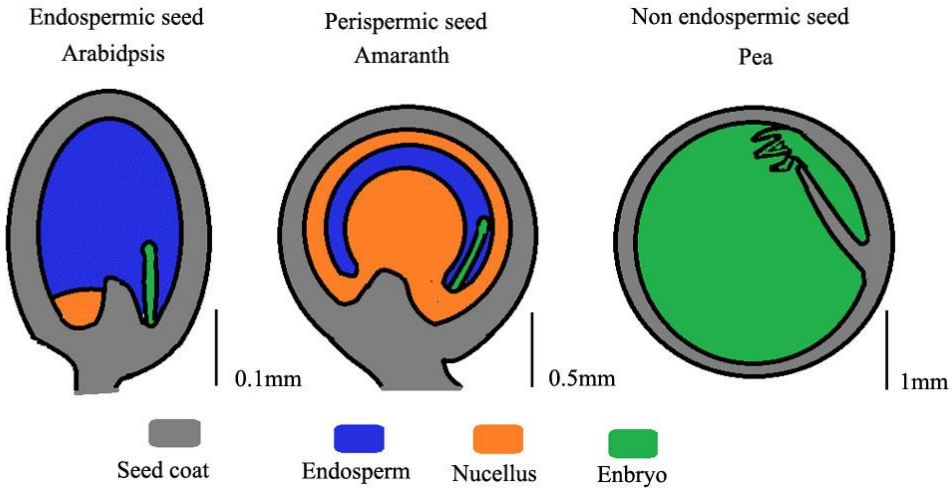

Figure 8. Angiosperm seed architectures.

In seed plants, the development of different seed architectures is achieved by the complete elimination of unwanted cells (cell elimination) of one tissue in favor of another tissue. Compared to other cell death programs that are ‘structure-conserving’, cell elimination results in the complete vanishing of cells, including the cell wall (Daneva et al., 2016; Ingram, 2017; van Doorn et al., 2011). In seeds, cell elimination has been observed in different tissues during development (Ingram, 2017). For example, the elimination of three out of four megaspores after meiosis and the degeneration of female gametophyte cells during or shortly after fertilization are observed in most angiosperm species. Cell elimination takes also place in maternal sporophyte tissues, as the nucellus or the integuments. After fertilization, cell elimination can also occur in the fertilization products, endosperm and the suspensor tissue of the embryo. Despite being a recurrent theme in seed development, cell elimination is still a poorly understood mechanism both at the biochemical and molecular level.

Seed nutrient partitioning

Tissue and nutrient partitioning are two inextricably linked processes. Such a diverse panorama of seed structures, described above, correlates therefore with an equally broad spectrum

of nutrient-storing strategies. Different seed tissues tend to accumulate different nutrients, thus different seed architectures may vary considerably in their nutrient content. The endosperm, which is rich in carbohydrates (mainly starch), accounts for most of the volume of the endospermic seed of the *Poaceae* family. As a consequence, about 80% of the nutrient composition of such grains is made of starch (Beloshapka et al., 2016). By contrast, non-endospermic seeds like soybeans, which carry a relatively large embryo, accumulate predominantly proteins and lipids (Yazdi - Samadi et al., 1977).

Sugars, lipids and proteins reserves in seeds

Starch is the main polysaccharide stored in seeds and it is the main source of carbohydrates for human nutrition. The biosynthesis of starch in both photosynthetic organs like leaves and in heterotrophic sink organs like seeds has been described. In plants, the photosynthetic tissues produce sucrose (Suc) as the major form of carbohydrates to be transported to the heterotrophic sink tissues (flower, root, seed, fruit) through the phloem (Fig 9). Once Suc reaches the sink tissue, it can be stored in vacuoles, further transported, hydrolyzed by invertases (INVs) or sucrose synthases (SuSys) into hexoses (glucose, Glc, and fructose, Fru), or metabolize into fructans by fructosyl transferases (FTs) (Ruan, 2014) (Fig 9). After being phosphorylated, hexoses are used for starch synthesis in the Amyloplasts or undergo glycolysis in Mitochondria (Stein and Granot, 2019) (Fig 9).

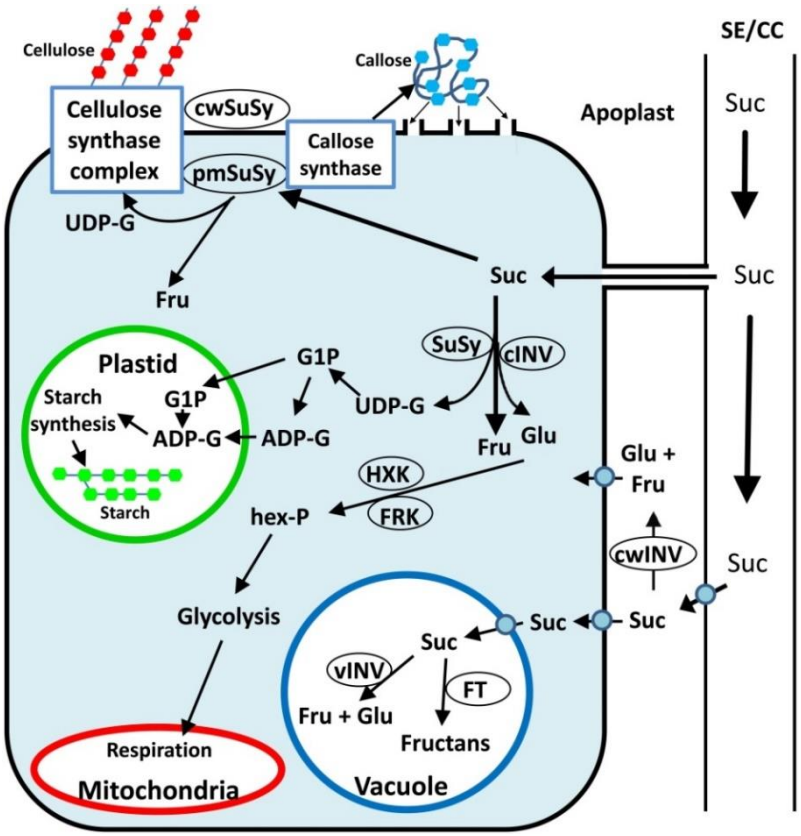

Figure 9 Sugar metabolism in sink tissue cells (Stein and Granot, 2019)

Oils are synthesized and stored in many plant tissues, where they act as energy reserves (Baud and Lepiniec, 2008). The storage lipids of seeds consist mainly of triacylglycerols (TAGs). The fatty acid profiles of seed oil vary greatly from one species to another (Voelker and Kinney, 2001). TAGs are stored in subcellular structures called oil bodies (Murphy, 1990). The abundance and location of these oil bodies in seed tissues vary considerably among species. For example, the embryos of soybean (*Glycinemax*) and sunflower (*Helianthus annuus*) is rich in oil bodies(Baud and Lepiniec, 2010). However, in castor bean, oils are mostly accumulated in the endosperm (Chen et al., 2007; Marriott and Northcote, 1975).

Plants provide most (around 58%) of the dietary protein for human nutrition compared to animal (Van Vliet et al., 2015). In seeds, proteins account for from 10% of dry seed weight in cereals to 40% in some legumes and oilseeds. With the exception of proteins with metabolic or structural roles, seed storage proteins (SSPs) are accumulated in the cotyledon and embryo in dicotyledonous plants and of the endosperm in monocotyledonous plants (Krishnan and Coe, 2001). SSPs have been classified into four main groups according to their solubility: water (albumins), dilute saline (globulins), alcohol-water mixtures (prolamines) and dilute acid or alkali (glutelins) (Osborne, 1924). SSPs serve as source of nitrogen, carbon and sulfur during seed germination and seedling growth.

Nutrients accumulation in the Arabidopsis seed

Arabidopsis seed development can be divided into three stages: the early embryogenesis stage, which is from fertilization until 7 days after flowering (DAF), the maturation phase, which is from 8DAF to 16 DAF, and the late maturation stage, which is from 17 DAF until dry seeds (Baud et al., 2002b). Developing Arabidopsis seeds initially accumulate starch, at 12 DAF the starch level reaches a peak and at the same time seed have very low content of lipids and protein. Starch accumulation decreases after 12DAF and the synthesis of lipids and proteins increases rapidly during the maturation phase (Andriotis et al., 2010; Baud et al., 2002b; Hills, 2004). In the late maturation stage, starch is hardly detectable, whereas lipids and proteins account for 45% of the seed weight (Andriotis et al., 2009; O'Neill et al., 2003).

The distribution of starch in seed coat and embryo was examined by iodine staining. In the seed coat, starch is visible at 4DAF (Fig. 10). The staining intensity increases until the cotyledon embryo stage. After the cotyledon embryo stage, starch in the seed coat decreases and is not detectable in mature seeds. At the torpedo embryo stage, starch granules are detectable in two regions, one is above the tip of the radicle and a second one is in the hypocotyl (Fig. 10). At the walking stick embryo stage, starch accumulates both in radicle and hypocotyl. At the early cotyledon embryo stage, starch is present in cotyledons as well as in radicle and hypocotyl. After 12

DAF, starch accumulation in the embryo decreases rapidly. At the expanded cotyledon embryo stage, starch is present only in the radicle tip (Andriotis et al., 2010).

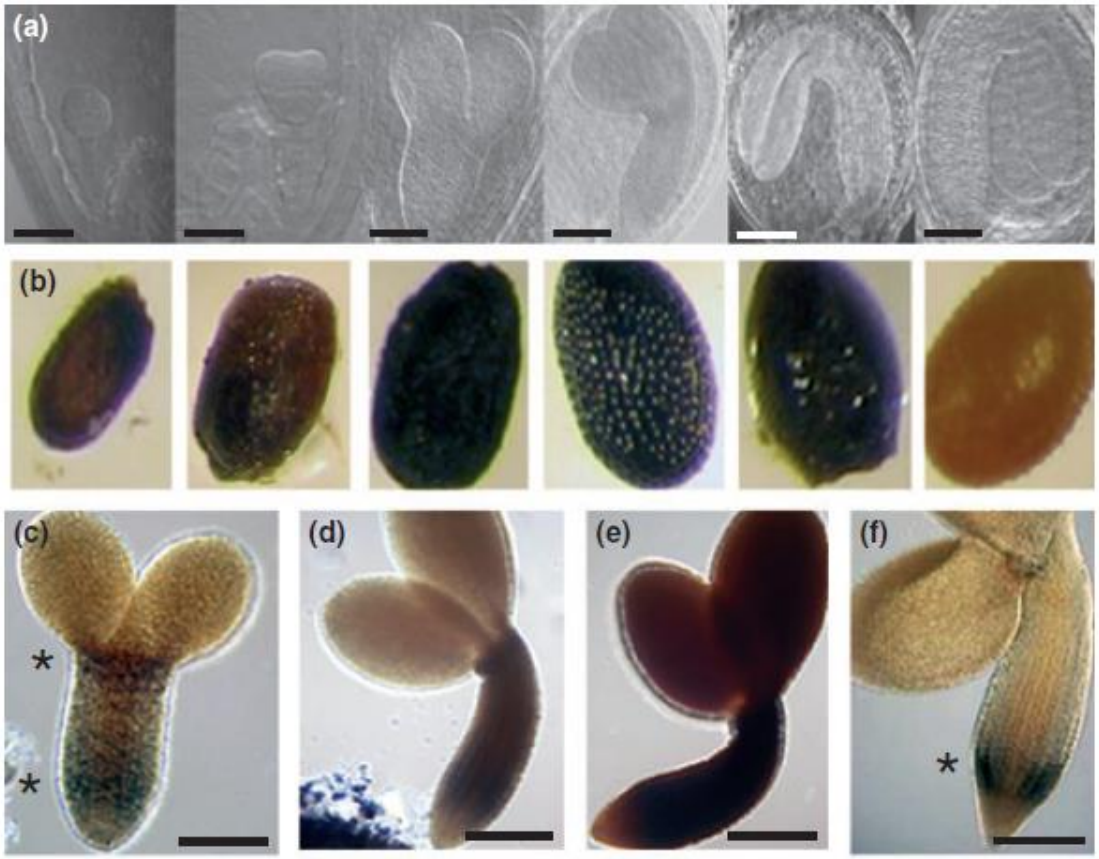

Figure 10 Starch accumulation during seed development (Andriotis et al., 2010).
(a) The embryo developmental stages. From left to right: globular embryo, heart embryo, torpedo embryo, walking stick embryo, and expanded cotyledon embryo. Bars, 50 μm for the first four stages; 100 μm for the last two stages. (b) Starch accumulation in seeds, corresponding to the embryo stages above, stained with iodine solution. (c)-(f) dissected embryos stained with iodine solution to visualize starch and observed under DIC optics. (c) Embryo at the torpedo stage, 8 DAF. (d) Embryo at the walking stick stage, 10 DAF. (e) Embryo at the early cotyledon stage, 12 DAF. (f) Embryo at the expanded cotyledon stage.

Nutrient transport in seeds

What all angiosperm seeds have in common is the allocation of resources from the placental maternal tissue, through the chalaza, to the storage tissues following a source-sink nutrient gradient (Patrick and Offler, 2001). In most angiosperms, vascularization arrests at the chalaza, and nutrients follow a combination of symplastic and apoplastic pathways to reach the sink tissues. Though, there are examples of nucellar tracheids, an ancestral character also observed in extinct gymnosperms, and vascularized seed coats (Johri et al., 2013).

Symplastic pathway

Seed maternal tissues have been shown to be symplastically connected in most studied species. Therefore, nutrients supposed to be unloaded from the phloem symplastically and move along the

maternal tissues through plasmodesmata (Ellis et al., 1992; Offler and Patrick, 1984; Stadler et al., 2005; Thorne, 1985). In Arabidopsis, GFP mobility assays have shown that sucrose reaches the seed through the funicular phloem, which is symplastic connected to the seed coat (Stadler et al., 2005). However endosperm and embryo are not symplastic connected to the surrounding maternal tissues and are, therefore, supposed to receive nutrients from the apoplast (Stadler et al., 2005).

Apoplastic pathway

Apoplast transport has been broadly described in angiosperms seeds. Many studies have shown that transporters allow the release of nutrients from the maternal tissues into the apoplast and the uptake of the same nutrients by the fertilization products. The SWEET passive facilitators, have been shown to allow the export of sucrose and hexoses from seed maternal tissue cells into the apoplast in different species (Chen et al., 2012b; Sosso et al., 2015; Yang et al., 2018a). Plant SWEET transporters have seven transmembrane helixes (TMs) arranged into two triple-helix-bundles (THBs) (Fig. 11). The two THBs are organised in a parallel orientation and function as a single unit. The THBs at the N and C terminus of the protein are fused together by the TM4 linker helix (Chen et al., 2010). Plant SWEET transporters originated from gene duplication of prokaryotic semiSWEETs, which contain only one THB (Chen et al., 2010; Xuan et al., 2013) (Fig. 11).

There are 17 *SWEET* genes in Arabidopsis: *AtSWEET1/2/4/5/7/8/13/17* are monosaccharide transporters (Chardon et al., 2013; Chen et al., 2010) whereas *AtSWEET9/10/11/12/14/15* function as sucrose transporters(Chen et al., 2015; Chen et al., 2012b; Kanno et al., 2016; Lin et al., 2014; Rottmann et al., 2018). *AtSWEET16* is able to transport sucrose, fructose, glucose(Klemens et al., 2013). *AtSWEET15*, *AtSWEET12*, and *AtSWEET11* are expressed in the seed coat and release sucrose in the apoplast (Chen et al., 2015). *AtSWEET 11* is also expressed in the endosperm, thus suggesting that it might play a role in reimporting sucrose in the endosperm. Triple *sweet11;12;15* mutant seeds showed retarded embryo development, reduced seed weight, and reduced starch and lipid content, causing a "wrinkled" seed phenotype, thus indicating their paramount importance in sugar allocation to the seed (Chen et al., 2015).

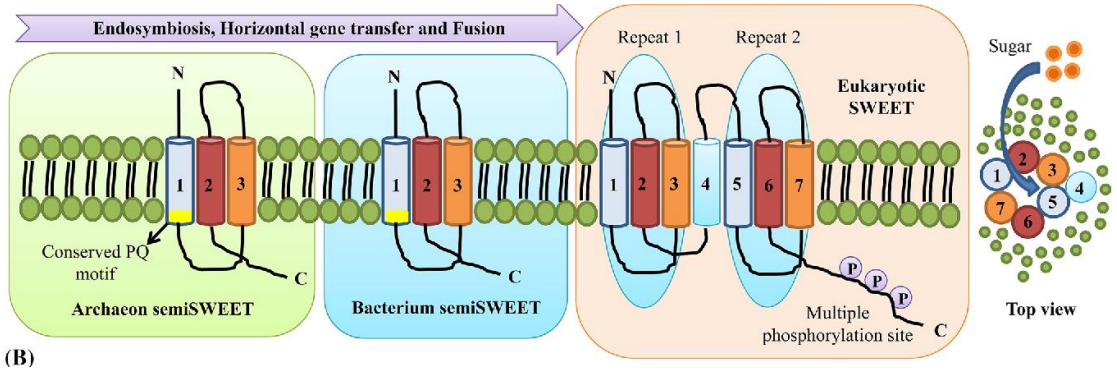

Figure 11 SWEETs domain structure (Jeena et al., 2019)

Apoplastic sucrose can be then converted into hexoses by the action of INVERTASEs (INVs) (Ruan et al., 2010). In maize, the export of sucrose in the endosperm apoplast by the ZmSWEET4c facilitator and its further hydroxylation into hexoses by a cell wall INV has been shown to be a key step in seed filling (Sosso et al., 2015). Loss of an endosperm specific cell wall INV results, indeed, in a miniature seed phenotype (Miller and Chourey, 1992).

Seed tissue and nutrient partitioning a case for nucellus

(Lu and Magnani, 2018) , attached below

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REVIEW

Seed tissue and nutrient partitioning, a case for the nucellus

Jing Lu[1,2] · **Enrico Magnani**[1]

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Abstract
Flowering plants display a large spectrum of seed architectures. The volume ratio of maternal versus zygotic seed tissues changes considerably among species and underlies different nutrient-storing strategies. Such diversity arose through the evolution of cell elimination programs that regulate the relative growth of one tissue over another to become the major storage compartment. The elimination of the nucellus maternal tissue is regulated by developmental programs that marked the origin of angiosperms and outlined the most ancient seed architectures. This review focuses on such a defining mechanism for seed evolution and discusses the role of nucellus development in seed tissues and nutrient partitioning at the light of novel discoveries on its molecular regulation.

Keywords Ovule · Seed · Nucellus · Perisperm · Endosperm · Partitioning

Introduction

Tissue partitioning is the driving force that shapes the development of different seed structures. The relative contribution of each tissue to the final seed mass varies considerably among species and underlies different nutrient-storing strategies. Tissue partitioning is achieved through cell elimination programs that regulate the degeneration of one tissue in favor of another (Ingram 2017). The nucellus, the most distal maternal tissue of the ovule primordium (the seed precursor) responsible for the formation of the female gametophyte, plays a key role in defining the seed structure together with the fertilization product/s. In gymnosperms, most of the nucellus is eliminated and replaced by the female gametophyte, the main storage tissue, which will be in turn absorbed by the developing embryo, the only fertilization product (Fig. 1) (Linkies et al. 2010). Angiosperm seeds have been classified into three major architectures according to the relative volumes of the fertilization products, embryo and endosperm, and the nucellus (Fig. 1). In mature endospermic seeds (e.g., cereals), the endosperm surrounds the embryo and plays an important role in nutrient storing (Sreenivasulu and Wobus 2013). By contrast, the endosperm of non-endospermic seeds (e.g., most legumes) is completely consumed by the embryo, which becomes the primary storage tissue (Weber et al. 2005). Finally, perispermic seeds (e.g., pseudocereals such as amaranth and quinoa) develop a large perisperm, a tissue originating from the nucellus, along with a minute endosperm (Burrieza et al. 2014). The ancestral condition of angiosperm seeds is still debated between endospermic and perispermic as basal angiosperms display either a large nucellus or endosperm as primary seed storage compartment (Friedman and Bachelier 2013). Plants shifted several times between the endospermic and perispermic seed condition highlighting the antagonistic development of endosperm and nucellus as a defining mechanism for seed evolution.

Recent discoveries on the molecular regulation of nucellus elimination have given an insight into the process of seed tissues partitioning. Here, we discuss them in the context of angiosperm seed natural diversity. Finally, we review nutrient transport and accumulation in the nucellus across different seed architectures to present seed tissue and nutrient partitioning as two coherent and inextricably linked aspects of seed development.

A contribution to the special issue ‘Seed Biology’.

Communicated by L. Lepiniec, H. North, G. Ingram.

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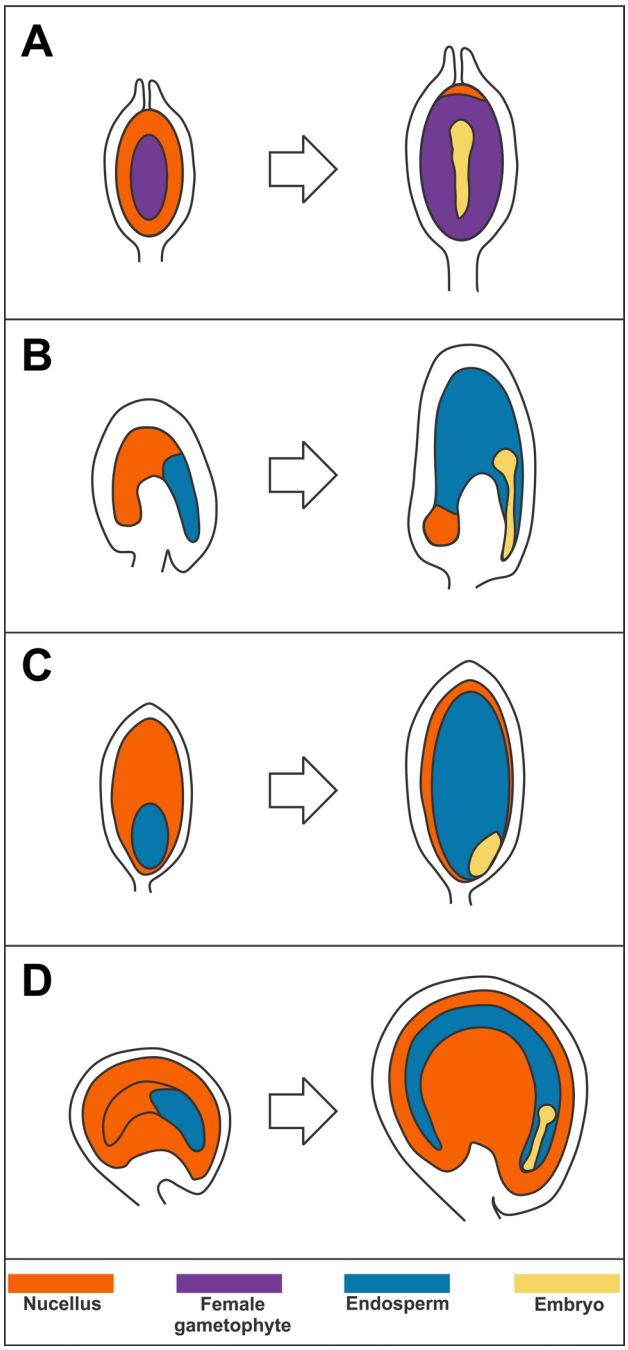

Fig. 1 Seed architectures. Diagrammatic representation in longitudinal sections of pine (gymnosperm) (**a**), Arabidopsis (angiosperm, endospermic) (**b**), rice (angiosperm, endospermic) (**c**), and quinoa (angiosperm, perispermic) (**d**) seeds right after fertilization and at an early embryogenesis stage. The figure is not in scale. Female gametophyte, nucellus, endosperm, and embryo are highlighted in violet, orange, blue, and yellow, respectively

Natural diversity in nucellus morphology

Angiosperm ovules have been classified according to their nucellus position and thickness (Endress 2011). A first general distinction is made between ovules that bear nucellus hypodermal cells above the megaspore mother cell (MMC) (crassinucellar) and those that display only distal epidermal nucellus cells (tenuinucellar). Crassinucellar ovules are considered ancestral to tenuinucellar, as they are present in basal angiosperms, magnoliids, most monocots, and basal and part of the core eudicots. They are further classified into (1) truly crassinucellar, if they carry two or more distal hypodermal nucellus cell layers, (2) weakly crassinucellar when they display only one hypodermal cell layer, or (3) pseudo-crassinucellar if the distal nucellus epidermal cell layer divides periclinally to form additional cell layers, in the absence of hypodermal cells. The tenuinucellar condition, observed in several monocots and part of the core eudicots, includes (4) incompletely tenuinucellar ovules, which display hypodermal nucellus cells proximal and/or lateral to the MMC, (5) truly tenuinucellar ovules, without any hypodermal nucellus cell, and (6) reduced tenuinucellar ovules, when the proximal region of the MMC is not fully enclosed by the nucellus. Further terminology has been created to describe specific nucellus regions. In pseudo-crassinucellar ovules, the dermal layers of the nucellus apex (at the micropylar region) undergoing periclinal cell divisions are called "nucellar cap." In extreme cases, the nucellus apex divides massively to form a "nucellar beak" that can extend outside the seed coat and define the micropyle. Nucellus epidermal cells can also elongate radially around the female gametophyte to form a so-called nucellar pad (Johri et al. 2013). A persistent nucellus base, at the chalazal side, is instead referred to as "podium" or "postament" if only its axial part persists (Johri et al. 2013). Overall, this classification highlights the great natural diversity in ovule nucellus size, which sets the premises for tissue partitioning programs later on in development.

Nucellus architecture changes during ovule and seed development. The female gametophyte grows at the expense of the nucellus which is partially eliminated, a process that is still almost completely unexplored (Johri et al. 2013). After fertilization, the nucellus of endospermic and non-endospermic seeds partially degenerates to make space to endosperm and embryo growth. By contrast, perispermic seeds display a large central nucellus (perisperm) that grows to become the main storage tissue along a minute endosperm. Variations have been observed in between these extreme seed architectures. A retard in the elimination of the nucellus is a hallmark of coffee grains. In coffee, the nucellus grows to define seed size and is then replaced by the endosperm (Alves et al. 2016; Mayne 1937). Similarly, the nucellus of *Austrobaileya scandens* seeds drives early seed growth and is then eliminated by the endosperm, whose further development determines final seed size (Losada et al. 2017). By contrast, nucellus and endosperm coexist and display a similar volume in *Acorus calamus* seeds (Floyd and Friedman 2000).

Furthermore, the structure of *Malpighiaceae* seeds appears perispermic during early seed development but the nucellus is fully eliminated by the embryo at later stages (Souto and Oliveira 2014). Finally, *Podostemaceae* ovules do not undergo central cell fertilization and lack an endosperm. In these species, the nucellus cell walls proximal to the female gametophyte break down to produce a multinucleate cytoplasmic structure termed "nucellar plasmodium" (Arekal and Nagendran 1975, 1977).

A mechanical role for the nucellus has also been hypothesized. The anticlinal cell walls of the rice nucellus epidermis, surrounding the endosperm, are uniquely thickened with cellulosic material and have been speculated to provide mechanical support (Krishnan and Dayanandan 2003). Similarly, the chalazal or micropylar nucellus cells can differentiate into the so-called hypostase and epistase, respectively. The cell walls of these nucellar structures thicken and accumulate cutin, suberin, lignin, or callose. Hypostase and epistase have not been assigned a clear function yet but are thought to play a mechanical role or work as apoplastic barriers (Johri et al. 2013).

Tissue partitioning

Nucellus elimination in Arabidopsis

In Arabidopsis seeds, nucellus elimination begins 2 days after flowering (DAF) and progresses in a distal–proximal fashion to achieve the loss of 50% of its cells by 8 DAF. A few layers of proximal nucellus cells persist and expand with the rest of the ovules to form a gate between chalaza and endosperm till embryo maturity (Xu et al. 2016).

Elimination of the nucellus, as well as seed coat growth, is triggered by the endosperm (Fig. 2) (Roszak and Kohler 2011; Xu et al. 2016). Single fertilization of the central cell is necessary and sufficient to initiate nucellus degeneration. The MADS box transcription factor AGAMOUS LIKE 62 (AGL62) is specifically expressed in the endosperm and essential for nucellus–endosperm communication. *agl62* mutant seeds display precocious endosperm cellularization and fail to undergo nucellus degeneration and seed coat differentiation. Figueiredo and co-workers have recently proposed that AGL62 regulates auxin efflux, considered the fertilization signal that coordinates the development of endosperm and maternal tissues (Figueiredo et al. 2016). Nevertheless, this model has been tested solely on seed coat growth and not on nucellus degeneration. Two alternative scenarios have been proposed to explain nucellus elimination: The endosperm might generate mechanical signals while growing against the nucellus or act as strong nutrient sink, thus triggering death of neighboring tissues by nutrient deprivation (Ingram 2017). It has been argued that the latter two models are less favorable to explain endosperm-maternal tissue developmental coordination as *titan 2* mutant seeds, which undergo early endosperm arrest comparable to *agl62* (Liu and Meinke 1998), show signs of seed coat growth (Roszak and Kohler 2011) and nucellus degeneration (personal observations).

Regardless of the nature of the signaling mechanism, it has been shown that endosperm growth relieves the repressive action mediated by Fertilization-Independent Seed (FIS) Polycomb Group (PcG) proteins on nucellus degeneration (Xu et al. 2016). Compared to other FIS genes that are solely

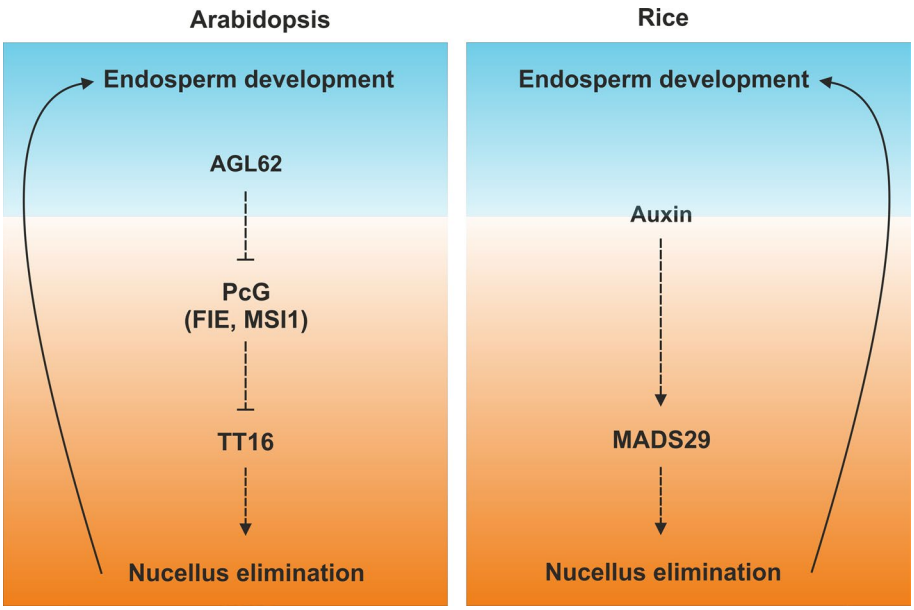

Fig. 2 Signaling pathways underlying nucellus and endosperm antagonistic development. Arrows indicate functional relationships

expressed in the ovule central cell, *Fertilization-Independent Endosperm* (*FIE*) and *Multicopy Suppressor of IRA1* (*MSI1*) are also expressed in the nucellus and seed coat (Kohler et al. 2003; Xu et al. 2016). Both *fie*/+ and *msi1*/+ mutants display high penetrance of autonomous seed coat growth (Roszak and Kohler 2011) and nucellus degeneration (Xu et al. 2016) in the absence of fertilization. Downstream of PcG proteins, TRANSPARENT TESTA 16 (TT16) and GORDITA (GOA) MADS BOX transcription factors promote nucellus elimination and inhibit cell division (Xu et al. 2016). TT16 regulates nucellus cell elimination in part by repressing the expression of *HVA22d*, which inhibits gibberellin-mediated programmed cell death (PCD) and autophagy. Furthermore, a papain-type KDEL-tailed cysteine endopeptidase (CysEP), involved in PCD of vegetative tissues, has been shown to be expressed in the distal nucellus undergoing degeneration (Zhou et al. 2016). Nevertheless, nucellus elimination has not been entirely assigned to any known cell death program. As in vacuolar PCD (van Doorn et al. 2011), nucellus cells undergo autophagy. By contrast, the nucellus displays protoplast shrinkage and largely unprocessed cell corpses, which are hallmarks of necrosis (van Doorn et al. 2011). Another example of PCD that combines signs of vacuolar and necrotic cell death is induced by the successful recognition of pathogens during hypersensitive response (HR) (van Doorn et al. 2011). Nevertheless, PCD associated with HR does not exhibit degradation of the cell wall as in the nucellus. Furthermore, mutations in the *METACASPASE1* and *LESION SIMULATING DISEASE1* genes, which encode components of the HR-PCD machinery (Coll et al. 2010), do not affect nucellus development (Xu et al. 2016).

As endosperm growth is necessary to initiate nucellus elimination, the persistence of the nucellus in *tt16* mutant seeds negatively affects endosperm development revealing an antagonistic development of endosperm and nucellus (Xu et al. 2016). This antagonism is reflected in the evolution of the two most ancient seed structures, perispermic and endospermic, which rely on nucellus or endosperm as major storage tissue, respectively.

Nucellus elimination in cereals

In cereals, the nucellus accounts for most of the grain volume at anthesis and it is eliminated after fertilization in a centripetal fashion. At grain filling, only the outermost nucellus cell layer (nucellus epidermis) and a few nucellus cell layers overlaying the ovule vascular trace at the chalazal side are retained and undergo PCD more or less rapidly according to the species. The chalazal nucellus of maize grains appears as compact layers of dead cells with limited plasmodesmata connections (Felker and Shannon 1980; Kladnik et al. 2004). In sorghum, the chalazal nucellus consists of a few large cell layers which are reduced to one during development and whose symplastic connection with the chalaza is interrupted (Dwivedi et al. 2014; Maness and McBee 1986; Wang et al. 2012). Rice, *Brachypodium*, barley, and wheat grains develop instead the so-called nucellar projection, a tissue dedicated to nutrient transport simplastically connected to the placenta (Krishnan and Dayanandan 2003; Opanowicz et al. 2011; Oparka and Gates 1981; Radchuk et al. 2009, 2011; Wang et al. 1994a, b, 1995; Zheng and Wang 2011). The nucellar projection of barley and wheat grains is more developed and has been divided into different regions based on cell morphology: (starting from the integument inward) (1) actively dividing cells, (2) elongating cells, (3) transfer cells with wall ingrowth, and (4) cell debris (Radchuk et al. 2006; Thiel et al. 2008; Wang et al. 1994a; Zheng and Wang 2011). By contrast, the nucellus epidermis of rice and *Brachypodium* appears larger and more persistent, compared to other cereals (Ellis and Chaffey 1987; Opanowicz et al. 2011; Oparka and Gates 1981). Finally, the chalazal nucellus physically touches the endosperm in maze, *Brachypodium,* and rice, while it is separated by a cavity filled with nucellar lysate (referred to as "endosperm or nucellar cavity" or "placental sac") in wheat, barley, and sorghum.

The Arabidopsis signaling pathway underlying nucellus development is partially conserved in rice grains (Fig. 2). The rice *TT16* orthologous gene, *MADS29*, is expressed in the nucellus and nucellar projection and promotes cell elimination (Nayar et al. 2013; Yang et al. 2012; Yin and Xue 2012). Compared to Arabidopsis, *MADS29* is also expressed in the embryo and the protein has been detected in nucellus epidermis, embryo, and endosperm but not in the nucellar projection (Nayar et al. 2013). MADS29 directly activates the expression of nucleotide-binding site–leucine-rich repeat proteins and Cys proteases (Yin and Xue 2012). In line with the Arabidopsis endosperm-maternal tissue signaling model (Figueiredo et al. 2016), *MADS29* expression is induced by auxin and regulates auxin–cytokinin homeostasis (Nayar et al. 2013; Yin and Xue 2012). Furthermore, antagonistic development of nucellus and endosperm has been observed also in rice as suppression of *MADS29* expression impairs starch accumulation and endosperm growth (Nayar et al. 2013; Yang et al. 2012; Yin and Xue 2012).

In barley grains, nucellus elimination correlates with the expression of genes encoding for Asp protease-like protein nucellin, vacuolar processing enzyme nucellain, Cys and Asp endopeptidases, subtilisin-like Ser proteinases, and JEKYLL protein, all known to play a role in PCD (Chen and Foolad 1997; Linnestad et al. 1998; Radchuk et al. 2006, 2011, 2018; Thiel et al. 2008; Tran et al. 2014). Down-regulation of *jekyll* by RNA interference affects nucellus elimination and nucellar projection differentiation and, indirectly, endosperm development and starch accumulation (Radchuk et al. 2006). Furthermore, the differentiation gradient along the barley nucellar projection is also regulated by a

gibberellin-to-abscisic acid balance, with gibberellin promoting differentiation (Weier et al. 2014).

Morphological analyses of nucellus parenchymal cells in wheat revealed fragmentation of the cytoplasm, vacuolization, disruption of the nuclear envelope and plasma membrane, and mitochondrion structural alterations (Dominguez et al. 2001). Nevertheless, the authors of this study might have erroneously located nucellus cells as what it is indicated as nucellus parenchymal cell in Fig. 2b appears to be integument cells. In line with this interpretation, the cells analyzed do not undergo degeneration of the cell wall. Nucellus epidermis and nucellar projection of wheat grains have been shown to express genes encoding for carboxypeptidase III, thiol protease, nucellain, and nucellin, some of which are also implicated in aleurone death during germination (Domınguez and Cejudo 1998; Drea et al. 2005). A parallel has been drawn between wheat and *Brachypodium* nucellus, which also expresses nucellain during its elimination (Opanowicz et al. 2011).

Finally, the study of the maize invertase *Miniature 1* (*Mn1*) gene revealed a mechanical interaction between nucellus and endosperm. Maize grains mutated for the *Mn1* gene show a gap between the nucellus cells, which are rapidly emptied of their nuclear and cytoplasmic material, and the endosperm. Such a gap is not due to cell death but to an underdeveloped endosperm that results in over-expanded nucellus cells, thus suggesting that the endosperm exercises a mechanical force on the nucellus (Kladnik et al. 2004).

Overall, these data indicate that a protease-dependent cell death machinery is shared by cereals to achieve nucellus degeneration. These same types of proteases, even though not necessarily the same genes, appear to drive endosperm cell death. On the other hand, more data are necessary to highlight variations in the nucellus elimination pathways responsible for the slightly different nucellus fates observed in different cereals.

Nucellus elimination in other angiosperms

Similar to Arabidopsis and cereals, a number of other angiosperm seeds have been shown to undergo early nucellus elimination in a progressive fashion starting from the nucellus–endosperm border toward the chalazal region. Proteomics and genetic analyses revealed the presence of Cys endopeptidases and other peptidases associated with PCD in the nucellus of castor bean seeds (Greenwood et al. 2005; Nogueira et al. 2012). Cys endopeptidases are accumulated in ricinosomes, organelles derived from the endoplasmic reticulum that collapse upon nucellus degeneration releasing their content in the cytoplasm and contributing to the digestion of proteinaceous debris (Greenwood et al. 2005). In *Sechium edule*, nucellus elimination correlates with the induction of caspase-like proteases and high levels of hydrogen peroxide, nitric oxide, and ethylene, which has been proposed as the signaling molecule between endosperm and nucellus (Lombardi et al. 2007, 2010, 2012). High level of indole acetic acid has also been detected in endosperm and nucellus of *Sechium edule* seeds but its role in nucellus development is still unclear (Lombardi et al. 2012). By contrast, the nucellus of peach seeds displays a pick of abscisic acid after anthesis, thus suggesting that different hormones might play a role in nucellus degeneration in different species (Piaggesi et al. 1991).

Nucellus retention

Perispermic seeds such as quinoa, amaranth, *Peperomia*, spinach, and *Nymphaeales* display a large nucellus, which defines seed size and becomes the major storage tissue, along a minute endosperm. The process has been well studied in quinoa. At anthesis, the nucellus reaches its final number of cells as its mitotic activity arrests. After fertilization, a relatively small endosperm grows at the expense of part of the nucellus and leads the way to embryo development which in turn consumes most of the endosperm and part of the nucellus. The central nucellus, termed perisperm, is not eliminated and undergoes cell expansion, endoreduplication, reserve accumulation, and PCD. Nucellus cell death involves nuclease and proteolytic activity but not cell wall degeneration, a process comparable to endosperm cell death in endospermic seeds (Burrieza et al. 2014; Lopez-Fernandez and Maldonado 2013).

Nutrient partitioning

Tissue and nutrient partitioning are two inextricably linked processes. Such a diverse panorama of seed structures correlates therefore with an equally broad spectrum of nutrient-storing strategies. What all angiosperm seeds have in common is the allocation of resources from the placental maternal tissue, through the chalaza, to the storage tissues following a source-sink nutrient gradient (Patrick and Offler 2001). In most angiosperms, vascularization arrests at the chalaza, and nutrients follow a combination of symplastic and apoplastic pathways to reach the sink tissues. Nevertheless, there are examples of nucellar tracheids, an ancestral character also observed in extinct gymnosperms, and vascularized seed coats (Johri et al. 2013).

Sugar transport in endospermic seeds

The role of the nucellus in nutrient transport has been mostly addressed studying cereal grain filling. In cereals, nutrients are supposed to travel simplastically from the phloem through the maternal tissues of the chalazal region to then

being released into the apoplast. The endosperm, which is not simplastically connected to the maternal tissues, uploads nutrients from the apoplast and accumulates mostly starch while undergoing PCD (Thorne 1985). The nucellus lies at the interface of maternal and endosperm tissues and can play a role in nutrient transfer.

In maize grains, sucrose moves simplastically from the phloem to the chalaza and is then released into the apoplast where cell wall-bound invertases convert it into hexoses, glucose and fructose (Felker and Shannon 1980; McLaughlin and Boyer 2004; Porter et al. 1985; Shannon 1972a, b; Tang and Boyer 2013). The nucellus is not simplastically connected to the chalaza and imports glucose during the first stages of grain development while being eliminated by endosperm growth (McLaughlin and Boyer 2004; Tang and Boyer 2013). Later in development, persistent nucellus cells undergo PCD (Felker and Shannon 1980; Kladnik et al. 2004), thus suggesting that nutrients cross the nucellus apoplastically. A similar path of sugar transport probably occurs in sorghum grains as they accumulate hexoses in the placental sac and display symplastic disconnection of chalaza and nucellus (Dwivedi et al. 2014; Maness and McBee 1986; Wang et al. 2012).

By contrast, the nucellus of wheat and barley is simplastically connected to the placenta and the nucellar projection develops transfer cells, thus expanding the nutrient unloading zone and facilitating transfer (Radchuk et al. 2009, 2011; Wang et al. 1994a, b, 1995; Zheng and Wang 2011). At the beginning of barley seed development, starch accumulates mostly in the pericarp, which acts as a short-term sink, and only transiently in the nucellus. Alpha amylase 4 is expressed in degenerating nucellus tissue facilitating mobilization of starch toward the endosperm during nucellus elimination (Radchuk et al. 2006, 2009). At barley grain filling, ^{13}C sucrose analyses revealed a flow of sucrose from the nucellar projection toward the endosperm (Melkus et al. 2011; Rolletschek et al. 2011). The nucellus projection of barley grains expresses a cell wall-bound invertase, indicating that hexoses are also released into the endosperm cavity (Weschke et al. 2003). Furthermore, barley nucellar projection and epidermis express members of the aquaporin family, which may play a role in nutrient efflux (Thiel et al. 2008). Interestingly, transfer cells of the nucellar projection and endosperm of barley and wheat express the same sucrose symporter (*SUT*) genes responsible for sucrose import in sink tissues (Bagnall et al. 2000; Weschke et al. 2000). The role of SUT proteins in the nucellus is not clear, and it might allow sucrose scavenging, work as sucrose passive port along concentration gradient or be an evolutionary relic of perispermic seeds. Impaired development of the barley nucellar projection leads to starch accumulation in maternal tissues at the expense of the endosperm, thus further proving the importance of this tissue in nutrient partitioning (Melkus et al. 2011; Radchuk et al. 2006; Rolletschek et al. 2011).

Rice and *Brachypodium* grains develop a smaller nucellar projection than barley and wheat, but display a thicker nucellus epidermis which has been proposed to play an active role in nutrient transport (Ellis and Chaffey 1987; Opanowicz et al. 2011; Oparka and Gates 1981). Defective starch synthesis in the endosperm has been observed in rice grains with suppressed *MADS29* expression, highlighting the active role of the rice nucellus in transferring nutrients to the endosperm (Nayar et al. 2013; Yin and Xue 2012). During nutrient transfer, the nucellus might act as a short-term sink as MADS29 has been found to promote the differentiation of proplastids in amyloplasts likely by regulating cytokinin biosynthesis (Nayar et al. 2013). Finally, SWEET sucrose exporters have been found in all rice nucellar tissues, indicating that the nucellus engages in apoplastic seed filling (Yang et al. 2018).

Sugar transport in perispermic seeds

The role of the nucellus in perispermic seeds changes from nutrient-transport facilitator to long-term nutrient sink. The perisperm of quinoa seeds accumulates mostly starch while undergoing PCD (Lopez-Fernandez and Maldonado 2013). Starch accumulation follows an apical–basal pattern, with the chalazal side being the last to be filled. Such a pattern might be the result of sugars transport from the chalaza toward the perisperm while maximizing seed filling by following a source-sink gradient. Alternatively, nutrient transport through the seed coat might also explain such a nutrient accumulation pattern. At seed maturity, perisperm cells appear as thin walled and completely filled with starch grains, similar to cereal starchy endosperm cells (Lopez-Fernandez and Maldonado 2013; Prego et al. 1998). By contrast, the role of the few endosperm cells that persist at the micropylar region is less clear.

Conclusive remarks

The evolution of seed storage tissues in angiosperms has been a "battle" between endosperm and nucellus development. Both tissues can store starch and become the main source of energy for embryo germination. Indeed, the nucellus of perispermic seeds parallels the endosperm of endospermic seeds at both morphological and functional levels. Nevertheless, most angiosperm seeds evolved mutually exclusive growth of nucellus and endosperm. The nucellus offers an easier system of nutrient storage simplastically connected to the placenta. By contrast, the endosperm couples

nutrient storing to fertilization, thus possibly avoiding energy waste in case of unsuccessful fertilization.

Whereas we have a better understanding on seed nutrient transport and tissue elimination, the next challenge will be to address how nutrient and tissue partitioning are coordinated at the molecular level.

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The end of the introduction

A change in nucellus cell fate led to the evolution of seed plants from non-seed plants and, subsequently, drove the evolution of different seed architectures. When I started my thesis, we had a general understanding of the nucellus elimination process in Arabidopsis, driven by the action of the MADS domain transcription factor TRANSPARENT TESTA 16 (TT16). The process of nucellus cell elimination was known to be conserved in cereals and, at least in rice, under the control of the *TT16* orthologue gene *MADS29*. As we had a good understanding of the process of nucellus elimination, nothing was known on its mechanism of arrest in both Arabidopsis and cereals as well as unknown was the opposite process of nucellus growth in perispermic seeds. Furthermore, the physiological role of the persistent nucellus had been explored in cereals, endospermic seeds, and in the quinoa perispermic seed. In rice, for example, the persistent nucellus specializes in the transport of nutrients to the endosperm and expresses SWEET facilitators to facilitate the transit of sugars into the apoplast. In perispermic seeds, the nucellus was instead known to accumulate nutrients. Despite light was shed on the mechanisms of both nutrient and tissue partitioning in the nucellus, these processes had been studied independently and little was known on their co-regulation.

I first asked what the role of the Arabidopsis nucellus is in nutrient partitioning and how it relates to nucellus elimination. As the nucellus is located between the nutrient sink (endosperm/embryo) and source (chalaza) tissues, I wondered if it played a function in nutrient transport, as shown in cereals, or accumulation, as shown in perispermic seeds. In other words, does the nucellus works as a sink or source tissue? I was also curious about the causal relationship, if any, between tissue and nutrient partitioning. Nutrient partitioning has been always described as following tissue partitioning and the hypothesis that the transient nucellus might be eliminated by a starving mechanism had never been addressed before. Finally, as tissue and nutrient portioning are two linked mechanisms, I was interested in testing if TT16 might co-regulate both processes.

I then asked how the process of nucellus is contained. The elimination of the transient nucellus happens cell layer by cell layer in a centripetal fashion, both in cereals and Arabidopsis. This mechanism is consistent with the spreading of a cell death signal or toxic materials responsible for the elimination of the nucellus. Nevertheless, the most proximal cell layers of the nucellus, the persistent nucellus, are not eliminated, thus indicating that the there is a mechanism for blocking the spreading of such hypothetical death signal. I therefore investigated if a symplastic block exists between persistent and transient nucellus. In addition, I wondered if the elimination and survival of the nucellus is genetically regulated.

I finally focused my attention on the opposite cell fate of the nucellus, growth. Compared to all other known seeds, which undergo nucellus elimination, perispermic seeds, such as amaranth,

display nucellus growth. During evolution, plants shifted several times between an endospermic (dominated by the elimination of the nucellus) and perispermic (characterized by nucellus growth) seed architecture, suggesting that few genes might be responsible for such a change. I therefore asked if a change in TT16 expression or function might explain the growth of the nucellus in the amaranth perispermic seed.

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Chapter II - *The Arabidopsis nucellus, between cell elimination and sugar transport*

The Arabidopsis nucellus, between cell elimination and sugar transport

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Short title: Sugar transport in the nucellus

One sentence summary: This study identifies a novel path of sugar transport through the nucellus maternal tissue of the Arabidopsis seed and its coordination with the process of nucellus cell elimination.

Author contribution:

J.L. performed the research, analyzed the data, and helped to write the article. D.G. performed the embryo analysis and part of the expression analysis, O.C. performed part of the expression analysis, R.LH performed sugar quantifications, S.J. performed oil and protein quantifications, C.P. performed transmission electron microscopy analyses. E.M. designed the research, analyzed the data and wrote the article.

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ABSTRACT

The architecture of the seed is shaped by the processes of tissue partitioning, which determines the volume ratio of maternal and zygotic tissues, and nutrient partitioning, which regulates nutrient distribution among tissues. In angiosperms, early seed development is characterized by the antagonistic development of the nucellus maternal tissue and the endosperm fertilization product to become the main sugar sink. Such a process marked the evolution of angiosperms and outline the most ancient seed architectures. In Arabidopsis, the endosperm partially eliminates the nucellus and imports sugars from the seed coat. Here, we show that the nucellus is symplasmically connected to the chalaza, the seed nutrient unloading zone, and works both as sugar sink and source alongside the seed coat. The transient nucellus accumulates starch early on after fertilization and releases it in the apoplasmic space during its elimination. By contrast, the persistent nucellus exports sugars toward the endosperm through the SWEET4 hexose facilitator. Finally, we analyze sugar metabolism and transport in the *transparent testa 16* mutant, which fails to undergo nucellus cell elimination, and shed light on the coordination between tissue and nutrient partitioning. Overall, this study identifies a novel path of sugar transport in the Arabidopsis seed and describes its link to the nucellus cell-elimination program.

INTRODUCTION

Nutrient accumulation in the seed helps the growth of the embryo and the establishment of the seedling in adverse environmental conditions. Seeds evolved different nutrient storing strategies alongside different seed structures. Seed nutrient and tissue partitioning are indeed deeply intertwined mechanisms. Cell elimination programs are put in place in the seed to eliminate unwanted tissues in favor of others that will accumulate and store nutrients, two processes whose sequential order is not fully understood (Ingram, 2017; Lu and Magnani, 2018). In angiosperms, the nucellus maternal tissue and the endosperm fertilization product grow antagonistically to become the main nutrient sink during early seed development (Lu and Magnani, 2018). Such a process defines different seed architectures (Lu and Magnani, 2018) and marked the evolution of angiosperm seeds (Magnani, 2018).

In early Arabidopsis seed development, the endosperm triggers the partial elimination of the nucellus through a signaling pathway that converges on the MADS Box transcription factor TRANSPARENT TESTA 16 (TT16) (Xu et al., 2016) and becomes the main sugar sink. Sucrose is transported from the placental tissue to the chalaza, the seed maternal tissue where vascularization ends, through the phloem. GFP mobility assays demonstrated that chalaza and seed coat maternal tissues are symplasmically connected, thus suggesting that sucrose diffuses from one tissue to the other through the plasmodesmata (Stadler et al., 2005). Nevertheless, maternal tissues and fertilization products are symplasmically isolated (Stadler et al., 2005). The discovery of SWEET sucrose facilitators on the plasma membrane of seed coat cells indicates that sucrose is exported to the apoplasm where it diffuses to the fertilization products (Chen et al., 2015). Finally, previous studies have shown that SWEET and SUCROSE-PROTON SYMPORTER (SUC) proteins allow the endosperm to reimport sucrose from the apoplasm (Baud et al., 2005; Chen et al., 2015).

Similar to Arabidopsis, the rice endosperm dominates early seed development and partially eliminates the nucellus through the action of the MADS29 transcription factor, TT16 orthologue in rice (Nayar et al., 2013; Yang et al., 2012). By contrast, the rice nucellus takes the role of the Arabidopsis seed coat in sugar transport. The nucellus cells in between endosperm and chalaza develop into the so-called nucellar projection, a tissue dedicated to sugar transport symplasmically connected to the chalaza (Krishnan and Dayanandan, 2003; Wu et al., 2016). *OsSWEET11* and *OsSWEET15* sucrose transporters are located on the plasma membrane of the nucellar projection and facilitate the export of sucrose to the apoplasm (Yang et al., 2018b). Cell wall bound invertases then hydrolyze part of the apoplasmic sucrose into hexoses (glucose and fructose) (Wang et al., 2008). Finally, sucrose and glucose are reimported in the endosperm cells by the *OsSWEET11*, *OsSWEET15*, and *OsSWEET4* sugar facilitators (Sosso et al., 2015; Yang et al., 2018b).

The Arabidopsis nucellus is conveniently positioned in between the nutrient unloading zone and the sink tissue (Fig. 1A) and suggests a role in nutrient transport or accumulation alongside the

seed coat. In line with this hypothesis, *in silico* analyses by Hedhly and coworkers indicate that the seed chalazal area is a hot-spot for sugar metabolism and transport during early seed development (Hedhly et al., 2016). Here, we demonstrate that chalaza and nucellus are symplasmically connected, thus suggesting that sugars can diffuse toward the nucellus. The transient nucellus is indeed an early sugar sink in Arabidopsis seeds while releasing starch granules in the apoplasmic space during its elimination. By contrast, the persistent nucellus exports sugars to the apoplasm through the SWEET4 sugar facilitator. Finally, we investigate the link between the processes of tissue and nutrient partitioning in the nucellus by analyzing *tt16* seeds, which do not undergo nucellus elimination.

RESULTS

The nucellus is an early sugar sink

In Arabidopsis, a few nucellus cell layers persist in between the chalaza, where nutrients are unloaded from the phloem, and the endosperm, a strong nutrient sink (Fig. 1A) (Xu et al., 2016). To test if the nucellus accumulates sugars, we monitored the starch (the main storage carbohydrate) content in the nucellus across fertilization. At the end of ovule development, Lugol staining (a starch indicator) revealed starch build-up at the boundary between nucellus and chalaza, in the female gametophyte and in proximal integument cells (Fig. 1B). After fertilization, the distal cells of the nucellus (transient nucellus, undergoing cell elimination, Fig. 1A), the boundary between nucellus and chalaza/proximal inner integument (ii), and the outer integument (oi) accumulated starch (Fig. 1C). Furthermore, we detected fainter Lugol staining in proximal nucellus cells (persistent nucellus, not undergoing cell elimination, Fig. 1A) and in distal ii cells (Fig. 1C). From the globular embryo stage of seed development on, we observed strong Lugol staining everywhere in the seed (Sup. Fig. 1), thus losing tissue resolution.

To better characterize the pattern of starch accumulation in different nucellus domains we adapted the modified pseudo-Schiff propidium iodide imaging (mPS-PI) technique (Truernit et al., 2008b; Xu et al., 2016) to the analysis of both cell wall and starch in seeds (starch-mPS-PI, see methods and Sup. Fig. 2). In line with Lugol staining analyses, we detected starch in the transient nucellus but not in the persistent nucellus, at early embryogenesis (Fig. 1D). Furthermore, we observed propidium iodide staining in the chalaza and ii cells surrounding the nucellus and in the oi2 (Fig. 1D). Starch-mPS-PI analyses revealed that the persistent nucellus does not accumulate starch even later in seed development, when most of the seed maternal tissues, with the exception of the ii1'cell layer, appear full of starch (Fig. 1E). These results indicate that the faint Lugol staining observed in the persistent nucellus is possibly due to diffusion of stained material during the seed clearing process (see methods).

We further confirmed our results by transmission electron microscopy, which showed starch

granules in the apoplasmic space in between nucellus and endosperm (nucellar cavity, which contains cell corpses and debris resulting from the process of cell elimination) and in transient nucellus cells but not in the persistent nucellus (Fig. 1F).

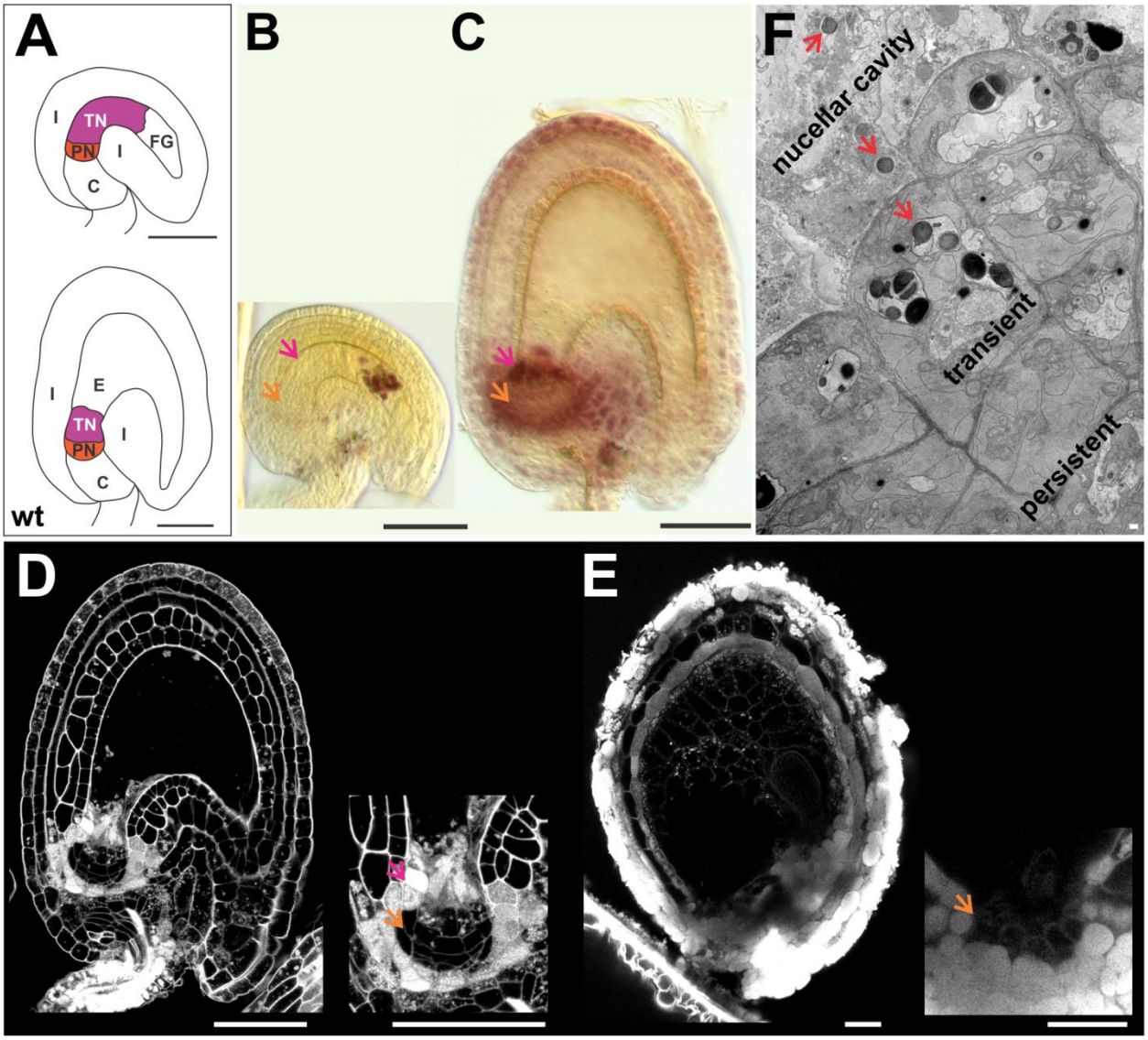


Figure 1. The transient nucellus is a sugar sink
(A) Diagrammatic representation in longitudinal sections of a wild type ovule at stage 3-VI (Schneitz et al., 1995) (upper) and seed at pre-globular embryo stage (lower). I, integuments; C, chalaza; PN, persistent nucellus (orange); TN, transient nucellus (purple); FG, female gametophyte; E, endosperm. Bars = 50 µm.
(B) and **(C)** Cleared whole mounts of a Lugol stained wild type ovule at stage 3-VI (B) and seed at pre-globular embryo stage (C). Orange and purple arrows indicate persistent and transient nucellus, respectively. Bars = 50 µm.
(D) and **(E)** Longitudinal mid-planes of wild type seeds at pre-globular (D) and torpedo (E) embryo stages, imaged using the starch-mPS-PI technique. White fluorescence inside the cells indicate the presence of starch. Close-up images of the nucellus are at the right of each seed image. Orange and purple arrows indicate persistent and transient nucellus, respectively. Bars = 50 µm.
(F) Transmission electron microscopy image of the nucellus of a wild type seed at pre-globular stage. Red arrows indicate starch granules. Bar = 0.5 µm.

Finally, we analyzed starch biosynthesis by fusing the promoter region of the *GLUCOSE-1-PHOSPHATE ADENYLYLTRANSFERASE LARGE SUBUNIT* 3 (*APL3*) gene, which encodes the

enzyme that catalyzes the first limiting step reaction in starch biosynthesis (Crevillen et al., 2003; Crevillen et al., 2005), to the chimeric *GFP-GUS* reporter gene (*ProAPL3:GFP-GUS*). In ovules, we detected GFP fluorescence in the oi1 and ii2 cell layers and the female gametophyte (Fig. 2A). After fertilization, the promoter was active in the transient nucellus, chalaza, oi1, ii2, and endosperm of pre-globular embryo stage seeds (Fig. 2B). At bent cotyledon embryo stage, we detected fluorescence solely in the oi (Fig. 2C). Overall, GFP fluorescence in the nucellus recapitulated starch deposition as observed by Lugol, starch-mPS-PI and transmission electron microscopy analyses. These data indicate that the transient nucellus is an early sugar sink in Arabidopsis seeds.

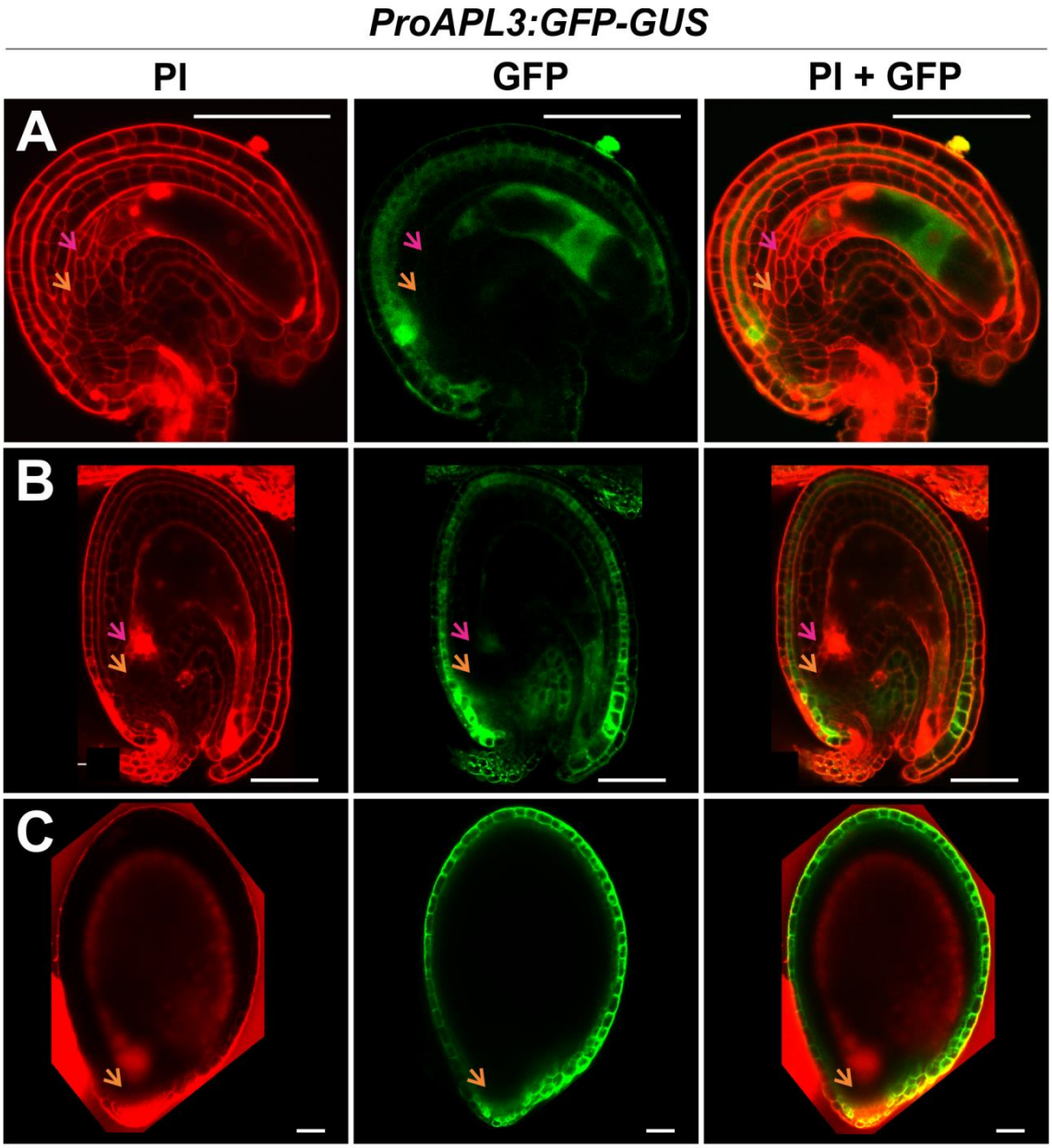


Figure 2. The expression pattern of the *APL3* promoter
(A) to **(C)** Propidium iodide (PI), GFP, and PI-GFP superimposed fluorescence images of *ProAPL3:GFP-GUS* ovule at stage 3-VI (A), and seeds at pre-globular (B) and bent cotyledons (C) embryo stages. Orange and purple arrows indicate persistent and transient nucellus, respectively. Bars = 50 µm.

The nucellus is symplasmically connected to the chalaza

The finding of starch in the nucellus prompted us to test if sugars can move symplasmically from the chalaza, the seed nutrient unloading zone, to the nucellus. To this mean, we conducted GFP mobility assays by expressing *GFP* or *GFP-sporamin* translational fusion in the companion cells of the chalazal phloem under the control of the *SUCROSE-PROTON SYMPORTER 2* (*SUC2*) promoter region. It has been previously shown that the size exclusion limit of the chalazal plasmodesmata allows GFP (27 kD), but not GFP-sporamin (47 kD), to diffuse to the neighboring seed coat cells (Stadler et al., 2005). At 1-cell embryo stage, GFP-sporamin was confined in a few chalazal cells (Fig. 3A). By contrast, we detected free GFP fluorescence in chalaza, oi, proximal ii and nucellus cells at the same developmental stage (Fig. 3B). These data demonstrate that the nucellus is symplasmically connected to the chalaza and suggest that nutrients diffuse from the chalaza to the nucellus in a symplasmic fashion.

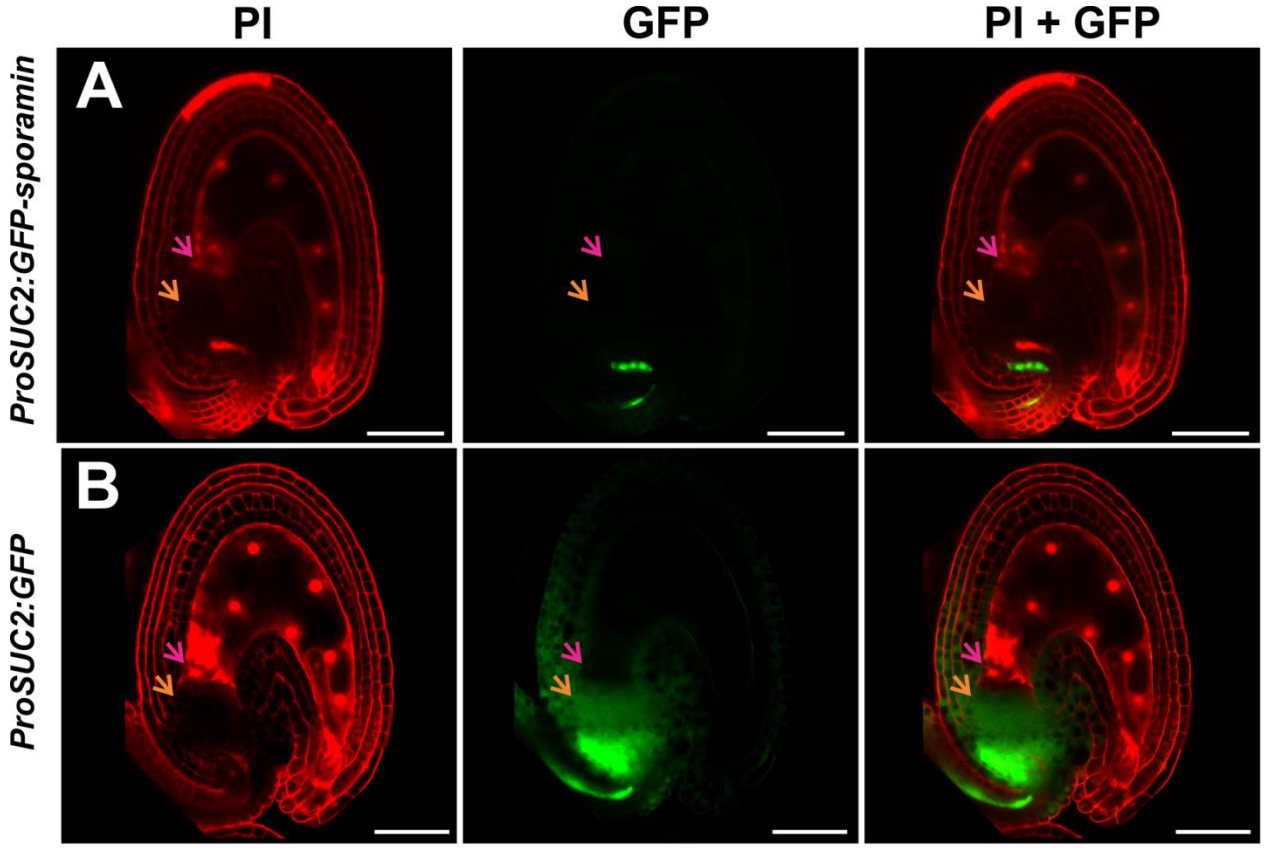


Figure 3. The nucellus is symplasmically connected to the chalaza
(A) and **(B)** Propidium iodide (PI), GFP, and PI-GFP superimposed fluorescence images of *ProSUC2:GFP-sporamin* (A) and *ProSUC2:GFP* (B) seeds at pre-globular embryo stage. Orange and purple arrows indicate persistent and transient nucellus, respectively. Bars = 50 µm.

SWEET4 sugar facilitator is expressed in the nucellus

Nutrients are supposed to reach the fertilization products via the apoplasm as maternal and zygotic tissues are not symplasmically connected (Stadler et al., 2005). In Arabidopsis, SWEET transporters in the seed coat facilitate sucrose export to the apoplasm (Chen et al., 2015). By

contrast, rice grains express *SWEET* genes in nucellar cells (Yang et al., 2018b), which play an important role in nutrient transfer to the endosperm. To verify if the Arabidopsis nucellus also plays a role in sugar apoplasmic transport, we tested if members of the *SWEET* gene family are expressed in such tissues. According to microarray data by Belmonte and coworkers (Belmonte et al., 2013), the *SWEET4* glucose and fructose facilitator gene (Chen et al., 2010; Liu et al., 2016) is specifically expressed in the chalazal region (including chalaza and nucellus) of Arabidopsis seeds throughout seed development (Sup. Fig. 3). To better characterize its expression pattern, we fused *SWEET4* promoter region to the chimeric *GFP-GUS* reporter gene. In *ProSWEET4:GFP-GUS* ovules and seeds, we detected GFP fluorescence in the most proximal nucellus cell layer and first 1-3 cells of the ii (Fig. 4A-4C). Our results indicate that in Arabidopsis the persistent nucellus plays a role in sugar transport.

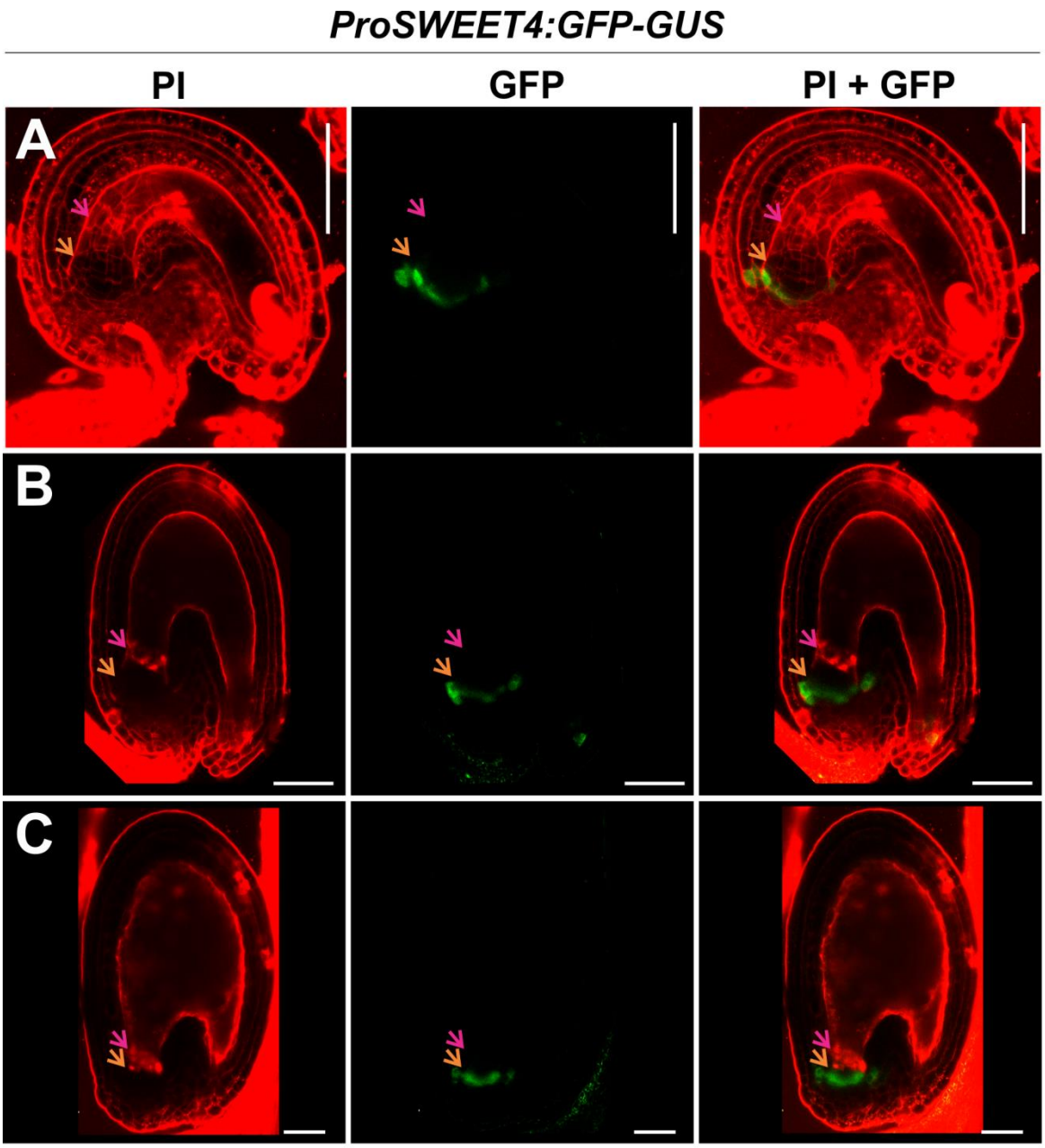


Figure 4. *SWEET4* is expressed in the nucellus
(A) to **(C)** Propidium iodide (PI), GFP, and PI-GFP superimposed fluorescence images of *ProSWEET4:GFP-GUS* ovule at stage 3-VI (A), and seeds at pre-globular (B) and globular (C) embryo stages. Orange and purple arrows indicate persistent and transient nucellus, respectively. Bars = 50 µm.

SWEET4 localizes to the plasma membrane

To test if SWEET4 facilitate transport across the plasma membrane, we studied its sub-cellular localization. The SWEET4-GFP chimeric protein has been shown to localize to the plasma membranes of protoplasts, when expressed under the control of the *35S* promoter sequence (Liu et al., 2016). To test SWEET4 localization *in planta*, we imaged cells of *Pro35S:SWEET4-GFP* seeds. We detected GFP fluorescence in the plasma membrane, in proximity to propidium iodide signal marking the cell wall, and in vesicles (Fig. 5A). In a number of cells we also detected fluorescence in the vacuole (Sup. Fig. 4). SWEET4-GFP localization in the vacuole might be due to incorrect

protein folding or overcrowding of the secretory pathway in the transgenic lines. Overall, these data indicate that SWEET4 facilitates sugar export to the apoplasm.

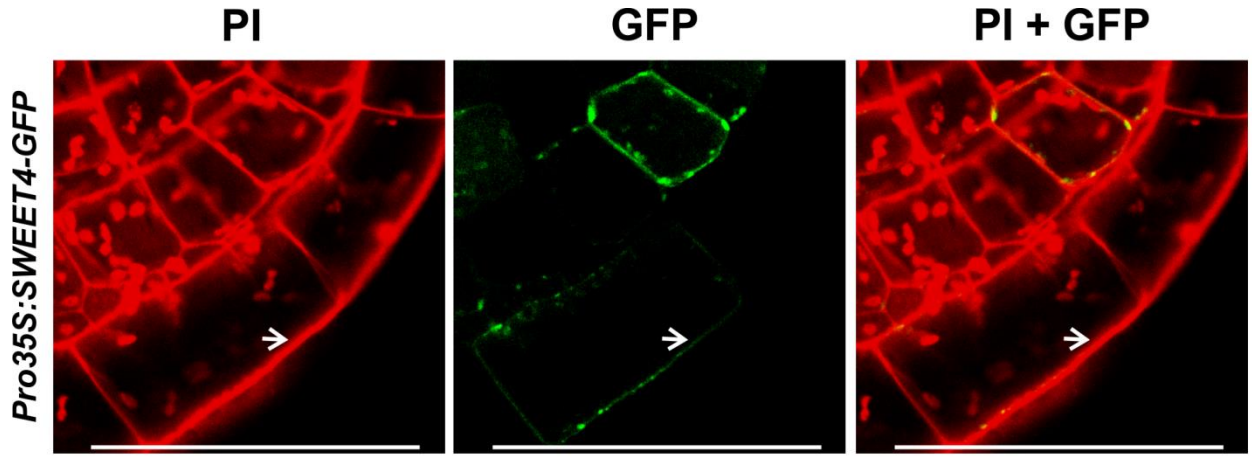


Figure 5. SWEET4 localizes to the plasma membrane
Propidium iodide (PI), GFP, and PI-GFP superimposed fluorescence images of a *Pro35S:SWEET4-GFP* seed at pre-globular stage. White arrows indicate plasma membranes. Bars = 25 µm.

SWEET4 affects seed sugar content

A number of *sweet* mutants have been shown to transiently and locally over-accumulate sugars as a consequence of impaired sugar export, while negatively affecting the sugar content of the sink tissues in the long run. For example, mutations in the *SWEET11*, *SWEET12*, and *SWEET15* genes lead to excessive starch accumulation in the seed coat and low starch levels in the embryo, when compared to the wild type (Chen et al., 2015). In line with these data, we observed stronger Lugol staining in *sweet4* mutant (see methods and Sup. Fig. 5) nucellus, chalaza and seed coat, when compared to the wild type (Fig. 6A-6C). Nevertheless, Lugol staining assays are not quantitative and we detected a certain degree of variability among seeds sharing the same genetic background. We therefore quantified sugars content in wild type and mutant seeds. On average, *sweet4* seeds at four and six days after flowering (DAF) accumulated higher levels of glucose, fructose, total soluble sugars and starch, when compared to the wild type (Fig. 6D and 6E). By contrast, *sweet4* dry seeds showed a slightly lower level of sucrose and soluble sugars than wild type seeds (Fig. 6F). Finally, we showed that the perturbed sugar transport in *sweet4* seeds does not affect oil and protein accumulation (Fig. 6G).

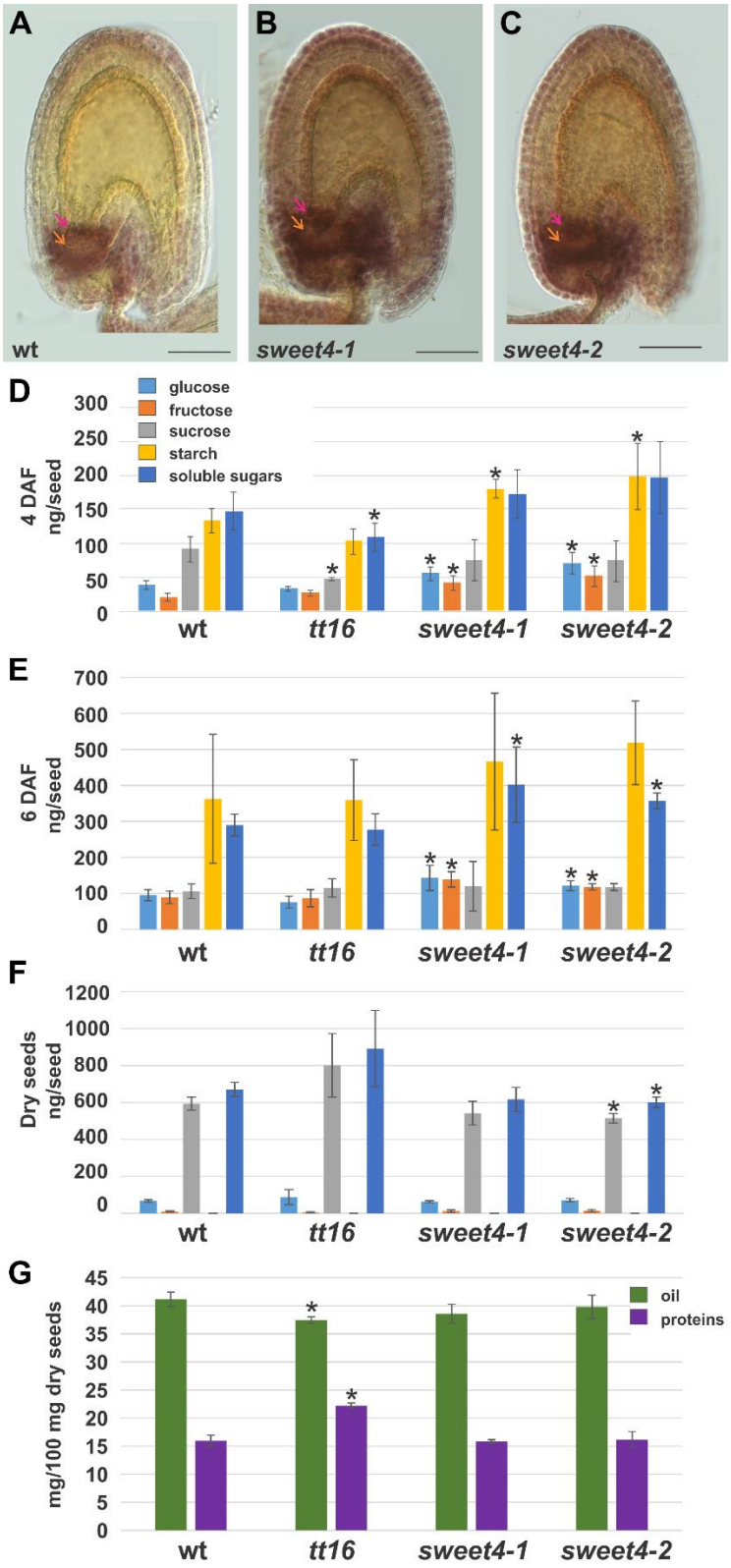


Figure 6. The *sweet4* and *tt16* mutations affect nutrient accumulation in seeds
(A) to **(C)** Cleared whole mounts of Lugol stained wild type (wt) and mutant seeds at pre-globular embryo stage. Orange and purple arrows indicate persistent and transient nucellus, respectively. Bars = 50 µm.
(D) to **(F)** Sugars quantification in wild type (wt) and mutant seeds at 4 (D) and 6 (E) days after flowering (DAF) and in dry seeds (F). Asterisks indicate statistical significance between wild type and mutant (two-tailed Student's *t*-test, $P<0.05$). Error bars indicate standard deviation.
(G) Oil and protein quantification in wild type (wt) and mutant dry seeds. Asterisks indicate statistical significance between wild type and mutant (two-tailed Student's *t*-test, $P<0.05$). Error bars indicate standard error.

When sugars are not efficiently transported from the seed coat to the fertilization products, as in the *sweet11*;*sweet12*;*sweet15* triple mutant, embryo development is delayed (Chen et al., 2015).

Similarly, we observed that the *sweet4* mutation impaired embryo growth (Fig. 7). Overall, these data support the hypothesis that SWEET4 facilitates sugar export to the apoplasm during early seed development.

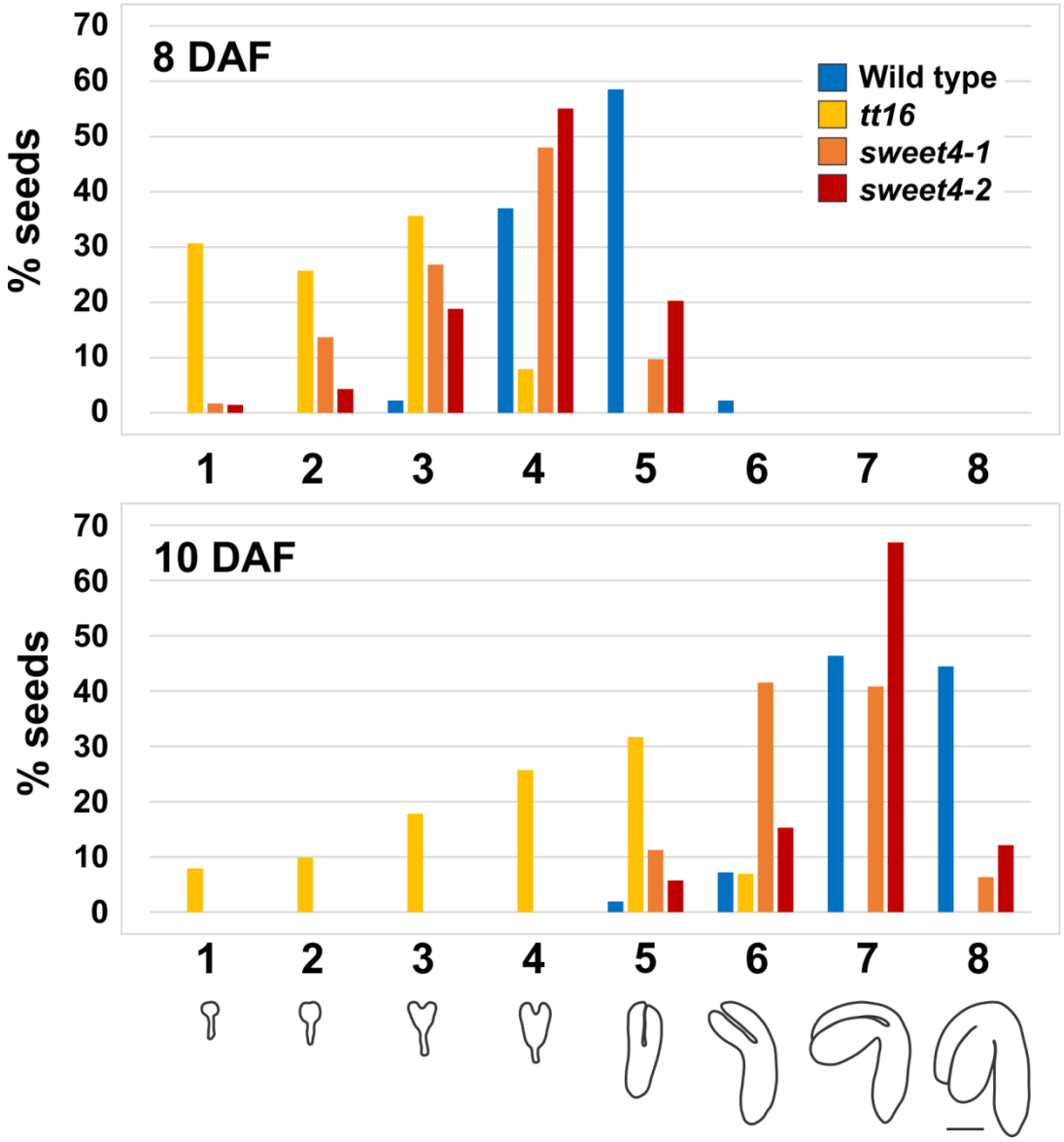


Figure 7. The *sweet4* and *tt16* mutations delay embryo development
Distribution of different embryo developmental stages (1: globular, 2: transition, 3: heart, 4: early torpedo, 5: late torpedo, 6: walking stick, 7: bent cotyledon, 8: mature embryo) in wild type and mutant seeds at 8 and 10 days after flowering (DAF). n > 100. Bar = 100 µm.

TT16 regulates cell elimination but not sugar export in the nucellus

We have previously shown that the B-sister MADS box transcription factor TT16 plays a role in seed tissue partitioning by promoting partial nucellus elimination in favor of endosperm growth (Xu et al., 2016). Since tissue and nutrient partitioning are known to be tightly linked mechanisms (Lu and Magnani, 2018), we investigated sugar transport and metabolism in *tt16* mutant seeds, whose transient nucellus (thereafter referred to as transient nucellus*, Fig. 8A) is not eliminated. Whereas starch accumulation in wild type and *tt16* ovules appeared indistinguishable (Fig. 1B and

8B), Lugol staining and starch-mPS-PI analyses highlighted three domains in the *tt16* nucellus: from proximal to distal, 1) the persistent nucellus, which did not accumulate starch, 2) the proximal cells of the transient nucellus*, which accumulated starch, and 3) the distal cells of the transient nucellus*, which did not accumulate starch (Fig. 8C and 8D). Nevertheless, the *ProAPL3:GFP-GUS* construct did not drive *GFP* expression in the nucellus of *tt16* ovules and seeds (Fig. 8E and 8F). Altogether, these data show that part of the transient nucellus maintains its sugar sink force if it is not eliminated.

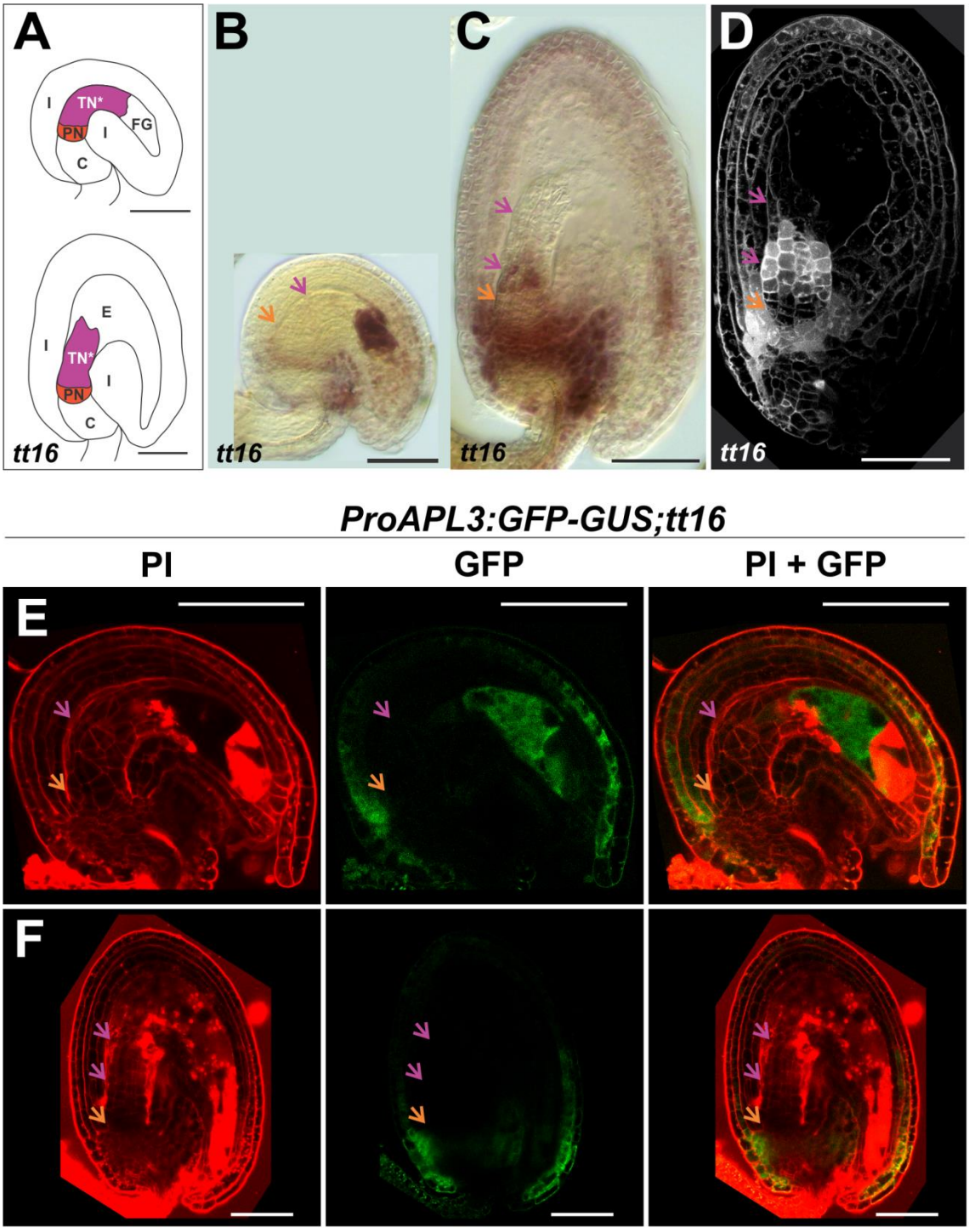


Figure 8. *tt16* nucellus accumulates starch

(A) Diagrammatic representation in longitudinal sections of a *tt16* ovule at stage 3-VI (upper) and seed at pre-globular embryo stage (lower). I, integuments; C, chalaza; PN, persistent nucellus (orange); TN*, transient nucellus* that is not eliminated in the *tt16* mutant (purple); FG, female gametophyte; E, endosperm. Bars = 50 µm.

(B) and **(C)** Cleared whole mounts of a Lugol stained *tt16* ovule at stage 3-VI (B) and seed at pre-globular embryo stage (C). Orange and purple arrows indicate persistent and transient nucellus*, respectively. Bars = 50 µm.

(D) Longitudinal plane of a *tt16* seed at pre-globular stage, imaged using the starch-mPS-PI technique. Orange and purple arrows indicate persistent and transient nucellus*, respectively. Bars = 50 µm.

(E) and **(F)** Propidium iodide (PI), GFP, and PI-GFP superimposed fluorescence images of *ProAPL3:GFP-GUS* ovule at stage 3-VI (E) and seed at pre-globular embryo stage (F). Orange and purple arrows indicate persistent and transient nucellus*, respectively. Bars = 50 µm.

Sugars quantification in *tt16* seeds revealed a lower level of sucrose and soluble sugars at 4 DAF and a higher level of the same sugars in dry seeds, when compared to the wild type (Fig. 6D-6F). Furthermore, *tt16* dry seeds contained less oil and more proteins than wild type seeds (Fig. 6G). Finally, embryo development was delayed in *tt16* seeds relative to the wild type (Fig. 7).

To test if TT16 affects nutrient transport by regulating *SWEET4* expression, we introgressed the *ProSWEET4:GFP-GUS* line in a *tt16* background. The *tt16* mutation did not perturb *SWEET4* spatial expression pattern (Fig. 9A to 9B), when compared to the wild type (Fig. 4). In line with these results, RT-qPCR analyses of *SWEET4* expression in *tt16* and wild type seeds at 2 and 11 DAF were statistically similar (Sup. Fig. 6). Altogether, these data suggest that TT16 regulates the elimination of the nucellus and, indirectly, the release of starch in the nucellar cavity but not the apoplasmic transport of sugar through the SWEET4 facilitator.

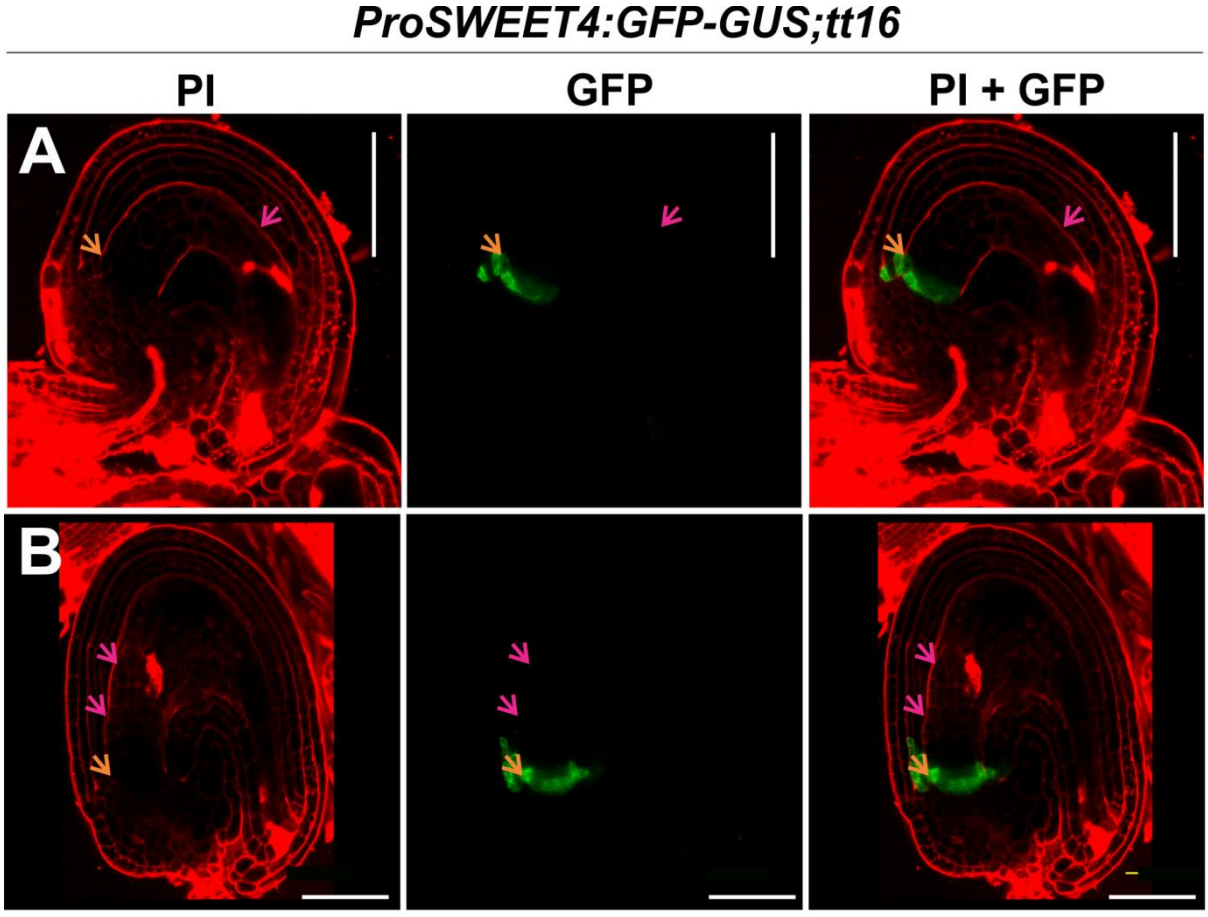


Figure 9. *SWEET4* expression in the *tt16* mutant
(A) and **(B)** Propidium iodide (PI), GFP, and PI-GFP superimposed fluorescence images of *ProSWEET4:GFP-GUS;tt16* ovule at stage 3-VI (A) and seed at pre-globular stage (B). Orange and purple arrows indicate persistent and transient nucellus*, respectively. Bars = 50 µm.

DISCUSSION

The nucellus path of nutrient transport

In Arabidopsis, it has been shown that sugars travel from the placental tissue to the chalaza through the phloem and then diffuse symplasmically towards the seed coat (Fig. 10) (Stadler et al.,

2005). The seed coat has been regarded as the only tissue responsible for sugar export to the apoplasm (Chen et al., 2015), a needed step to allow sugar diffusion toward the fertilization products that are symplasmically disconnected from the maternal tissues (Stadler et al., 2005). Here, we identified a novel path of sugar transport through the nucellus (Fig. 10). Our data indicate that the chalaza is symplasmically connected not only to the seed coat but also to the nucellus. Therefore, we speculate that chalazal sucrose and/or hexoses, resulting from the activity of cytoplasmic sucrose synthases present in the chalaza (Fallahi et al., 2008), diffuse from cell to cell to the nucellus. The most proximal cells of the persistent nucellus express the *SWEET4* glucose and fructose facilitator (Chen et al., 2010; Liu et al., 2016), indicating that part of the nucellar hexoses are exported to the apoplasmic space. We speculate that the apoplasmic glucose and fructose, provided by the nucellus, might diffuse toward the endosperm to be reimported by endosperm transporters (Baud et al., 2005; Chen et al., 2015). In line with our model, *sweet4* mutant lines displayed over-accumulation of soluble sugars during early seed development. Part of the excess of hexoses observed in the *sweet4* mutant might be converted into starch in the maternal tissues, as suggested by Lugol staining assays and starch quantification analyses. Later in seed development, the lower sugar content of *sweet4* seeds, when compared to the wild type, might be due to the compromised transport of sugars from the nucellus to the fertilization products, the ultimate sink tissues. A correlation between sugar and lipid content in seeds has been observed in a number of mutations that affect sugar metabolism. Nevertheless, the relationship between sugar and lipid metabolism has not been fully understood as other mutations, such as *sus2* and *sus3*, perturb starch but not lipid accumulation, similar to what observed in *sweet4* seeds (Angeles-Nunez and Tiessen, 2010; Barratt et al., 2009). Compared to the seed coat path, which is based on sucrose transport (Chen et al., 2015), our data indicate that hexoses are mostly transported through the nucellus path. We speculate that the seed-coat is involved in relatively longer distance transport of sugars and therefore favors the more energy-efficient and less reactive sucrose. By contrast, the nucellus offers a faster route toward the endosperm thus allowing the transport of readily available glucose and fructose.

Cell elimination as a way to recycle sugars.

We demonstrated that the transient nucellus accumulates starch across fertilization. Starch is then released into the nucellar cavity, the apoplasmic space in between nucellus and endosperm, which contains cell corpses and debris resulting from the process of cell elimination (Fig. 10). A similar phenomenon has been shown in cereals, which possess a larger transient nucellus that works as transient sugar sink and develops, in some species, a larger nucellar cavity in between nucellus and endosperm (Lu and Magnani, 2018). In Barley grains, alpha amylase 4 has been shown to be active in nucellus cells during cell elimination, thus indicating that the starch in the nucellus is

hydrolyzed to facilitate its mobilization to the endosperm (Radchuk et al., 2009). Similarly, microarray data suggest that the starch degradation pathway is active in the Arabidopsis chalazal area (Hedhly et al., 2016). In line with this hypothesis, starch granules in nucellus cells at an advanced stage of cell elimination appeared less contrasted than those in cells starting the process of elimination, when imaged by transmission electron microscopy (Fig. 1F). Therefore, the starch present in the Arabidopsis transient nucellus might be recycled in favor of the endosperm. The advantage, if any, of such a process in a relatively small nucellus is not clear. Alternatively, it might be an evolutionary relic. The nucellus, and not the endosperm, acts indeed as major sugar sink in perispermic seeds (Lopez-Fernandez and Maldonado, 2013), which might be the ancestral seed condition (Lu and Magnani, 2018; Magnani, 2018), a character that might have been retained by the Arabidopsis nucellus despite undergoing cell elimination. *tt16* seeds partially resemble perispermic seeds as they carry a large nucellus that accumulates starch, thus indicating a possible molecular scenario for the evolution of such different seed architectures.

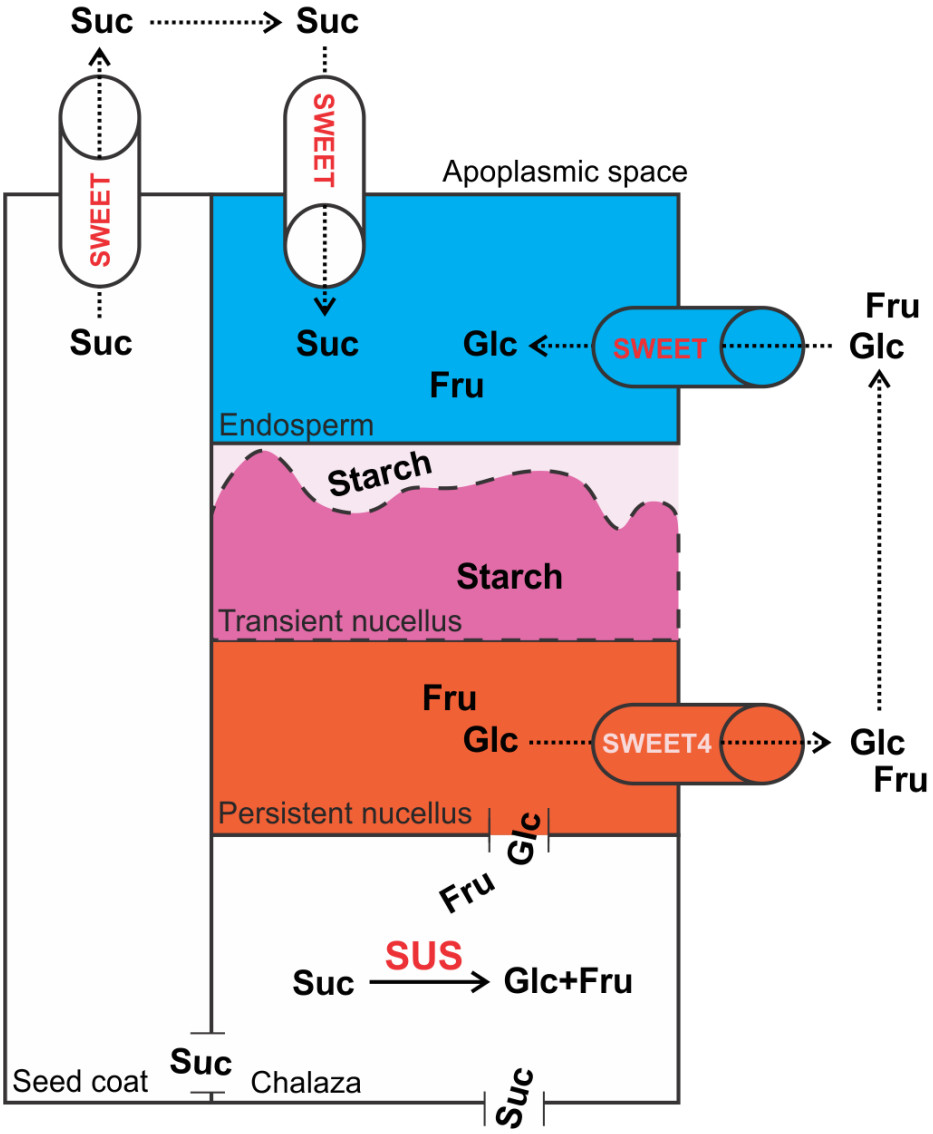


Figure 10. The nucellus path of sugar transport
Solid and dotted arrows indicate enzymatic reactions and diffusion paths, respectively. Dashed lines indicate cells undergoing cell elimination. SUS, SUCROSE SYNTHASE; Glc, glucose; Fru, fructose; Suc, sucrose.

Coordination of tissue and nutrient partitioning.

Tissue and nutrient partitioning are thought to be two inextricably linked processes. In early Arabidopsis seed development, the nucellus is eliminated to allow endosperm growth and nutrient accumulation. Later in seed development, most of the endosperm is eliminated to allow embryo growth, the ultimate nutrient sink. It is still debated if nutrient partitioning follows or precedes tissue partitioning (Ingram, 2017). It has been speculated that preventing nutrient transport to a tissue might indeed lead to its elimination. Our finding of starch accumulation in the transient nucellus, alongside sugar export towards the endosperm in the persistent nucellus, argues against this hypothesis. Furthermore, TT16 allows endosperm growth by eliminating the transient nucellus (Xu et al., 2016) but does not promote nutrient export to the endosperm by activating *SWEET4* expression in the persistent nucellus.

Nonetheless, the *tt16* mutation affected sugar, oil and protein content in seeds. TT16 might regulate, directly or indirectly, the expression of other nutrient transporters in the seed coat or in the chalaza. Indeed, the pleiotropic nature of the *tt16* seed phenotype, which shows defects in nucellus, seed coat, and chalaza development (Coen et al., 2017; Coen et al., 2019a; Coen et al., 2019b; Ehlers et al., 2016; Fiume et al., 2017a, b; Nesi et al., 2002a; Xu et al., 2016), does not allow us to conclusively interpret such data. Further experiments are necessary to understand the regulatory mechanisms underlying such physiological changes.

METHODS

Plant material

All *Arabidopsis thaliana* plants are in the Columbia accession. The *tt16-1* allele was isolated in the Wassilewskija accession from the INRA Versailles collection (Bechtold and Pelletier, 1998) and then backcrossed to Columbia (Coen et al., 2017; Nesi et al., 2002a). *sweet4-1* (SALK_072225) (Chong et al., 2014) allele is from the Salk collection (Alonso et al., 2003) whereas the *sweet4-2* (GK_858G02) (Chong et al., 2014) allele is from the GABI-KAT collection (Kleinboelting et al., 2012). *SWEET4* expression in *sweet4-1* and *sweet4-2* seeds, compared to the wild type, has been tested by RT-qPCR, as shown below, by using a set of primers (5'CCTCAATGGTGTCGTTTGGG3' and 5'TAGCTTGTCCACTGTTGCCA3') downstream of both T-DNA insertions (Sup. Fig. 5).

Cloning and Construction

PCR amplifications were performed using the gene-specific primers described below carrying the attB1 (5′GGGGACAAGTTTGTACAAAAAAGCAGGCT3′) and attB2 (5′GGGGACCACTTTGTACAAGAAAGCTGGGTC3′) Gateway recombination sites at the 5′-ends of the forward and reverse primers, respectively. All PCR products were amplified with the high-fidelity Phusion DNA polymerase (Thermo Fisher Scientific), recombined into the *pDONR207* or *pDONR201* vector (BP Gateway reaction) according to the manufacturer's instructions (Thermo Fisher Scientific), and then sequenced. The PCR products cloned into the *pDONR* vectors were then recombined into the appropriate destination vector (LR Gateway reaction) according to the manufacturer's instructions (Thermo Fisher Scientific). *SWEET4* 3.2 kb promoter region was amplified using the attB1-(5′TGGTTCGCATTTTGGATTCTTTGTTTAC3′) forward and attB2-(5′ TTCACTTCAAAAGAAAAATCCGAAC3') reverse primers. *SWEET4* genomic sequence without the stop codon was PCR amplified using the attB1- (5′CGCTCGCTCTCTTCTTTGTT3') forward and attB2-(5′AGCTGAAACTCGTTTAGCTTGTC3') reverse primers. *APL3* 1.8 kb promoter region was amplified using the attB1-(5′GTCGACGATGTTTGGTTTCTTTATCC3') forward and attB2-(5′CCATGGCTTTTTTTAGCTGGAATGA3') reverse primers.

The *ProSWEET4* sequence was recombined into the *pBGWFS7.0* binary vector (Karimi et al., 2002) whereas the *SWEET4* genomic sequence was recombined into the *pMDC83* binary vector (Curtis and Grossniklaus, 2003).

Constructs carrying *GFP* or *GFP-sporamin* under the control of the *SUCROSE-PROTON SYMPORTER 2* (*SUC2*) promoter region were previously described (Stadler et al., 2005).

Transgenic plants

The *Agrobacterium tumefaciens* strain C58C1 was used to stably transform Arabidopsis plants using the floral dip method (Clough and Bent, 1998). Transformants were selected with the appropriate resistance and subsequently transferred to soil for further characterization. More than 10 independent transgenic lines were tested for each construct transformed. One transgenic line for each construct is presented as representative of the majority of lines showing consistent results.

Expression analysis

At least three independent biological samples were used for each analysis. Each replicate comprised the content in seeds of ten siliques. Total RNA was extracted using the RNeasy Mini kit (Qiagen), including RNase-Free DNase Set (Qiagen) treatment during washing, according to the manufacturer's instructions. The Superscript Reverse Transcriptase II kit (Invitrogen) was used to generate cDNA from 1µg of total RNA. Quantitative PCRs were performed with the SYBR Green kit (Bio-Rad) on a Bio-Rad CFX real-time PCR machine. *SWEET4* was PCR amplified using the (5'CCTCAATGGTGTCGTTTGGG3') forward and (5'TAGCTTGTCCACTGTTGCCA3') reverse primers. *SWEET4* expression levels were normalized to the expression levels of three reference genes (AT4G12590, AT4G02080, and AT3G25800) (Dekkers et al., 2012).

Lugol staining

Harvested seeds and ovules were incubated in a 1% SDS, 0.2 N NaOH solution at 37℃ for 15 minutes to clear the tissue. Samples were rinsed in water and incubated in a 12.5% bleach solution (1.25% active Cl–) for 10 minutes. Samples were then rinsed in water and transferred to a 33% Lugol solution and incubated for 30 seconds. Finally, samples were analyzed by differential interference contrast microscopy with an Axioplan 2 microscope (Zeiss). More than 30 seeds were analyzed for each genotype and time point.

Confocal microscopy

Modified pseudo-schiff propidium iodide (mPS-PI) analyses were conducted as previously described (Xu et al., 2016). Starch-mPS-PI staining analyses were conducted following the mPS-PI protocol but omitting the α-amylase step. In starch-mPS-PI treated samples, propidium iodide stains

both cell walls and starch.

GFP expressing lines were analyzed one hour after mounting in a 100 µg/ml propidium iodide, 7% sucrose solution, as previously described (Figueiredo et al., 2016).

Samples were imaged by confocal laser scanning microscopy (Leica SP8). Three-dimensional Z-stack confocal laser scanning microscope images were analyzed through the Volume Viewer plug-in of the ImageJ software (Schneider et al., 2012). More than 30 seeds were analyzed for each genotype and time point.

Transmission electron microscopy

Transmission electron microscopy analyses were conducted as previously described (Coen et al., 2019a).

Enzymatic analyses

Sugars were extracted as previously described (Baud et al., 2002a) and quantified by using the Enzytech D-Glucose/Fructose/Sucrose kit (R-biopharm, https://r-biopharm.com/fr/). At least four biological replicates were performed for each genotype. 50 seeds were used for each replicate.

Seed oil and protein content by near-infrared spectroscopy

Seed samples were analyzed as previously described (Jasinski et al., 2016). Four biological replicates were performed for each genotype. 300 µl of seeds were used for each replicate.

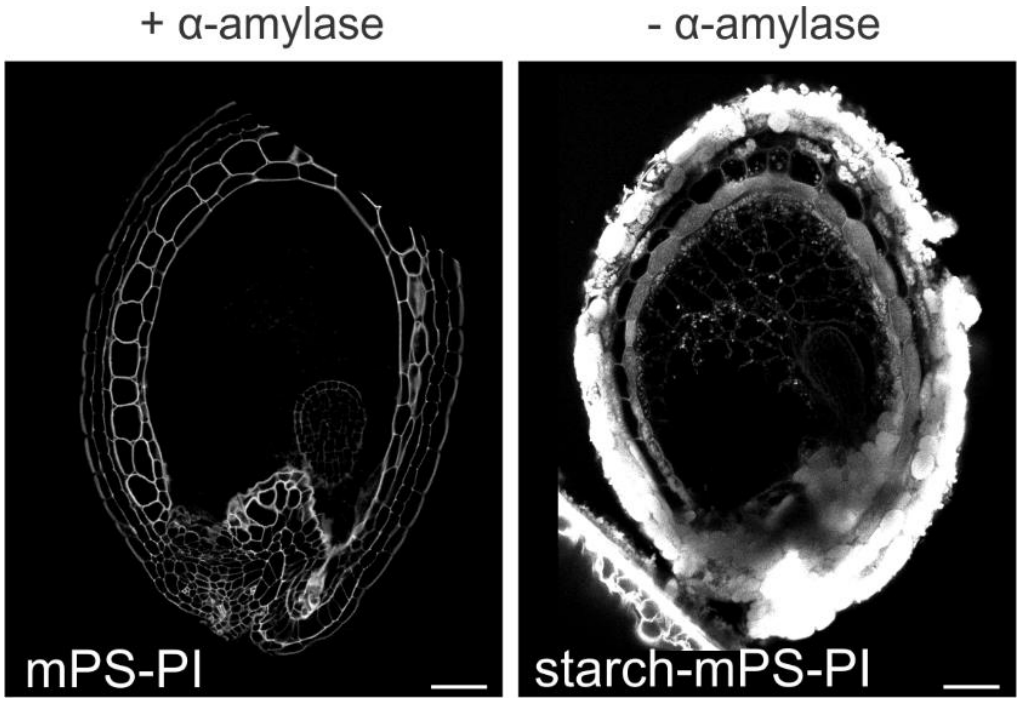
Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: *TT16* (AT5G23260), *SWEET4* (AT3G28007), *APL3* (AT4G39210).

SUPPLEMENTAL DATA

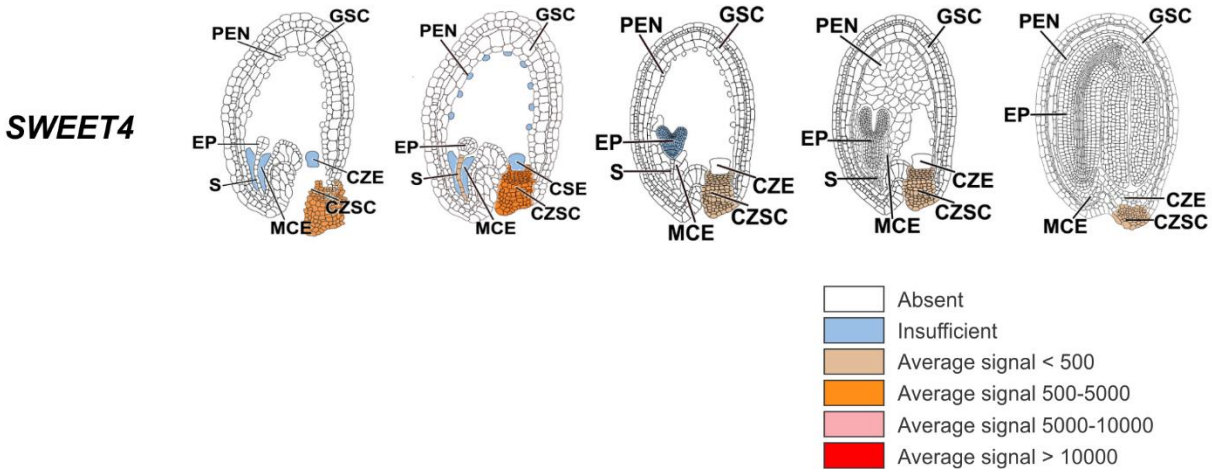
Supplemental Figure 1. Lugol staining of seeds at globular embryo stage

Cleared whole mount of a Lugol stained wild type seed at globular embryo stage. Orange and purple arrows indicate persistent and transient nucellus, respectively. Bar = 50 µm.



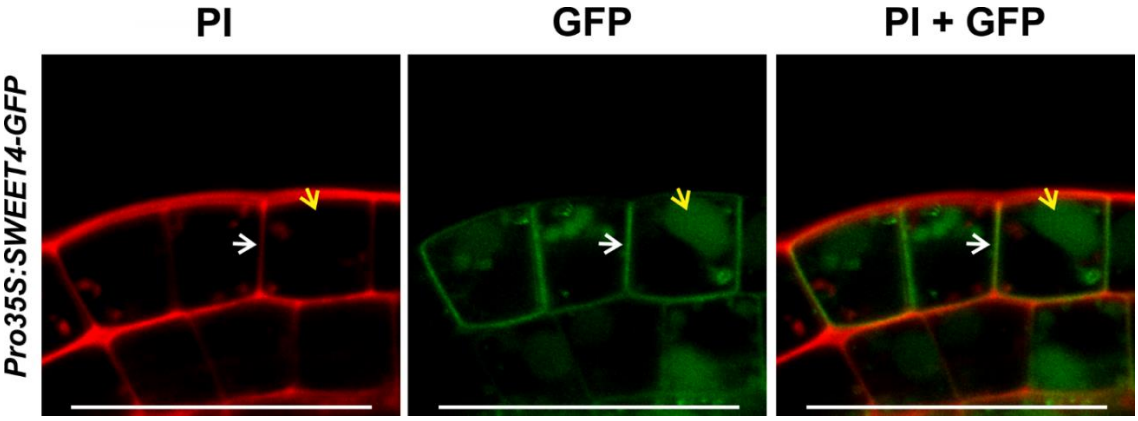
Supplemental Figure 2. The starch-modified pseudo-Schiff propidium iodide imaging technique

Longitudinal mid-planes of wild type seeds at torpedo embryo stage, imaged using the mPS-PI technique or the starch-mPS-PI technique. White fluorescence inside the cells indicate the presence of starch. Bars = 50 µm.



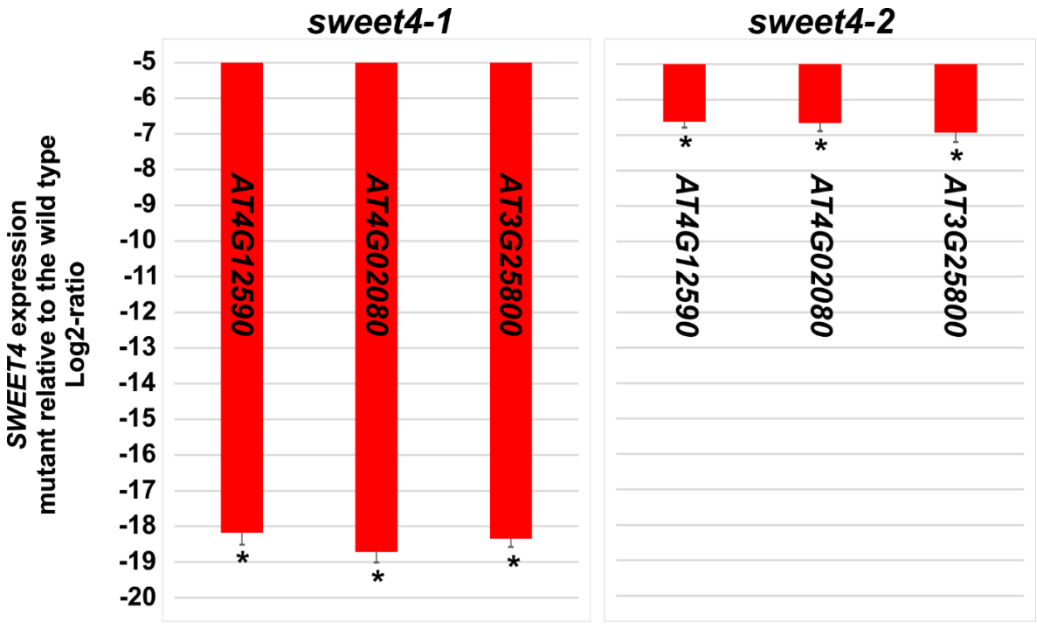
Supplemental Figure 3. SWEET4 expression in seeds

SWEET4 expression pattern in the seed chalazal region (including chalaza and nucellus) according to microarray data by Belmonte and coworkers (Belmonte et al., 2013). CZE, Chalazal Endosperm; CZSC, Chalazal Seed Coat (chalaza and nucellus); EP, Embryo Proper; GSC, General Seed Coat; MCE, Micropylar Endosperm; PEN, Peripheral Endosperm; S, Suspensor; WS, Whole Seed.



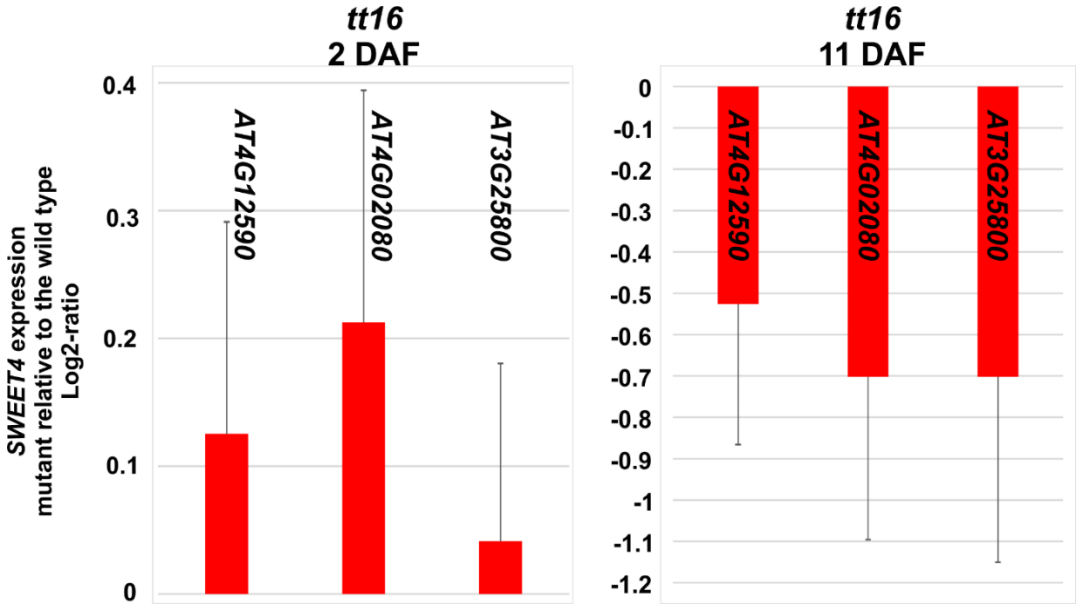
Supplemental Figure 4. SWEET4 localizes to the plasma membrane and vacuole

Propidium iodide (PI), GFP, and PI-GFP superimposed fluorescence images of a *Pro35S:SWEET4-GFP* seeds at pre-globular stage. White and yellow arrows indicate plasma membranes and vacuoles, respectively. Bars = 25 µm.



Supplemental Figure 5. *SWEET4* expression in *sweet4* mutant seeds

Quantitative RT-PCR analyses of *SWEET4* expression in *sweet4* mutant seeds at 11 days after flowering (DAF), relative to the wild type. *SWEET4* expression levels were normalized to the expression levels of three reference genes (AT4G12590, AT4G02080, and AT3G25800). Asterisks indicate statistical significance between wild type and mutant (two-tailed Student's *t*-test, P<0.05). Error bars indicate standard error.



Supplemental Figure 6. *SWEET4* expression in *tt16* mutant seeds

Quantitative RT-PCR analyses of *SWEET4* expression in *tt16* mutant seeds at 2 and 11 days after flowering (DAF) relative to the wild type. *SWEET4* expression levels were normalized to the expression levels of three reference genes (AT4G12590, AT4G02080, and AT3G25800). We did not observe statistical significance between wild type and mutant (two-tailed Student's *t*-test, P<0.05). Error bars indicate standard error.

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Nutrient transport in the chalazal region

INTRODUCTION

The chalaza has been shown to be the nutrient unloading zone, symplastically connected to the phloem, which provide nutrients to the seed, and to the seed coat and nucellus tissues, which export nutrients to the apoplastic space. In this study we challenged the hypothesis that the chalaza might also be involved in apoplastic sugar transport. We characterized the role of the SWEET10 transporter protein in facilitating sucrose export in the chalazal region (Chen et al., 2012a; Rottmann et al., 2018). The nature of the SWEET facilitator and our finding of a cell wall bound invertase in the chalazal area suggest that the chalaza provide sucrose and hexoses to the endosperm.

RESULTS

The *SWEET10* gene is expressed in the chalaza

We tested if members of the *SWEET* gene family are expressed in the chalzal tissue. According to microarray data by Belmonte and coworkers (Belmonte et al., 2013), the *SWEET10* sucrose facilitator gene (Rottmann et al., 2018) is specifically expressed in the chalazal region of Arabidopsis seeds throughout seed development. To better characterize its expression pattern, we fused *SWEET10* promoter region to the chimeric *GFP-GUS* reporter gene. In *ProSWEET10:GFP-GUS* ovules and seeds, we detected GFP fluorescence in the chalazal cells surrounding the vasculatures, in both ovules and seeds (Fig. 1). Our results indicate that the chalaza play a role in sugar apoplastic transport.

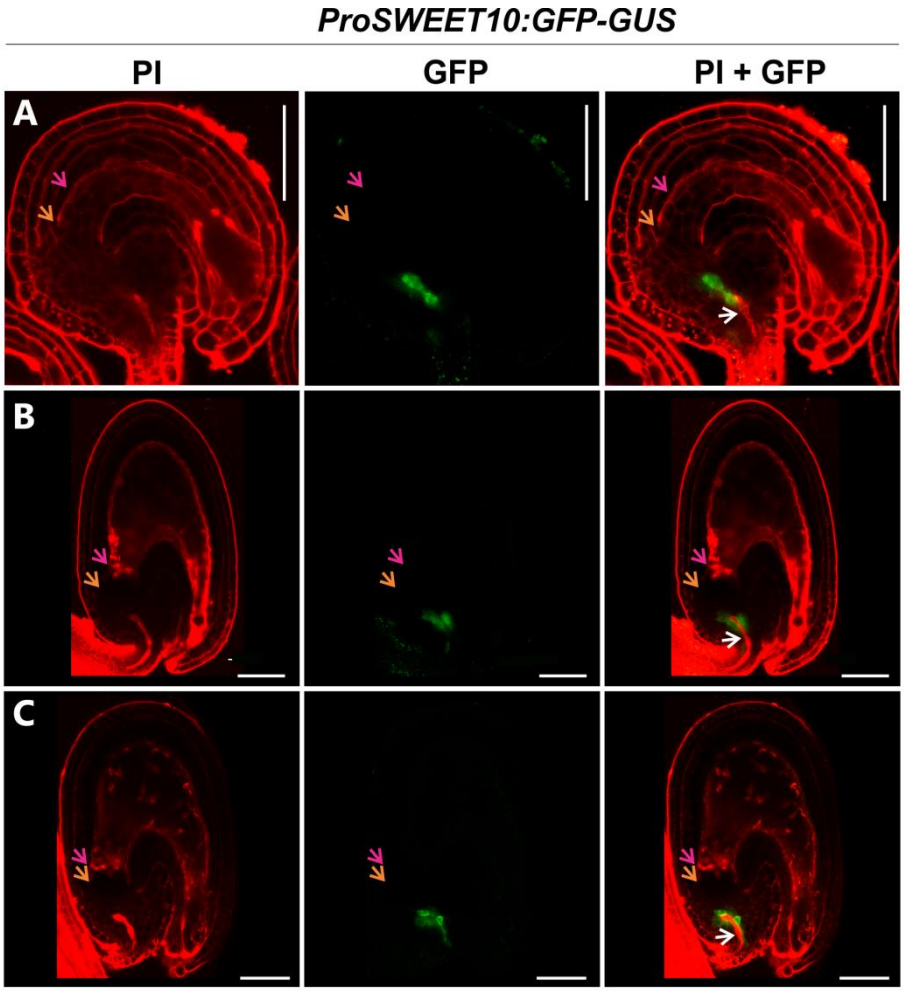

Figure 1 *SWEET10* is expressed in chalaza
(A) to (C) Propidium iodide(PI), GFP, and PI-GFP superimposed fluorescence images of *proSWEET10:GFP-GUS* ovule stage 3-VI(A), and seed at pre-globular (B), and globular (C) embryo stages. Orange, purple and white arrows indicate persistent and transient nucellus and vascular bundle, prospectively. Bars=50μm.

SWEET10 localize to the plasma membrane

To test if SWEET10 facilitate transport across the plasma membrane, we studied its sub-cellular localization. Orthologues of the Arabidopsis SWEET10 protein in sweet potato and grapes have been shown to localize to the plasma membrane (Li et al., 2017; Zhang et al., 2019). Similarly, we observed GFP fluorescence in the plasma membrane and vacuoles (Fig. 2A) or only in the plasma membrane (Fig. 2B) of chalazal cells expressing *SWEET10* promoter and genomic sequence translationally fused to *GFP*. These data indicate that SWEET10 facilitate sugar export to the apoplast. SWEET-GFP localization in the vacuole might be due to incorrect protein folding or overcrowding of the secretory pathway in the transgenic lines.

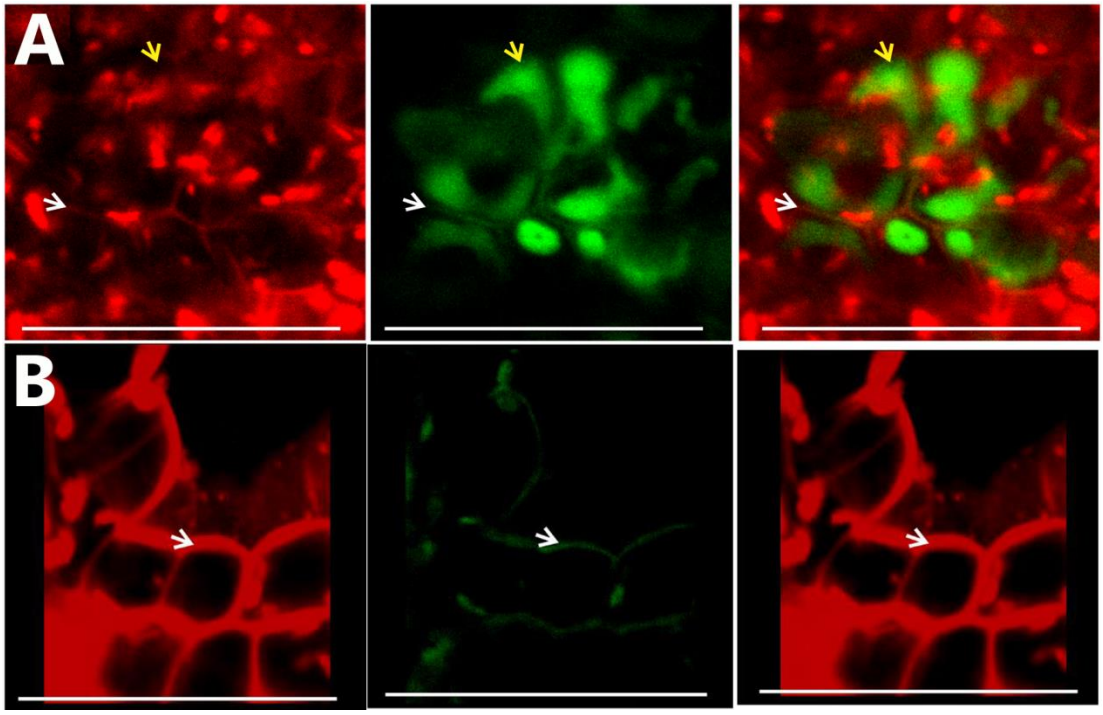

Figure 2 SWEET10 localize to the plasma membrane
(A) to (B) Propidium iodide (PI), GFP, and PI-GFP superimposed fluorescence images of *proSWEET10: SWEET10-GFP* seeds at pre-globular stage. White and yellow arrows indicate plasma membranes and vacuoles, respectively. Bars=25µm.

The chalaza provides hexoses to the endosperm

SWEET10 has been demonstrated to work as sucrose facilitator (Chen et al., 2012a; Rottmann et al., 2018). To test if the sucrose transported by SWEET10 to the apoplastic space is then converted into hexoses, we tested if cell wall bound invertases, which hydrolyze sucrose into hexoses, are expressed in the chalaza. According to microarray data by Belmonte and coworkers (Belmonte et al., 2013), the cell wall bound *INVERTASE4* (*INV4*) (Ruhlmann et al., 2010) is expressed in the chalazal region of Arabidopsis seeds throughout seed development. To better characterize its expression pattern, we translationally fused the *INV4* promoter and genomic sequence to *GFP*. *ProINV4:INV4-GFP* ovules and seeds displayed GFP fluorescence in the central domain of the chalaza (Fig. 3A-3C), an expression pattern similar to that observed in *ProSWEET10:GFP-GUS* lines. These data indicate that INV4 might hydrolyze the sucrose exported to the apoplast by SWEET10.

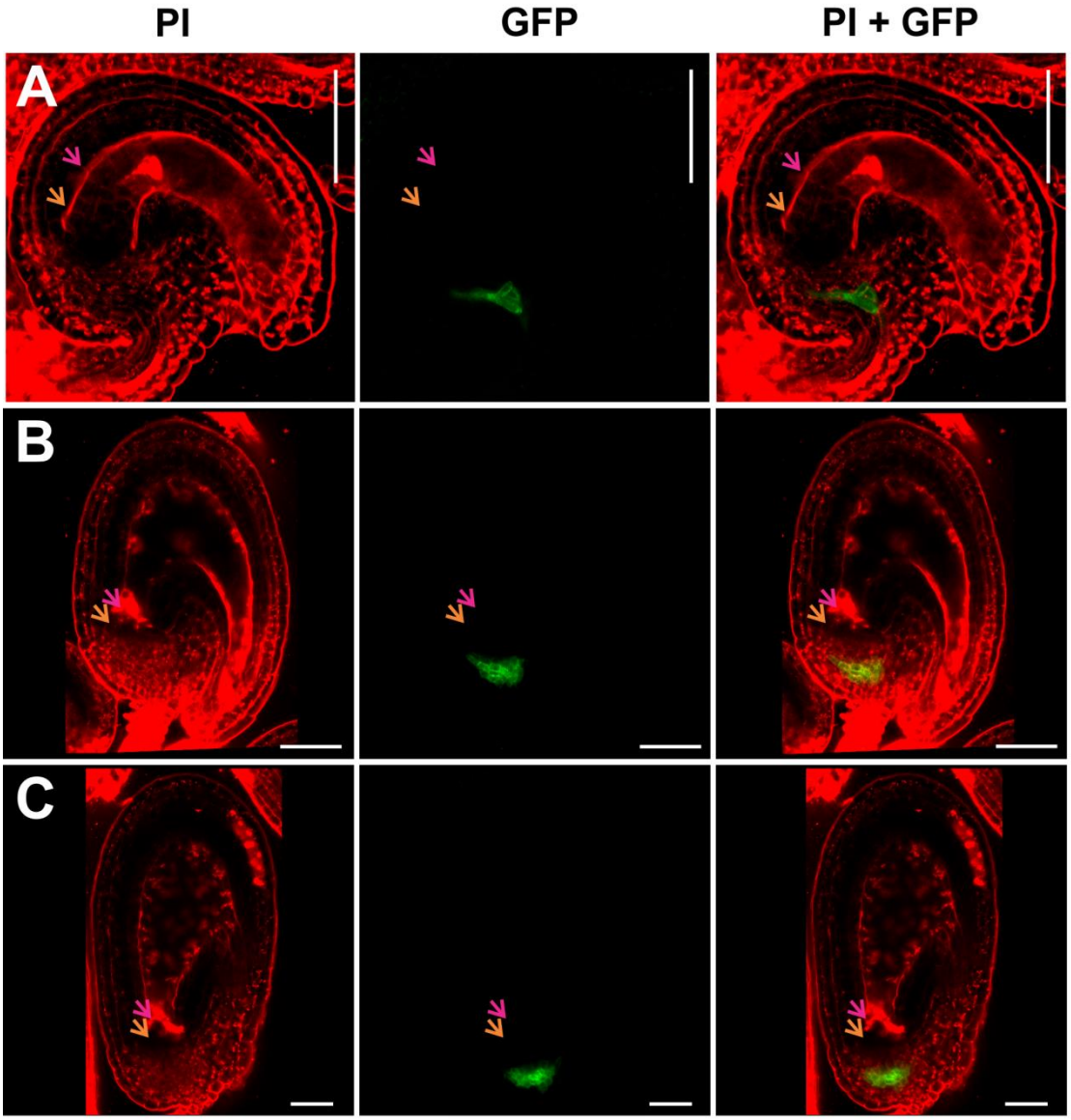

Figure 3 *INV4* is expressed in the chalaza
(A) to (C) Propidium iodide (PI), GFP, and PI-GFP superimposed fluorescence images of *proINV4: INV4-GFP* ovule at stage 3-VI (A), and seeds at pre-globular (B), and globular (C) embryo stages. Orange and purple arrows indicate persistent and transient nucellus, respectively. Bars=50μm.

The *sweet10* mutation delays embryo development

Similar to *sweet4*, we observed that the *sweet10* mutation impaired embryo growth (Fig. 4). The *sweet10* mutant embryo development was largely delayed. These data support the hypothesis that SWEET10 facilitates sugar export to the embryo.

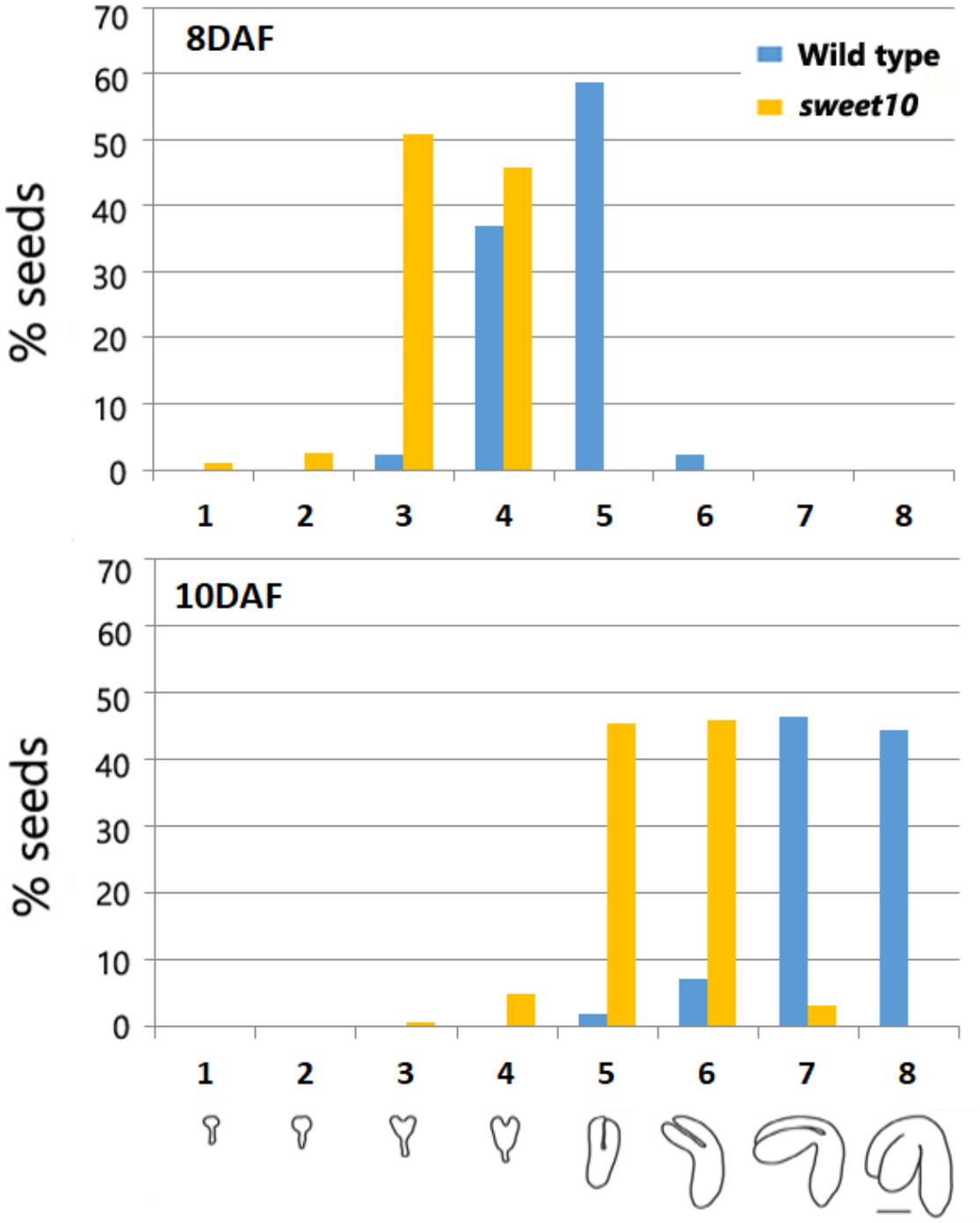

Figure 4 The *sweet10* mutation delays embryo development
Distribution of different embryo developmental stages (1: globular, 2: transition, 3: heart, 4: early torpedo, 5: late torpedo, 6: walking stick, 7: bent cotyledon, 8: mature embryo) in wild type and mutant seeds at 8 and 10 days after fertilization (DAF). N > 100. Bars=100µm.

TT16 regulates *SWEET10* expression pattern

To test if TT16 affects nutrient transport by regulating *SWEET10* expression, we introgressed *ProSWEET10:GFP-GUS* lines in a *tt16* background. *tt16* mutation did not appear to affect *SWEET10* spatial expression pattern (Fig. 5A to 5B), when compared to the wild type (Fig. 1). By contrast, RT-qPCR analyses revealed a 2.2 fold increase of *SWEET10* expression in *tt16* seeds at 2 DAF, when compared to the wild type (Fig. 5C). *TT16* is not expressed in the chalaza (Xu et al., 2016) but it has been shown to act non-autonomously on the development of the chalazal cells

(Fiume et al., 2017a).

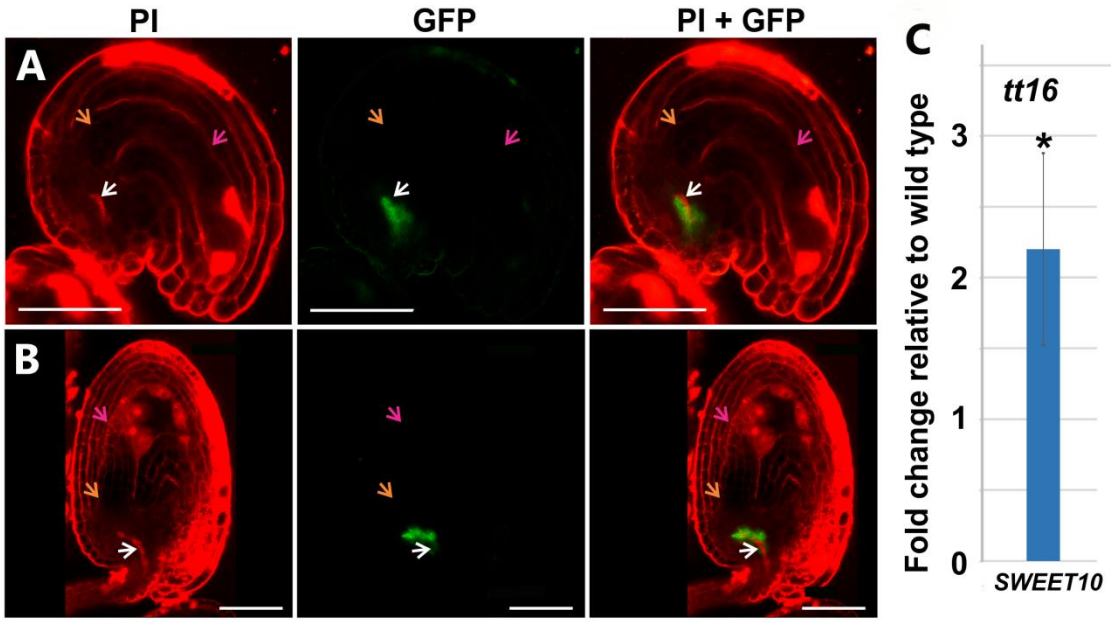

Figure 5 *TT16* regulates *SWEET4* expression pattern
(A) to (B) Propidium iodide (PI), GFP, and PI-GFP superimposed fluorescence images of *proSWEET10: SWEET10-GFP* ovule at stage 3-VI (A), and seeds at pre-globular (B), and globular. Orange and purple arrows indicate persistent and transient nucellus, respectively. Bars=50µm.
(C) Quantitative RT-PCR analyses of *SWEET10* expression in tt16 mutant relative to the wild type. Asterisks indicate statistical significance difference between wild type and mutant (two tailed Students'/-test, $P<0.05$). Error bar indicates standard deviation. Expression level was normalized and averaged from four independent biological samples.

DISCUSSION

The chalaza-nucellus path of nutrient transport

Our data indicate that part of the sucrose transported to the seed through the phloem is immediately exported to the apoplast by the SWEET10 sucrose exporter and converted into hexoses by the INV4 cell wall bound invertase (Fig. 6). We speculate that the apoplastic glucose, provided by nucellus and chalaza, might diffuse toward the endosperm to be reimported by endosperm SWEET facilitators. Compared to the seed coat pathway, which is based on sucrose transport, the chalaza-nucellus path offers a faster route toward the endosperm thus allowing the transport of readily available glucose and fructose.

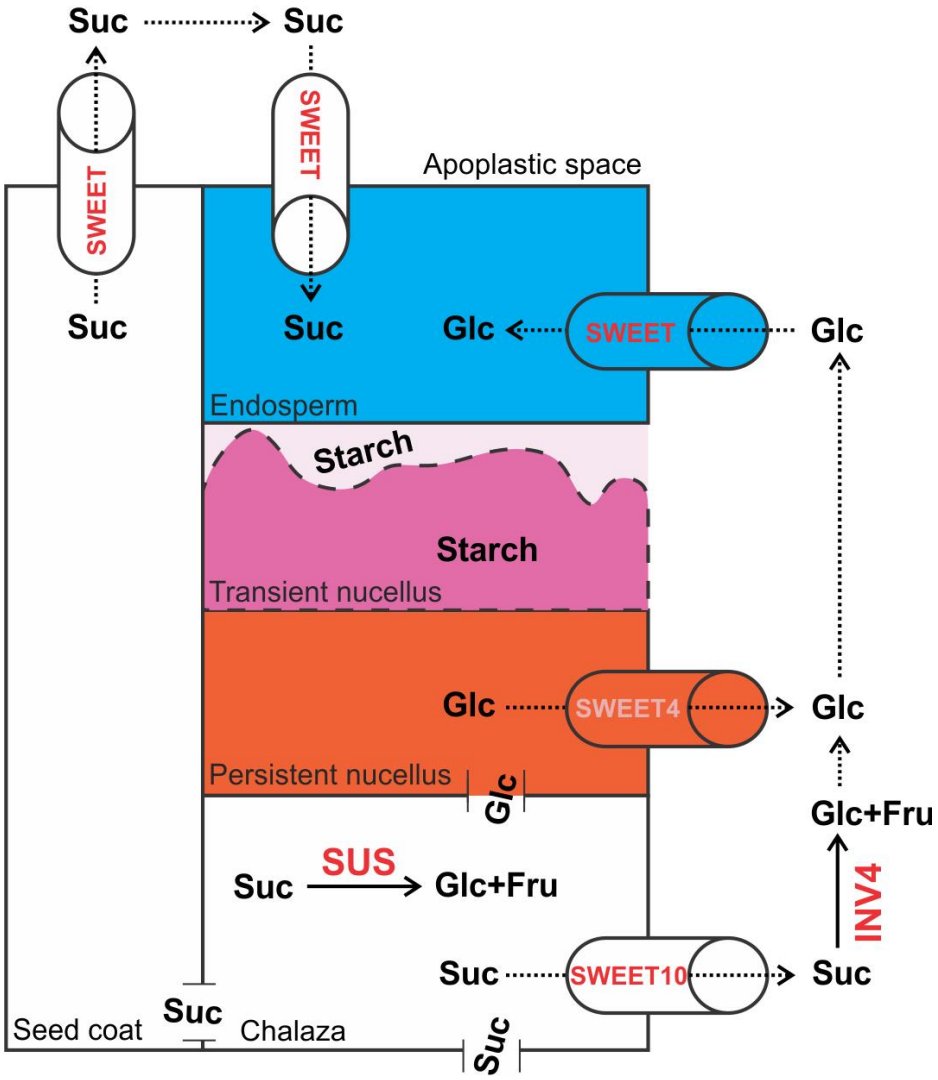

Figure 6 The chalaza-nucellus path of sugar transport
Solid and dotted arrows indicate enzymatic reactions and diffusion paths, respectively. Dashed lines indicate cells undergoing cell elimination. Glu, glucose; Fru, fructose; Suc, sucrose.

METHODS

Plant material

All *Arabidopsis thaliana* plants are in the Columbia accession. The *tt16-1* allele was isolated in the Wassilewskija accession from the INRA Versailles collection (Bechtold and Pelletier, 1998) and then backcrossed to Columbia (Coen et al., 2017; Nesi et al., 2002a). *sweet10-1* (SALK_144826) alleles are from the Salk collection (Alonso et al., 2003) whereas the *sweet4-2* (GK_858G02) (Chong et al., 2014) allele is from the GABI-KAT collection (Kleinboelting et al., 2012).

Cloning and Construction

PCR amplifications were performed using the gene-specific primers described below carrying the attB1 (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3′) and attB2 (5′-GGGGACCACTTTGTACAAGAAAGCTGGGTC- 3′) Gateway recombination sites at the 5′-ends

of the forward and reverse primers, respectively. All PCR products were amplified with the high-fidelity Phusion DNA polymerase (Thermo Fisher Scientific), recombined into the *pDONR207* or *pDONR201* vector (BP Gateway reaction) according to the manufacturer's instructions (Thermo Fisher Scientific), and then sequenced. The PCR products cloned into the *pDONR* vectors were then recombined into the appropriate destination vector (LR Gateway reaction) according to the manufacturer's instructions (Thermo Fisher Scientific). *SWEET10* 3.1 kb promoter region was amplified using the attB1-(5′ CCTAAAGATGATAGTATTGATGA 3') forward and attB2-(5′ TTTTATATCTCTCTCAAAGTAGTC 3') reverse primers. *SWEET10* 3.1 kb promoter region and genomic sequence without the stop codon was PCR amplified using the attB1-(5′ CCTAAAGATGATAGTATTGATGA3') forward and attB2-(5′ ATTCTTAGAAATGAGAAATACTTCTT3') reverse primers. *INV4* 3.2 kb promoter region and genomic sequence without the stop codon was PCR amplified using the attB1-(5′ATTTCTCGGCTGCAAAACAT3') forward and attB2-(5′AAGAGCTCCATCATTCATTTGCAG3') reverse primers.

ProSWEET10 sequences were recombined into the *pBGWFS7.0* binary vector (Karimi et al., 2002), *ProSWEET10:SWEET10* and *ProINV4:INV4* sequences were recombined into the *pMDC107* binary vector (Curtis and Grossniklaus, 2003).

Transgenic plants

The *Agrobacterium tumefaciens* strain C58C1 was used to stably transform Arabidopsis plants using the floral dip method (Clough and Bent, 1998). Transformants were selected with the appropriate resistance and subsequently transferred to soil for further characterization. More than 10 independent transgenic lines were tested for each construct transformed. One transgenic line for each construct is presented as representative of the majority of lines showing consistent results.

Expression analysis

Four independent biological samples were used for each analysis. Each replicate comprised the content in seeds of ten siliques. Total RNA was extracted using the RNeasy Mini kit (Qiagen), including RNase-Free DNase Set (Qiagen) treatment during washing, according to the manufacturer's instructions. The Superscript Reverse Transcriptase II kit (Invitrogen) was used to generate cDNA from 1µg of total RNA. Quantitative PCRs were performed with the SYBR Green kit (Bio-Rad) on a Bio-Rad CFX real-time PCR machine. *SWEET10* was PCR amplified using the (5'CCTTATCGGCTGTCATGTGG3') forward and (5' AAGCTTAATCCCTGGTGGCT3') reverse primers. Expression levels were first normalized to the geometrical mean of the expression levels of four reference genes (GAPDH, AT4G12590, AT4G02080 and AT3G25800) and subsequently normalized to the expression level of the adequate control.

Confocal microscopy (see chapter2)

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: *TT16* (AT5G23260), *SWEET4* (AT3G28007), *SWEET10* (AT5G50790), *INV4* (AT2G36190).

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Chapter III - *Symplastic block in the nucellus*

INTRODUCTION

How is cell elimination contained?

Our preliminary results suggest that cell elimination of the nucellus involves the degradation of cell walls and the release of cell components, including vacuole contents, into the apoplast. Such events undoubtedly involve the release of destructive molecules that might be expected to diffuse locally through the apoplast or through plasmodesmata. However, intriguingly, cell death has not been reported to spread to neighboring tissues in seeds. Furthermore, the nucellus is not completely eliminated. As a result, it seems clear that cell elimination is tightly spatially controlled both within and between the tissues of the developing seed.

Soon after fertilization the three genetic compartments of the seed (maternal tissues, endosperm and embryo), become symplastically isolated (Stadler et al., 2005), thus suggesting that the establishment of a symplastic barrier might be responsible of containing cell elimination.

The establishments of symplastic blocks

Previous studies have shown that the regulation of plasmodesmal connectivity among cells is a dynamic process that is tightly linked to developmental and physiological processes (Holdaway-Clarke et al., 2000; Tucker and Boss, 1996). Furthermore, various signal pathways have been shown to relay primary cellular signals to modify plasmodesmata (Sager and Lee, 2014). Two general classes of mechanisms have been shown to modify the permeability of plasmodesmata: callose dependent and callose independent mechanisms.

Callose (β-1,3-glucan) is a polysaccharide that is deposited at the cell plate during cytokinesis. When cell division is completed, callose is almost completely degraded (Northcote and Lay, 1989). Nevertheless, a small portion persists and plays a role in regulating the permeability of plasmodesmata. Callose accumulation around the neck region of plasmodesmata effectively reduces plasmodesmal permeability (Zavaliev et al., 2011a), and even lead to plasmodesmal closure (Fig 1). The level of callose accumulation at plasmodesmata is mediated mainly by the activity of two enzymes: β-1,3-glucan synthases and glucanases (Zavaliev et al., 2011a), which catalyze the synthesis and degradation of callose, respectively. In Arabidopsis, twelve genes are predicted to encode the catalytic subunit of the β-1,3-glucan synthase and are named *CALLOSE SYNTHASE* (*CALS*) or *GLUCAN SYNTHASE-LIKE* (*GSL*) genes (Saxena and Brown Jr, 2000; Verma and Hong, 2001). Furthermore, fifty *B-1,3-GLUCANASE* (*BGL*) genes were identified in Arabidopsis (Doxey et al., 2007; Zavaliev et al., 2011b).

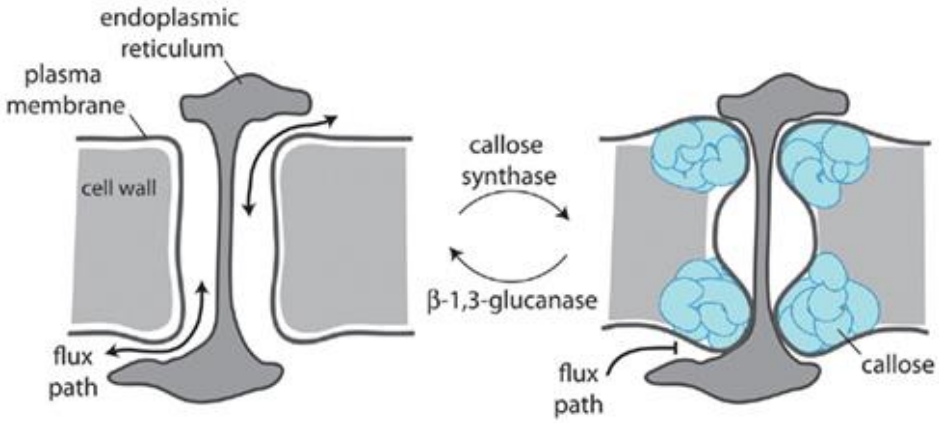


Fig 1 Callose dependent closure of plasmodesmata (Maule et al., 2012)

Callose-dependent modulation of plasmodesmata permeability has been described in various developmental processes and in response to different cellular and environmental conditions. It has been shown that the loss of symplastic connectivity between megaspore mother cell and neighboring nucellus cells in the ovule (Werner et al., 2011) is due to callose deposition around the megaspore mother cell (Schneitz et al., 1995c). Similarly, the tapetal cells, surrounding the pollen grains, secrete callose, thus leading to their symplastic isolation (Mamun et al., 2005a, b; Regan and Moffatt, 1990). As a consequence of the symplastic isolation of the tapetum cell layer, nutrients and metabolites are transported apoplastically to the developing pollens grains (Clément and Audran, 1995; Roschzttardtz et al., 2011). It is speculated that the symplastic isolation of the tapetum during pollen development is necessary to protect the surrounding cells from potentially detrimental molecules generated during the programmed cell death of the tapetum (Wu and Cheung, 2000). After fertilization, the embryo is symplastically isolated from the surrounding tissues. Only the suspensor and embryo proper are symplastically connected during early embryogenesis (Stadler et al., 2005). However at embryo heart stage, the suspensor become cytoplasmically isolated as its plasmodesmata increase in structural complexity and are occluded by a callose like substance (Kozieradzka-Kiszkurno and Płachno, 2012). The symplastic restriction escalates through embryo maturation, till an overall block in intercellular permeability in the late torpedo stage as the suspensor start degenerating (Stadler et al., 2005).

Besides the over mentioned developmental processes, callose modulates plasmodesmata closing in response to various cellular and environmental conditions, such as cold, wound, toxic metal stress, defense and dormancy. A number of callose modifying enzymes were shown to modulate plasmodesmata callose level in response to various environmental stimuli. The *cals1* mutant fails to accumulate callose in response to pathogenic bacterial infection, whereas the *cals8* mutant shows disrupted callose deposition in response to mechanical wounding (Cui and Lee, 2016). Chilling-induced dormancy breakage at the shoot apical meristem is marked by an increase in symplastic connectivity and shows up-regulation of *BGL* gene expression, responsible for

plasmodesmata callose degradation (Rinne et al., 2001).

Plasmodesmata can be blocked also through a callose-independent mechanism. Cytoskeletal elements like filamentous actin, myosin proteins and microtubules, have been found localized to or near plasmodesmata, and play a role in transporting viral movement proteins. For example, the cauliflower mosaic virus (CaMV) inclusion body protein P6 traffics intracellularly along microfilaments and actin disruption inhibits CaMV cell to cell movement (Harries et al., 2009). Plant MYOSIN VIII locates at the plasma membrane and in clusters near plasmodesmata and helps targeting viral cargoes to plasmodesmata (Baluška et al., 2004; Radford and White, 1998; Reichelt et al., 1999). It has been shown that the disruption of tobacco MYOSIN VIII impairs the targeting of the viral protein Hsp70 to plasmodesmata (Avisar et al., 2008). Furthermore, a study showed that increasing the concentration of cytosolic free calcium stimulates the rapid closure of plasmodesmata (Tucker, 1990) by affecting calcium-responsive proteins, including certrin and myosin, which associate with plasmodesmata (Geimer and Melkonian, 2005; Hu et al., 2004; Knight and Kendrick-Jones, 1993; Sanders and Salisbury, 1989). This rapid but transient contractions of the pore, which is physiologically relevant, takes only 10 seconds compared to the callose-dependent transient closure which takes hours (Holdaway-Clarke et al., 2000; Tucker and Boss, 1996). Based on their localization in plasmodesmata and detrimental effect on intercellular movement, it has been speculated that filamentous actin and myosin regulate the plasmodesmal size exclusion limit (Tilsner et al., 2016).

In addition to β-1,3-glucan synthases and glucanases, a number of other plasmodesmata-localized proteins have been identified to regulate the permeability of plasmodesmata through both callose-dependent and independent modes. For example, ectopic overexpression of the Arabidopsis *PLASMODESMATA CALLOSE BINDING PROTEIN1* (*PDCB1*) gene, which encodes an extracellular protein that contains a glycosylphosphatidylinositol anchor, increases callose deposition in the plasmodesmata (Simpson et al., 2009). Furthermore, *PLASMODESMATA-LOCATED PROTEIN 1* (*PDLP1*) and *5* genes encode receptor-like integral membrane proteins in Arabidopsis whose overexpression results in reduced plasmodesmata connectivity (Cui and Lee, 2016; Thomas et al., 2008). When cells are infected by pathogens, PDLP5 allows callose accumulation in plasmodesmata, eventually leading to the symplastic isolation of the infected cells. This response helps affected cells to isolate themselves from adjacent cells and safely execute programmed cell death (Lee et al., 2011).

RESULTS

Persistent and transient nucellus are symplastically isolated.

We have previously shown that the nucellus is symplastically connected to the chalaza by GFP mobility analyses. Free GFP, which is small enough to diffuse through the plasmodesmata, was

expressed in a few chalazal cell under the control of the *pSUC2* promoter and fluorescence was observed in chalaza, seed coat and persistent nucellus at 1-cell globular embryo stage (Fig. 2B). As a control, GFP-sporamin, which exceeds the plasmodesmata size exclusion limit, was confined in chalaza cells at the same development stage (Fig. 2A). Interestingly, we did not detect free GFP in the transient nucellus cells that have not undergone elimination yet (Fig. 2B). These data suggest that the transient nucellus is not symplastically connected to the persistent nucellus.

We than asked when such a symplastic barrier is established during seed development. In particular, if it is fertilization dependent, as many other seed developmental pathways. To address these questions we analyzed symplastic connections in mature ovules using the same *pSUC2:GFP* and *pSUC2:GFP-sporamin* lines described above. Free GFP was detected in chalaza, oi, proximal part of ii and persistent nucellus of mature ovules, but not in the transient nucellus (Fig. 2D). As control, GFP-sporamin was detected only in a few cells of the chalaza where the phloem ends, at the same develop stage (Fig. 2C). These results indicate that the symplastic barrier is already created between transient and persistent nucellus before fertilization.

To characterize the precise time point at which the symplastic block is established we looked earlier in ovule development. We detected GFP fluorescence in the all nucellus of ovule primordia (Supplementary Fig. 1). Interestingly, at stage 3-IV of ovule development (Schneitz et al., 1995b) the entire nucellus, including its proximal region, appeared symplastically disconnected from the chalaza (Supplementary Fig. 1). At stage 3-V we detected GFP in the persistent but not transient nucellus, thus indicating that the symplastic block is established at this stage of ovule development (Supplementary Fig. 1).

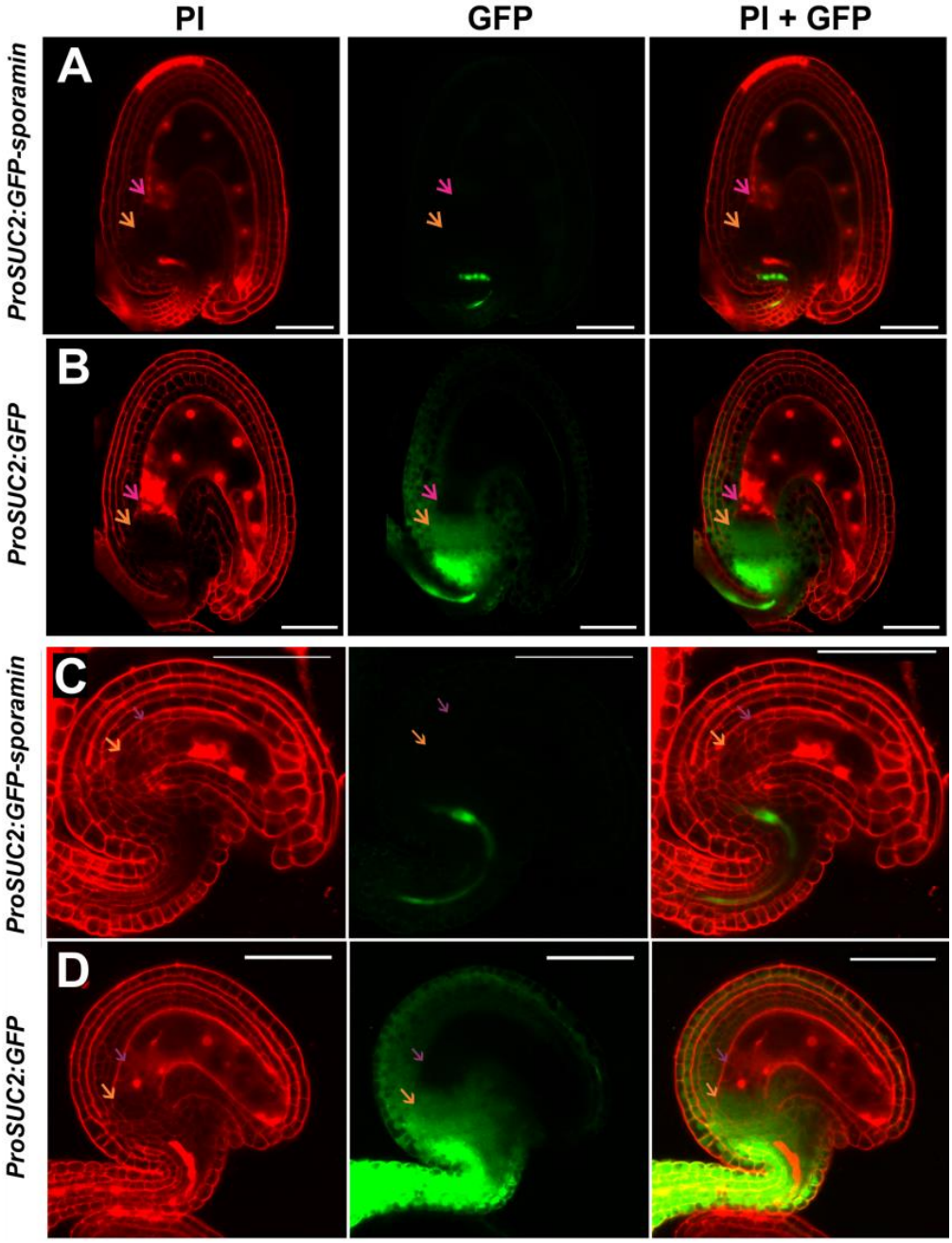


Figure 2 The transient nucellus is symplastically isolated from the persistent nucellus
(A) and (B) Propidium iodide (PI), GFP and PI-GFP superimposed fluorescence images of *ProSUC2:GFP-sporamin* (A) and *ProSUC2:GFP* (B) wild type seeds at pre-globular embryo stage. (C) and (D) Propidium iodide (PI), GFP and PI-GFP superimposed fluorescence images of *ProSUC2:GFP-sporamin* (C) and *ProSUC2:GFP* (D) wild type mature ovules. Orange and purple arrows indicate persistent and transient nucellus, respectively, Bars=50 μm.

TT16 does not regulate the nucellus symplastic block

Considering that TT16 promotes the elimination of the transient nucellus, we tested if the nucellus symplastic block is altered by the *tt16* mutation. We therefore introgressed *ProSUC2:GFP-sporamin* and *ProSUC2:GFP* lines in a *tt16* background. We found that the symplastic connection pattern in the *tt16* mutant is comparable to that of the wild type. Free GFP was not observed in the large transient nucellus, which does not undergo cell elimination, of *tt16* seeds (Fig. 3B). As a control, GFP-sporamin was confined only in a few cells of the chalaza at the

same development stage (Fig. 3A).

At the mature ovule stage, free GFP was not detected in the transient nucellus of *tt16* lines, as observed in the wild type (Fig. 3D). GFP-sporamin was detected in a few cells of the chalaza where the phloem ends (Fig. 3C). Wild type and *tt16* ovules appeared indistinguishable at earlier ovule stages (Supplementary Fig. 2). These data suggest that TT16 does not regulate the establishment of the symplastic block between persistent and transient nucellus.

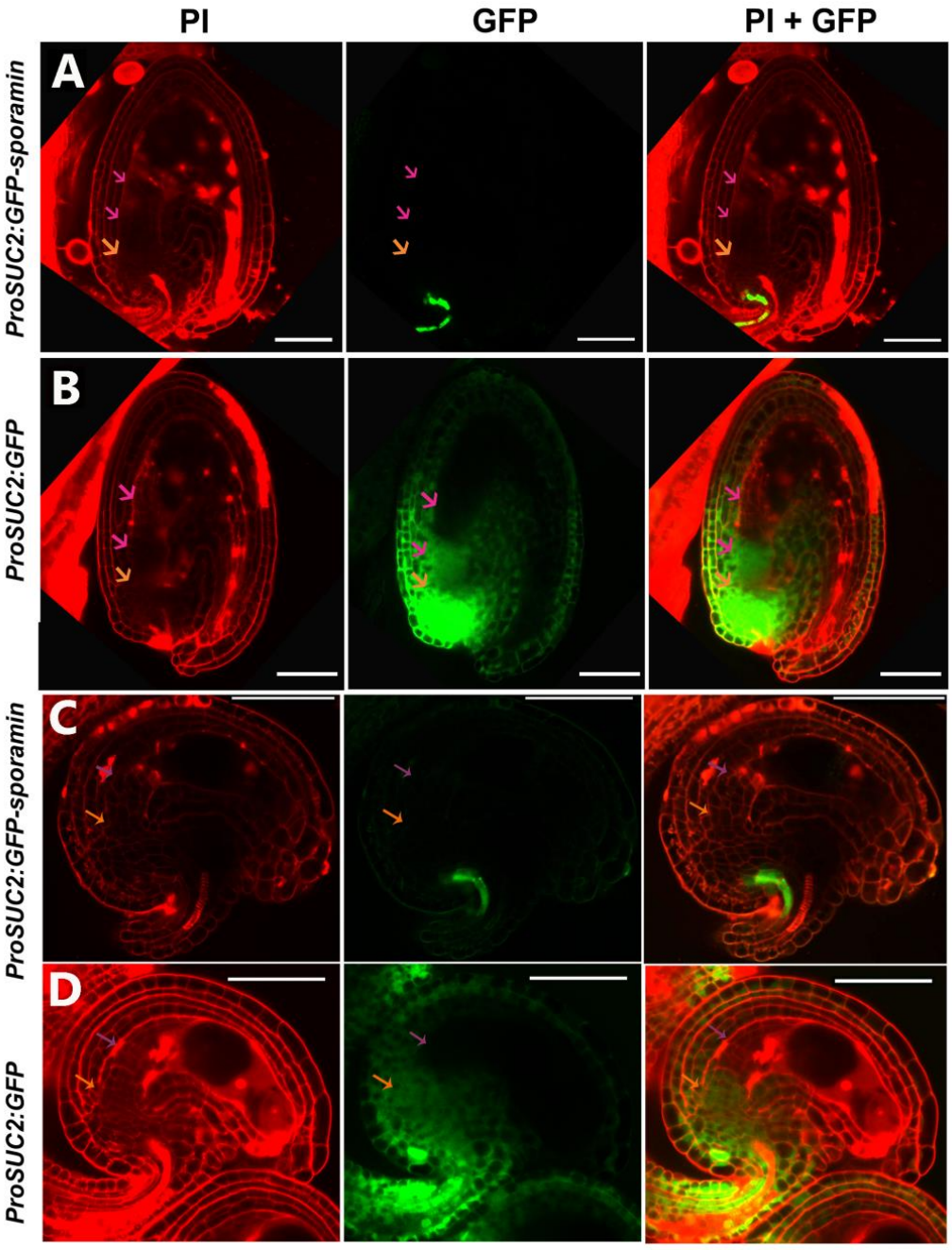


Figure 3 The nucellus symplastic block in the *tt16* mutant
(A) and (B) Propidium iodide (PI), GFP and PI-GFP superimposed fluorescence images of *ProSUC2:GFP-sporamin* (A) and *ProSUC2:GFP*(B) *tt16* seeds at the pre-globular embryo stage. (C) and (D) Propidium iodide (PI), GFP and PI-GFP superimposed fluorescence images of *ProSUC2:GFP-sporamin* (C) and *ProSUC2:GFP*(D) *tt16* mature ovules. Orange and purple arrows indicate persistent and transient nucellus, respectively, Bars=50 μm.

Callose deposition in the nucellus

Callose accumulation around the neck region of plasmodesmata can lead to the closure of plasmodesmata. To test whether callose deposition affects permeability of nucellus plasmodesmata, we used aniline blue, which is a visual marker of callose (Zavaliev and Epel, 2015). We detected a strong aniline blue signal in the distal part of transient nucellus in both mature ovules and seeds at the pre-globular embryo stage (Fig. 4). Similar data were obtained in *tt16* ovules and seeds (Supplementary Fig 3).

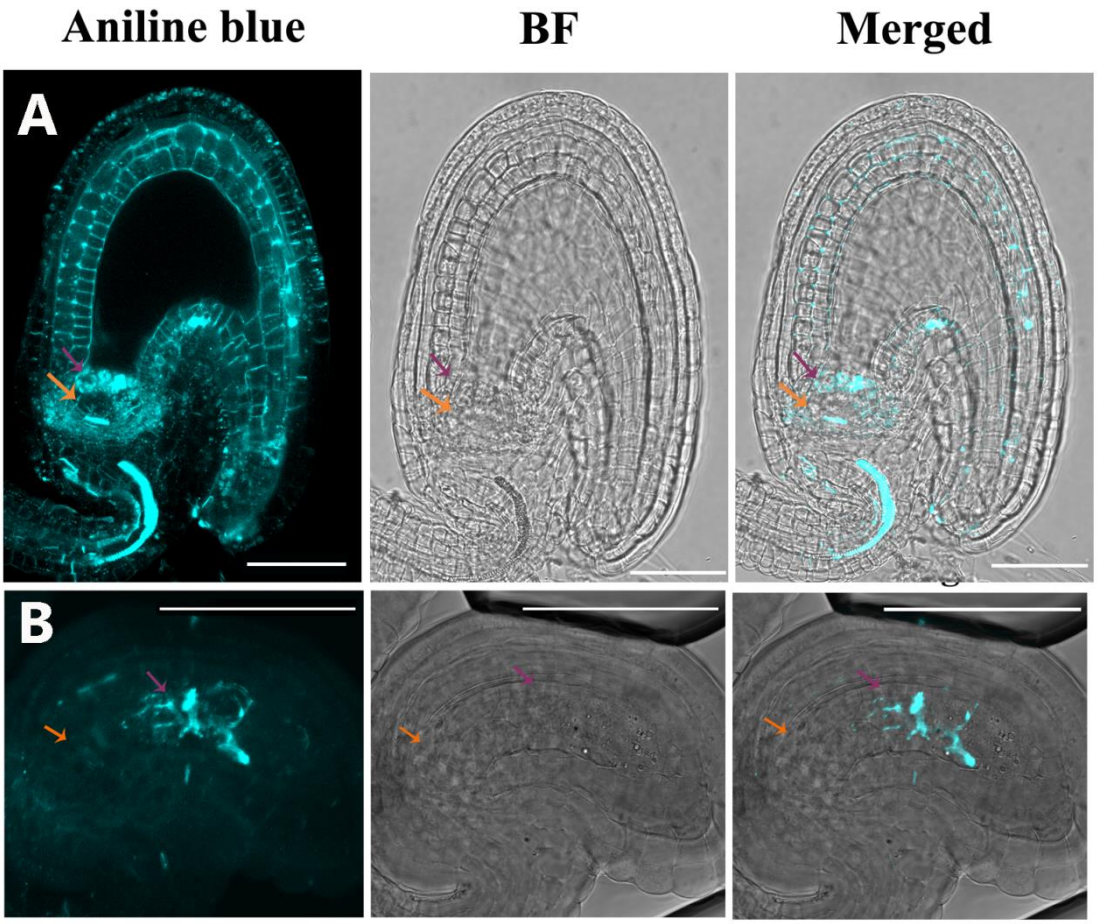


Figure 4 Callose deposition in the nucellus
(A) and (B) Aniline blue, bright field (BF), and superimposed images of a seed at pre-globular embryo stage and a mature ovule. Orange and purple arrows indicate persistent and transient nucellus, respectively, Bars=50 μm.

The morphology of plasmodesmata in the nucellus

Plasmodesmata that accumulate callose at their neck region have a characteristic “buldgy” appearance when observed at the transmission electron microscope (TEM). We, therefore, analyzed plasmodesmata by TEM in nucellus longitudinal sections of mature ovules (Fig. 5A). We found a relatively high number of plasmodesmata connecting the cells of the persistent nucellus (Fig. 5B). However, in the transient nucellus, we hardly found plasmodesmata, and the morphology of the relatively few ones that we detected resembled that of plasmodesmata closed by callose

accumulation (Fig. 5C).

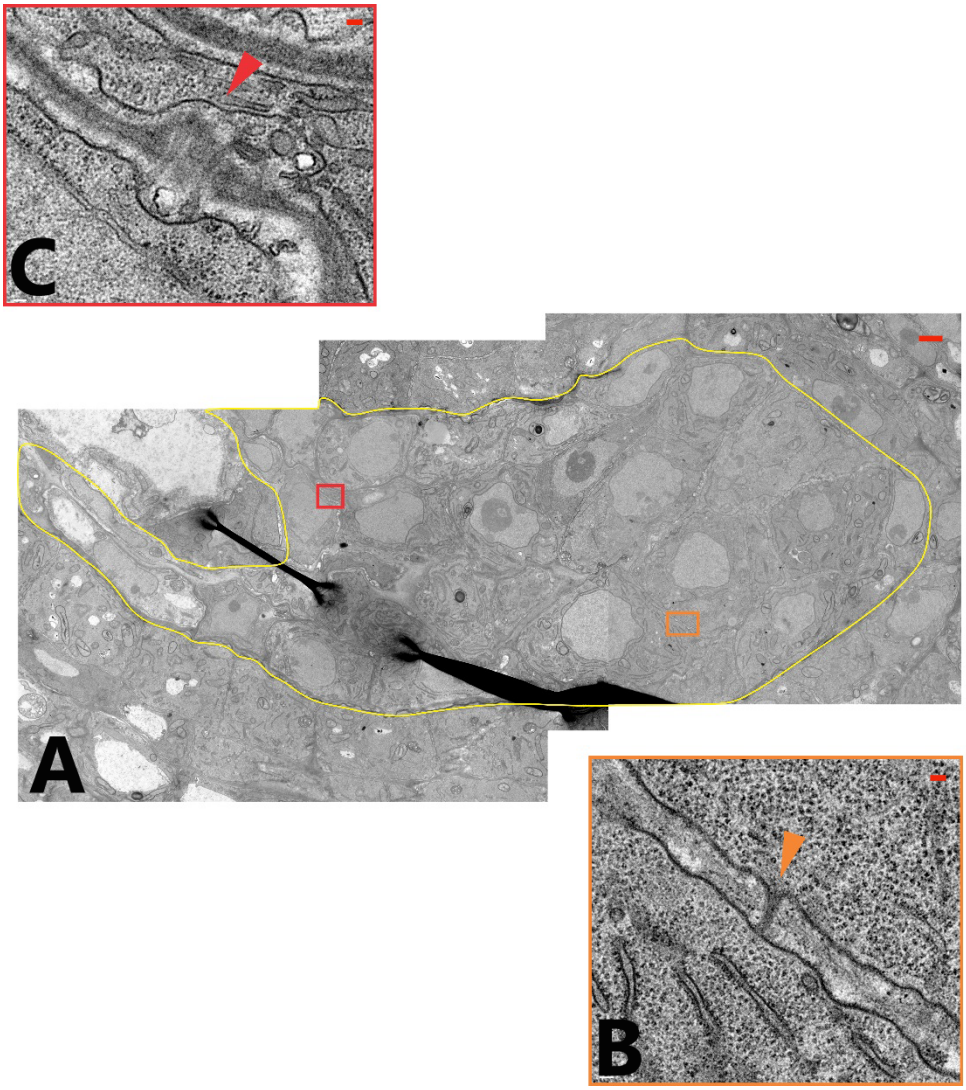


Figure 5 The morphology of plasmodesmata in the nucellus of mature ovule
(A) TEM image of a nucellus (highlighted in yellow) longitudinal section of a mature ovule. The distal region of the nucellus (transient nucellus) is on the left of the image A. (B and C) Close-up images of plasmodesmata in the persistent and transient nucellus, respectively. Orange and red arrowheads indicate plasmodesmata. Bars=10μm(A), 500=nm(B) and (C).

Expression of callose synthases in the nucellus

To determine if callose syntheses are expressed in the nucellus and potentially contribute to the formation of the nucellus symplastic block, we looked at the microarray data by Belmonte and coworkers (Belmonte et al., 2013) obtained from laser micro-dissected domains of the seed. The *GSL4* callose synthase gene was annotated as specifically expressed in the chalaza/nucellus area of the seed. To study its expression pattern, we generated a marker line carrying the *GSL4* -3kb promoter region fused to the uidA reporter gene, which encodes the GUS enzyme. The *GSL4* promoter drove GUS expression in the chalaza, but not the nucellus, throughout ovule and seed development (Fig.6).

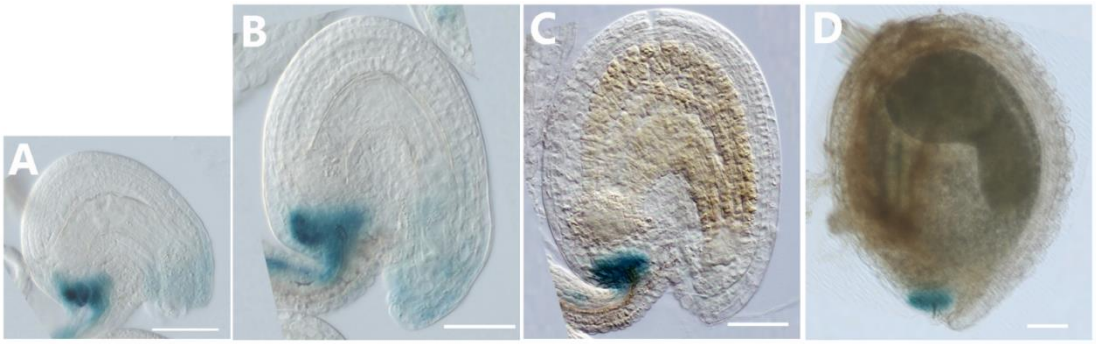


Figure 6 *GSL4* expression pattern.
GUS staining of *pGSL4:GUS* mature ovule (A), seed at early globular stage (B), seed at globular stage (C), and seed at mature embryo stage (D). Bars=50 µm.

MATERIAL AND METHODS

Plant materials

All *Arabidopsis thaliana* plants are in the Columbia accession. *ProSUC2:GFP* and *ProSUC2:GFP-sporamin* lines have been previously described (Stadler et al., 2005). The *tt16-1* allele was isolated in the Wassilewskija accession from the INRA Versailles collection (Bechtold and Pelletier, 1998) and then backcrossed to Columbia (Coen et al., 2017; Nesi et al., 2002a). We crossed *ProSUC2:GFP* and *ProSUC2:GFP-sporamin* lines into the *tt16-1* background.

Aniline blue staining

Harvested seeds and ovules were incubated in a 1% SDS, 0.2 N NaOH solution at 37 ℃ for 2hrs to clear the tissue. Samples were rinsed in water and incubated in a 12.5% bleach solution (1.25% active Cl–) for 10 minutes. Samples were then rinsed in water and transferred to the staining solution of 0.01% (w/v) aniline blue in water and incubated for 1hr at room temperature. Finally, samples were analyzed at the confocal microscope.

Confocal microscopy

GFP expressing lines were analyzed one hour after mounting in a 100 µg/ml propidium iodide diluted in a 7% sucrose solution (Figueiredo et al., 2016). Samples were imaged by confocal laser scanning microscopy (Leica SP8). More than 30 seeds were analyzed for each genotype and time point.

Transmission electron microscopy

For transmission electron microscopy (TEM), septums with seeds were immersed immediately after harvest in a fresh fixative solution which is Glutaraldehyde (Sigma, 2%), fresh formaldehyde (Sigma, 0.5%) in cacodylate buffer 0.1 M at pH 7. Fixation was performed for 4h at room temperature and 1 week at 4 ℃. Samples were subsequently contrasted with Oolong Tea Extract (OTE) (Delta Microscopies-France) 0.5% in cacodylate buffer 0.1M pH 7.4 for 1h, post-fixed with

1% osmium tetroxide containing 1.5% potassium cyanoferrate for 2h, gradually dehydrated in ethanol series (10% to 90%, 1h for each bath), and dehydrated twice for 1h in ethanol 100%. Samples were then gradually treated with mixtures of ethanol-epon (ratios of 1:2, 1:1 and 2:1, for 2h time) and finally transferred to pure epon (Delta Microscopie) overnight under vacuum. For embedding, ovules/seeds were spread on silanized glass slides. One drop of epon was added before overlaying with a second glass slide. After polymerisation (48h at 56℃), the epon layer was removed from the slide. Selected ovules and seeds were cut out and stuck on the top of Beem capsules (EMS) pre-filled with epon. Semi-thin sections (500 nm) were collected and colored with azure II/methylene blue to check tissue integrity before thin sections. Thin sections (70 nm) were collected onto either 125/200 mesh copper grids or slot grids and counter-stained with lead citrate. TEM samples were examined with Hitachi HT7700 electron microscope operated at 80 kV (Elexience-France), and images were acquired with a charge-coupled device camera (AMT).

Cloning

GSL4 -3kb promoter region was amplified using the attB1-(5′-GCGGCATGGAGATTATCAAC-3′) forward and attB2-(5′- GGGACGATTTCGTGAGACAT-3’) reverse primers. AttB1 (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3′) and attB2 (5′-GGGGACCACTTTGTACAAGAAAGCTGGGTC- 3′) carrying the Gateway recombination sites at the 5′-ends of the forward and reverse primers, respectively. The PCR products were amplified with the high-fidelity Phusion DNA polymerase (Thermo Fisher Scientific), recombined into the *Pdonr221* vector (BP Gateway reaction) according to the manufacturer’s instructions (Thermo Fisher Scientific), and then sequenced. The PCR products cloned into the *pDONR221* vectors were then recombined into the *pGWBFS7* binary vector (Curtis and Grossniklaus, 2003), (LR Gateway reaction) according to the manufacturer’s instructions (Thermo Fisher Scientific).

DISCUSSION

Why is the transient nucellus symplastically isolated from other tissues?

Cell elimination of the transient nucellus does not spread to the neighboring persistent nucellus, thus indicating that a mechanism to contain cell elimination is put in place. Our data indicate that the persistent nucellus is symplastically isolated from the transient nucellus already before fertilization, in mature ovules. We speculate that the symplastic block is put in place to stop the spreading of the cell elimination process that occurs in the transient nucellus. It could prevent the movement to the persistent nucellus of toxic compounds released from the degeneration of the transient nucellus cells. Furthermore, it might prevent the movement of death signals that might indicate which cells have to be eliminated. Nucellus cell elimination happens in a centripetal fashion, from distal to proximal, consistent with the diffusions of a death signal. Finally, the block

of the plasmodesmata of the persistent nucellus might be necessary to isolate the symplastic and apoplastic space of such cells once the transient nucellus is fully eliminated. Our data are based on GFP mobility assays and do not allow us to conclude if a full symplastic block or a reduction in the size exclusion limit of the nucellus plasmodesmata is put in place. Molecules smaller than 27 KD, the GFP size, might still be able to move through. We detected starch accumulation in the distal nucellus of *tt16* seeds, thus indicating the sucrose or hexoses might still move through the proximal-distal axis of the nucellus.

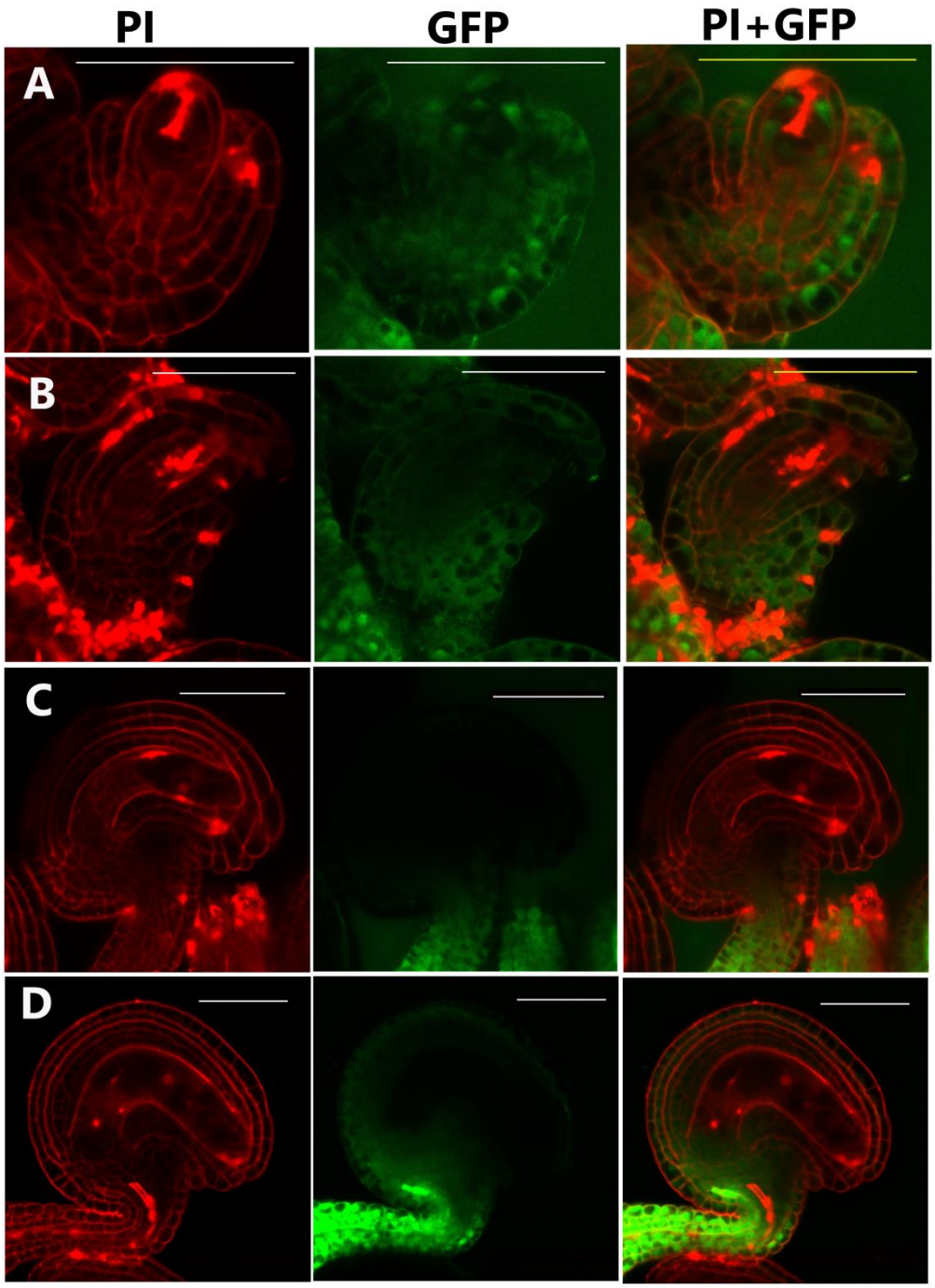
Callose deposition created the symplastic block in nucellus

Our aniline blue staining analyses suggest that the accumulation of callose in the plasmodesmata of the transient nucellus might lead to a reduction in their size exclusion limit. Nevertheless, the anline blue staining technique is limiting because does not allow the resolution needed to observe plasmodesmata and the fluorescence is easily quenched, thus requiring rapid imaging. TEM analysis support the hypothesis of a callose-dependent symplastic block as the distal nucellus plasmodesmata appeared swollen as observed in plasmodesmata clogged with callose. Further experiments are required to fully address this question.

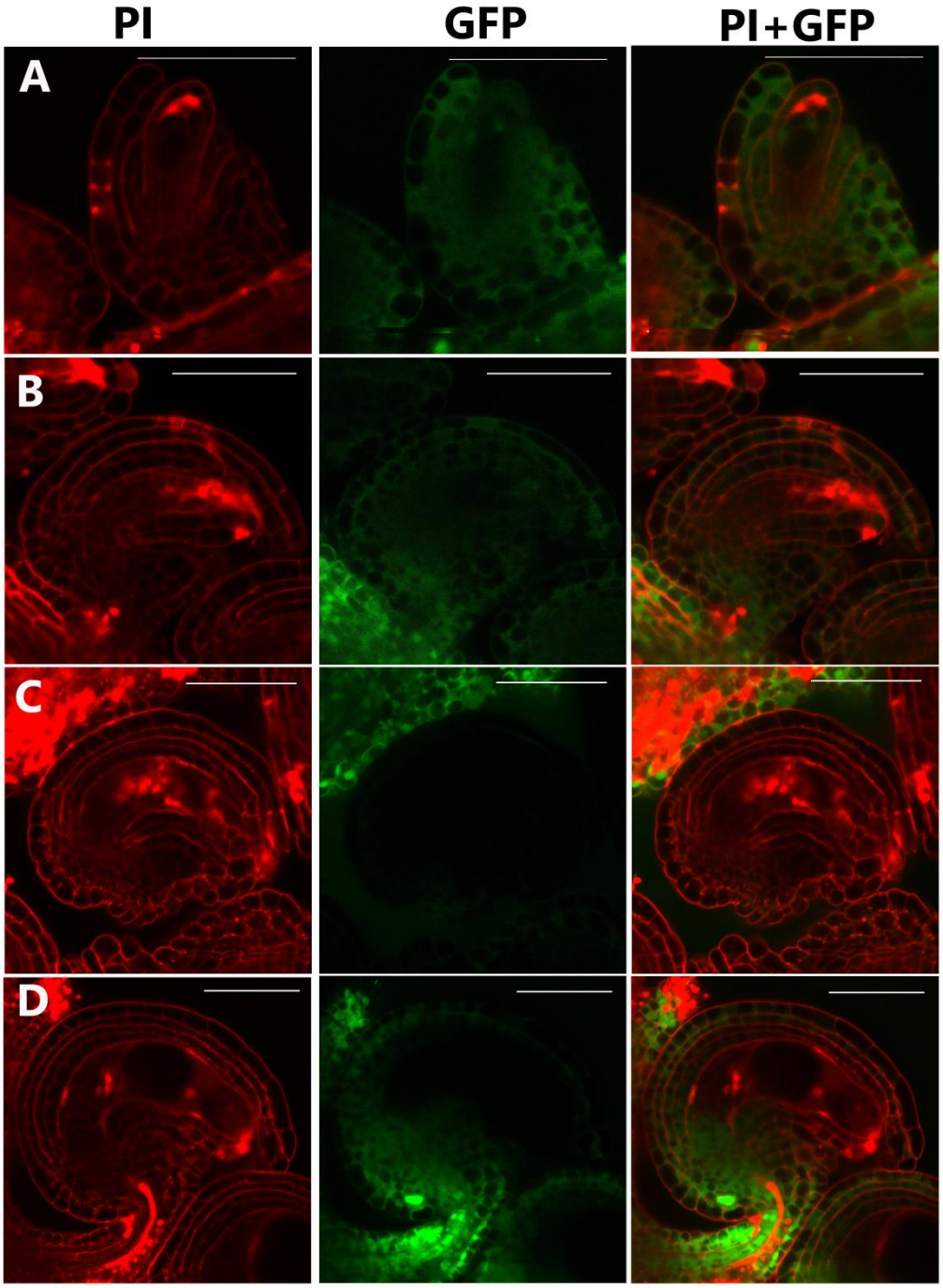
Future perspective

To confirm if callose is deposited in the plasmodesmata of the transient nucellus, we are planning to preform immunogold labeling assays using callose antibodies. Immunogold labeling could help us locate callose accurately and provide solid evidence of callose deposition in plasmodesmata. We will also keep testing the expression of members of the *GSL* gene family predicted to be expressed in the nucellus according to the microarray data by Belmonte and coworkers (Belmonte et al., 2013). The analysis of their mutant and over-expression lines will help us test hypotheses on the role of callose deposition in nucellus plasmodesmata and of the establishment of a symplastic block. Finally, we plan on testing if there is a decrease of plasmodesmata frequency in the transient nucellus. To address these questions we will visualize plasmodesmata during nucellus development by using fluorescent markers, such as endogenous plasmodesmal proteins fused to fluorescent proteins (e.g. members of the PDLP family (Lee et al., 2011; Thomas et al., 2008), PDCB1/2 (Simpson et al., 2009) and PdBG1/2(Benitez-Alfonso et al., 2013)). Furthermore, MP17, which is a movement protein of the potato leaf roll virus that binds to branched plasmodesmata, can be used as additional marker for plasmodesmata branching.

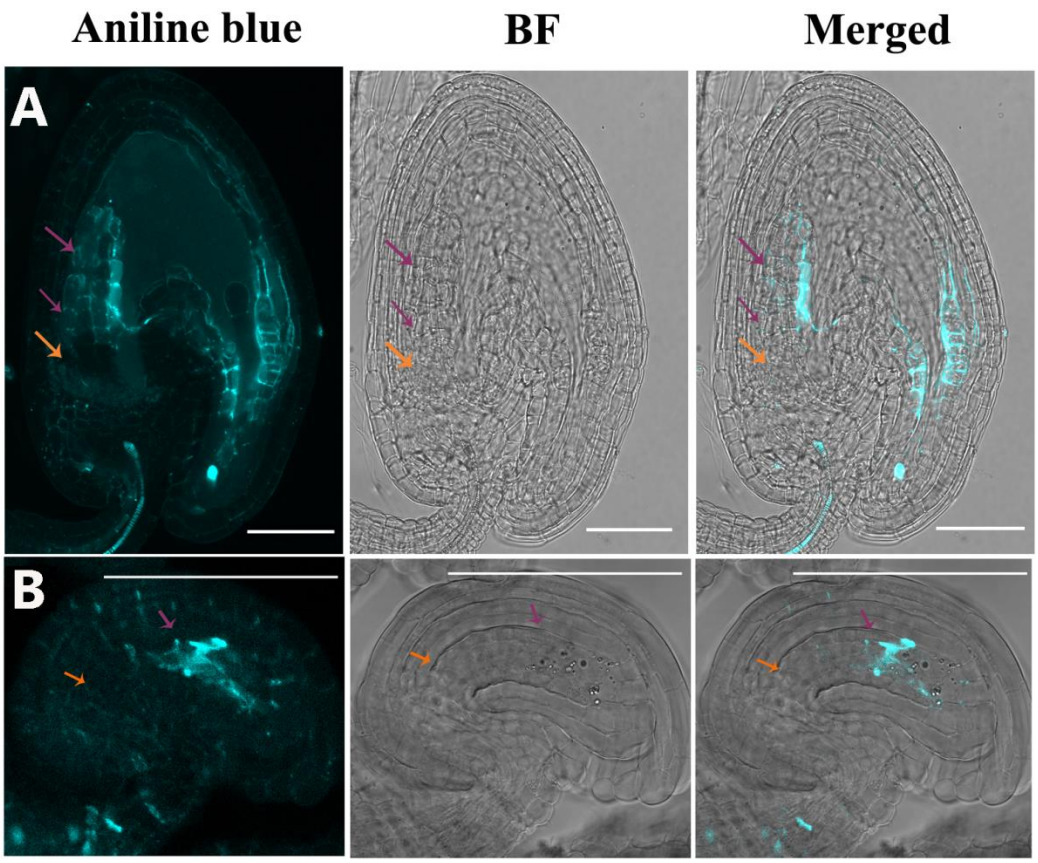
SUPPLEMENTARY DATA



Supplementary Figure 1 Nucellus symplastic connections during ovule development.
(A) to (D) Propidium iodide (PI), GFP and PI-GFP superimposed fluorescence images of *ProSUC2:GFP* ovules. (A) stage 2-V, (B) stage 3-I, (C) stage 3-IV, (D) stage 3-V. Bars=50 μm.



Supplementary Figure 2 Nucellus symplastic connections during *tt16* ovule development
(A) to (D) Propidium iodide (PI), GFP and PI-GFP superimposed fluorescence images of *ProSUC2:GFP*;*tt16* ovules. (A) stage 2-V, (B) stage 3-I, (C) stage 3-IV, (D) stage 3-V. Bars=50 µm.



Supplementary Figure 3 Callose deposition in the *tt16* nucellus
(A) and (B) Aniline blue, bright field (BF) and superimposed images of *tt16* seed at the pre-globular embryo stage and mature ovule. Orange and purple arrows indicate persistent and transient nucellus, respectively, Bars=50 μm.

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Chapter IV - *SHATTERPROOF MADS-box genes promote nucellus survival.*

INTRODUCTION

MADS-box transcription factors are key regulators of several plant development processes. The MADS-box gene family is characterized by the presence of the MADS box sequence motif, which encodes the DNA-binding MADS domain. The analysis of several MADS-domain proteins revealed that the 58 amino acids long MADS domain recognizes a DNA consensus sequence known as the CArG box [CC(A/T)6GG] (Hayes et al., 1988; Riechmann et al., 1996).

MADS-box genes have been identified in plants, fungi and metazoans. The phylogenetic analysis performed by Alvarez-Buylla et al.(Alvarez-Buylla et al., 2000), which included 44 Arabidopsis MADS domain sequences and several MYOCYTE ENHANCER FACTOR2–like (MEF2) and SERUM RESPONSE FACTOR-like (SRF) MADS box domains from fungi and animals, suggests that a gene duplication occurred before the divergence of plants and animals, giving rise to two main lineages of MADS-box genes: type I and type II (Fig.1). The plant type-I MADS-box transcription factors, together with the animal SRF-like domain proteins, forms the type-I lineage. The plant MIKC-type MADS-domain proteins, together with the animal MEF2-like proteins, form instead the type-II lineage (Parenicová et al., 2003).

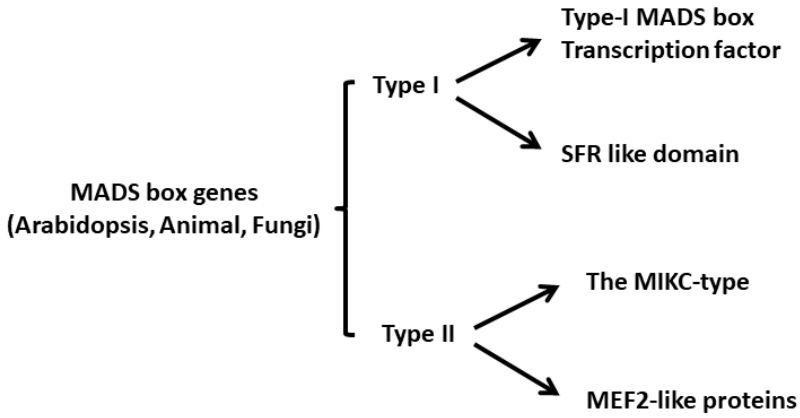

Figure 1 MADS-box genes have been identified in plants, fungi and metazoans.

Whereas plant type-I MADS-domain proteins share only a MADS domain, MIKC-type transcription factors (type-II) have a characteristic modular structure made of a (from the N to the C terminus of the protein) MADS (M), intervening (I), keratin-like (K), and C-terminal (C) domain (Fig.2). The I domain influences protein dimerization, which is driven by the K coiled-coil domain (Davies et al., 1996; Fan et al., 1997). The C domain is the least conserved domain; in some cases, it has been shown to contain a transactivation domain or to contribute to the formation of multimeric MADS-domain protein complexes (Egea‐Cortines et al., 1999; Honma and Goto, 2001). Furthermore, based on domain structure differences, MIKC-type MADS-box transcription factors have been classified into $MIKC^{C}$ and $MIKC^{*}$ types (Henschel et al., 2002). $MIKC^{*}$ type proteins appear to have an altered K domain structure (Kwantes et al., 2012).

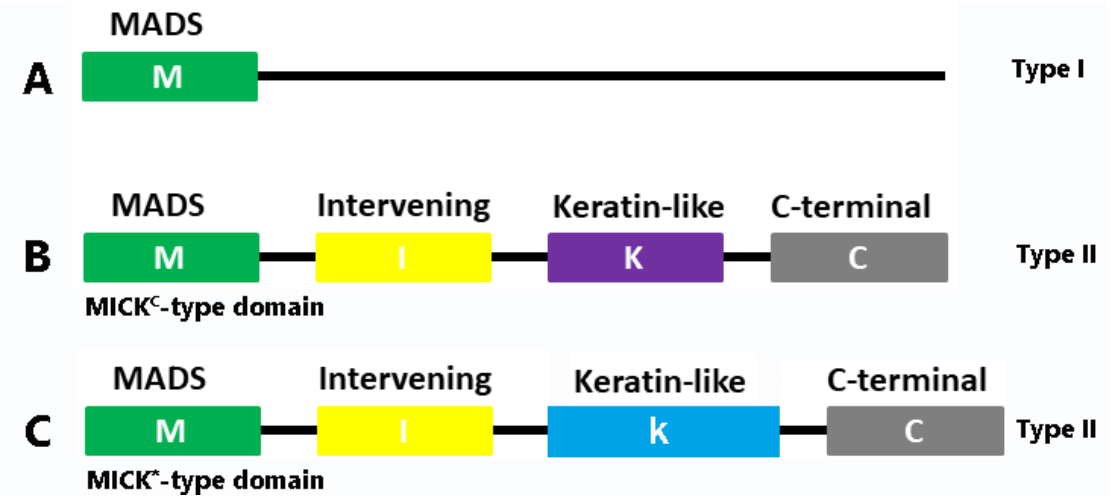

Figure 2 Domain structures of MADS-domain proteins.

Analyses of the Arabidopsis genome revealed 107 genes encoding MADS-domain proteins. Phylogenetic analyses indicated that the type I Arabidopsis MADS-box genes can be further subdivided into three subfamilies, named Mα, Mβ, Mγ (Fig.3) (Smaczniak et al., 2012). Based on the distinct sequence motifs in their C-terminal end, the Arabidopsis MIKC^C type genes are divided into several distinctive subfamilies (Vandenbussche et al., 2003): B-sister (GGM13), AGAMOUS (AG), AGL12, SOLANUM TUBEROSUM MADS-BOX 11 (STMADS11), AGL15, AGL17, TOMATO MADS-BOX3 (TM3), SOC1, TM8, SEPALLATA (SEP), AGL6, SQUAMOSA (SQUA), FLOWERING LOCUS C (FLC), DEFICIENS (DEF) and GLOBOSA (GLO) (Fig.4). Several members of the MIKC^C-type subfamilies have been shown to have conserved functions in different flowering plant species (Smaczniak et al., 2012).

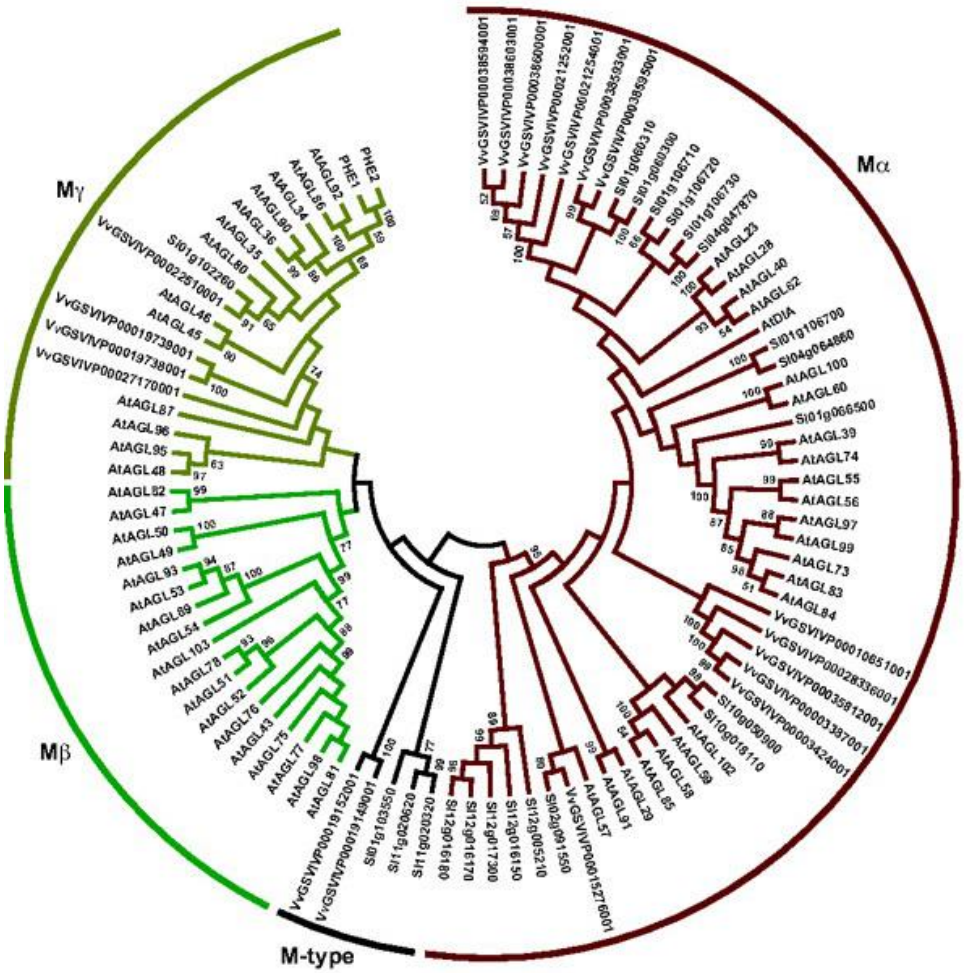

Figure3 Phylogenetic analysis of selected representatives of type I MADS-box transcription factors from thale cress (*Arabidopsis thaliana, At*), grape (*Vitis vinifera,Vv*) and tomato (*Solanum lycopersicum, Sl*) (Smaczniak et al., 2012).

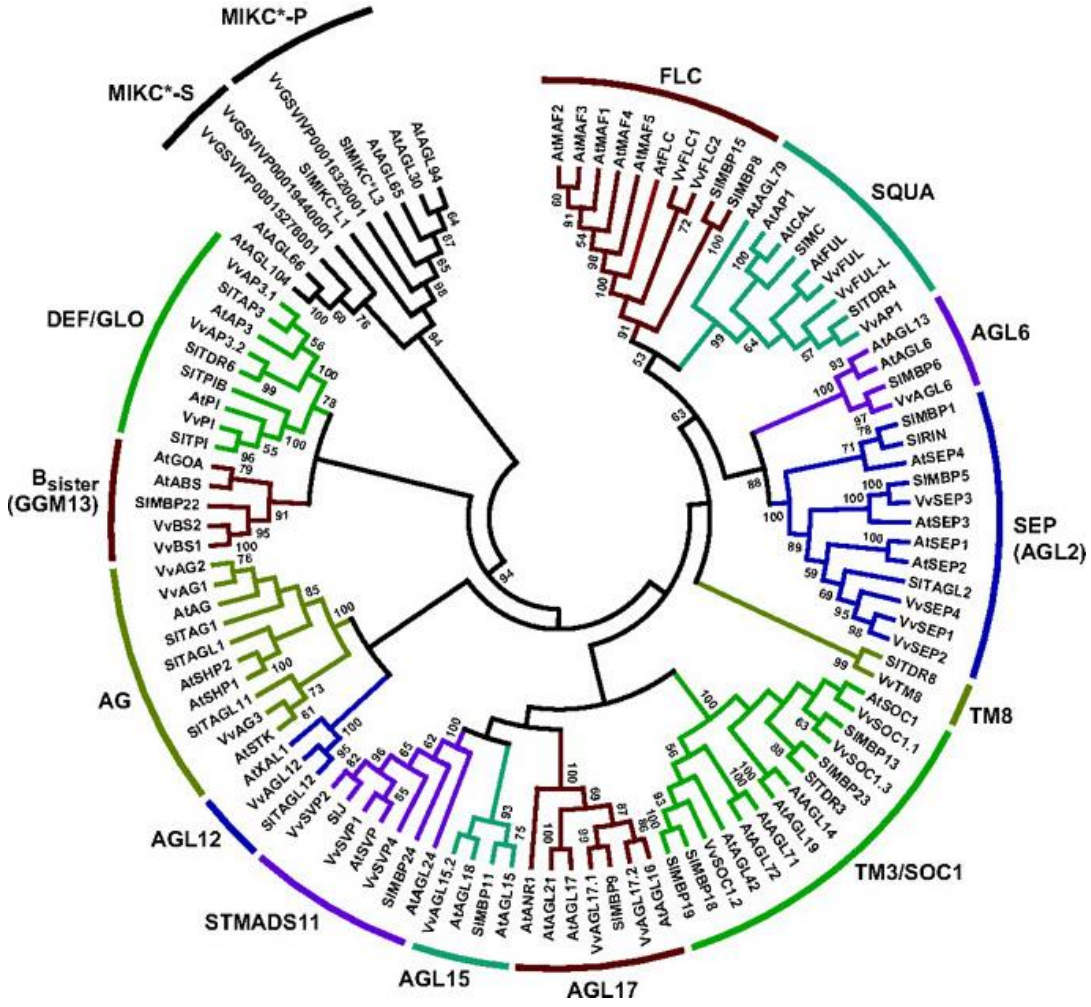

Figure4 Phylogenetic analysis of selected representatives of type II MADS-box transcription factors from thale cress(*Arabidopsis thaliana, At*), grape (*Vitis vinifera,Vv*) and tomato (*Solanum lycopersicum, Sl*) (Smaczniak et al., 2012).

B-sister (Bs) MADS box genes are the closest relatives of the homeotic B MADS box genes (DEF/GLO subfamily of MIKC^C type genes). Divergence of B and Bs lineages happened by the duplication of an ancestor gene between 400MYA (million years ago) and 300MYA, which corresponds to the divergence of fern and seed plant lineages (400MYA) and separation of gymnosperm and angiosperm lineages (300MYA). Expression analyses revealed that Bs genes are predominately expressed in female reproductive organs whereas B genes are mainly expressed in male reproductive organs (Becker et al., 2002).

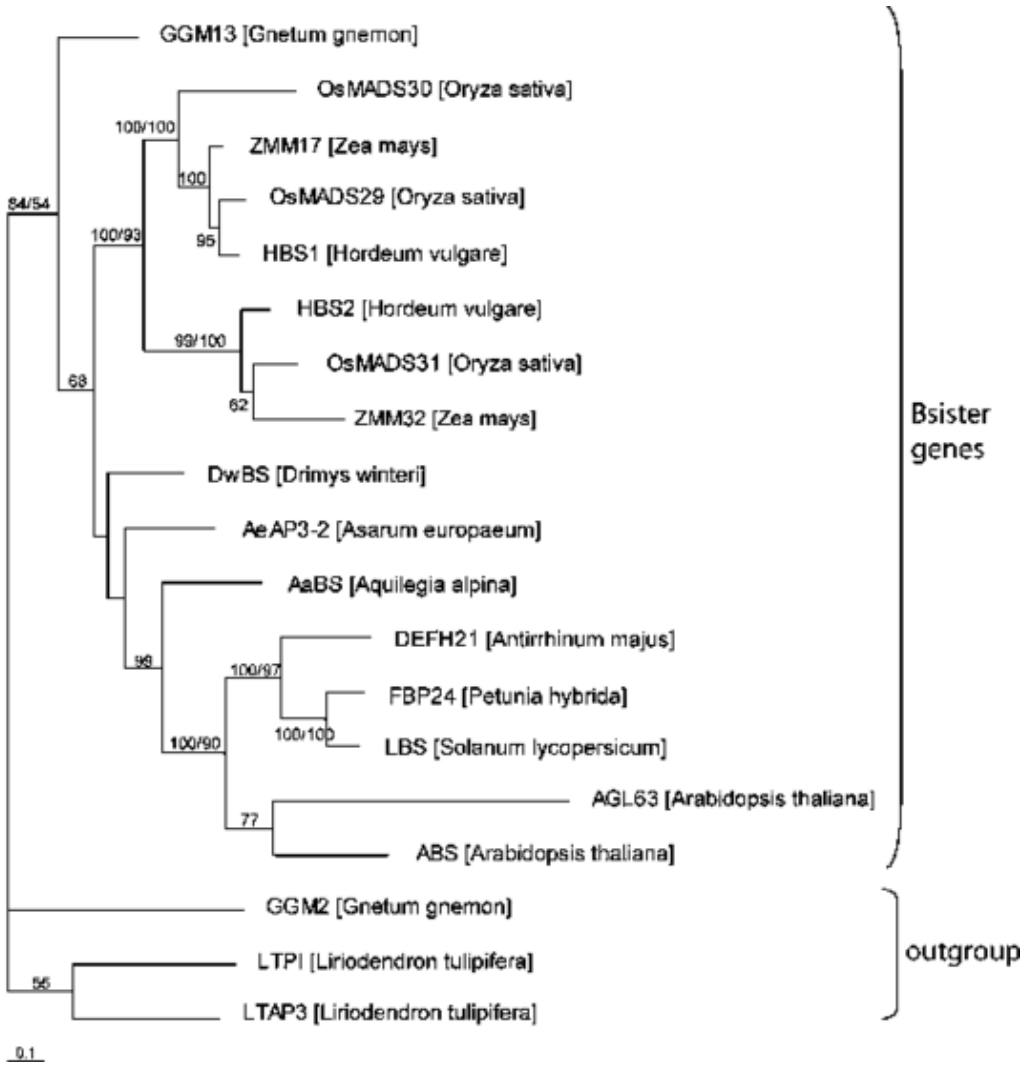

Figure 5 Identified homologue B-sister genes in plants (Kaufmann et al., 2005).

To date, Bs genes have been only isolated from seed plants and gymnosperm (Becker et al., 2002). In Arabidopsis there are two Bs genes (Fig 5): *TRANSPARENT TESTA 16* (*TT16*), also named *ARABIDOPSIS B-SISTER* (*ABS*), and its orthologue *GORDITA* (*GOA*), also named *AGL63* (Parenicová et al., 2003). *TT16* is involved in cell differentiation and pigmentation of the seed coat (Coen et al., 2017; Coen et al., 2019a; Coen et al., 2019c; Fiume et al., 2017a; Nesi et al., 2002b; Xu et al., 2016). It promotes cuticle deposition and flavonoid accumulation in the endothelium, the innermost cell layer of the seed coat (Coen et al., 2019a; Nesi et al., 2002b). Mutations in the *TT16* gene also affect the cell shape and polarity of the inner integument cell layers of the seed coat (Coen et al., 2017; Coen et al., 2019c; Fiume et al., 2017a; Nesi et al., 2002b). Finally, Xu et al. showed that *TT16* promotes nucellus elimination (Xu et al., 2016). Before fertilization, Fertilization-Independent Seed (FIS) Polycomb Group (PcG) proteins repress nucellus degeneration. After fertilization, the endosperm initiates a signal, through the action of the AGAMOUS-LIKE 62 MADS box transcription factor that relieves the repressive action mediated by the FIS PcG proteins. *TT16* functions downstream of PcG proteins to promote nucellus elimination. *TT16* is expressed in

the persistent nucellus and regulates non-cell autonomously the elimination of the transient nucellus in part by repressing the expression of the *HVA22d* gene, which inhibits gibberellin-mediated program cell death (PCD) and autophagy. However, nucellus elimination has not been entirely assigned to any known cell death program. TT16 paralogue, *GOA*, has been identified as regulator of fruit growth (Erdmann et al., 2010; Prasad et al., 2010a). Furthermore, *GOA* is expressed in the transient nucellus and acts redundantly with *TT16* in repressing nucellus cell division after fertilization (Xu et al., 2016) .

A handful of other Arabidopsis type-2 MADS box genes have been involved in ovule and seed development. WUSCHEL (WUS) acts non-cell-autonomously from the nucellus to induce integument growth from chalaza. *wus* mutant ovules fail to develop integuments and eventually degenerate (Gross-Hardt et al., 2002). *SHATTERPROOF1* (*SHP1*) and *SHP2*, first discovered for their redundant role in fruit dehiscence (Liljegren et al., 2000), have been shown to play an antagonistic role with *TT16* in seed coat development. *shp1* and *shp2* partially rescue the defects in the development of the inner integument of *tt16* seeds (Ehlers et al., 2016). *SEEDSTICK* (*STK*) acts redundantly with *SHP1* and *SHP2* to control ovule identity: *stk;shp1;shp2* triple mutant ovules develop as leaf-like or carpel-like structures (Favaro et al., 2003; Pinyopich et al., 2003; Rodr ǵuez-Cazorla et al., 2018). Furthermore, *STK* and *TT16* redundantly regulate the development of the inner integument, as a total absence of the inner integument 1' cell layer is observed in the *tt16;stk* double mutant (Coen et al., 2017; Mizzotti et al., 2012). In the *stk* single mutant, ovules display a relatively larger funiculus and seeds do not detach from the silique once matured (Pinyopich et al., 2003). Finally, the SEPALLATA (SEP) MADS-domain proteins have been detected in reproductive organs and act as bridges in the formation of MADS multimeric protein complexes. SEP3 has been shown to enable the interaction of TT16 with STK, SHP1 and SHP2 (Favaro et al., 2003; Kaufmann et al., 2005). *SEP3* RNA was detected in ovule and seed maternal tissues (Mandel and Yanofsky, 1998). These results suggest a possible role of *SEP3* in ovule and seed development.

In this study, we have characterized SHP2 and SEP3 expression pattern in ovules and seeds and found out a spatial-temporal overlap with TT16 in ovules. We also found that *shp1*;*shp2* double mutation can rescue the nucellus phenotype of *tt16;goa* seeds. We therefore, speculate that SEP3 allows TT16 and SHP physical interaction, timing and regulating nucellus elimination.

RESULTS

MADS box genes expression pattern in the nucellus

To test if additional MADS-box genes are involved in nucellus development, we further characterized the expression pattern of MADS box genes known to be expressed in ovules or seeds. It was shown that TT16 can interact *in vitro* with SHP1 or SHP2 through SEP3 (Kaufmann et al., 2005). To test if such an interaction might happen in the nucellus, we studied the expression pattern

of *SHP* genes. We cloned *SHP2* 3kb promoter and genomic sequence and translationally fused to *GFP*. The transgene was transferred into both wild type and *tt16* plants. We detected the expression of *SHP2* in both inner (ii) and outer integuments (oi), chalaza and nucellus, with the only exception of the most distal nucellus cells above the tetrad, in wild type and *tt16* ovule primordia at stage 2-V (Fig.6A and 6D). In ovules at stage 3-V, *SHP2* was expressed in the proximal part of the ii and oi, in the most distal cells of the oi, chalaza and nucellus in both wild type and *tt16* plants (Fig.6B and 6E). In pre-globular embryo seeds, we detected stronger GFP fluorescence in the proximal oi and everywhere in the nucellus, and a weaker signal in the proximal ii and in the chalaza in both wild type and *tt16* plants (Fig.6C and 6F). Cloning of *SHP1* promoter and genomic sequence is currently underway.

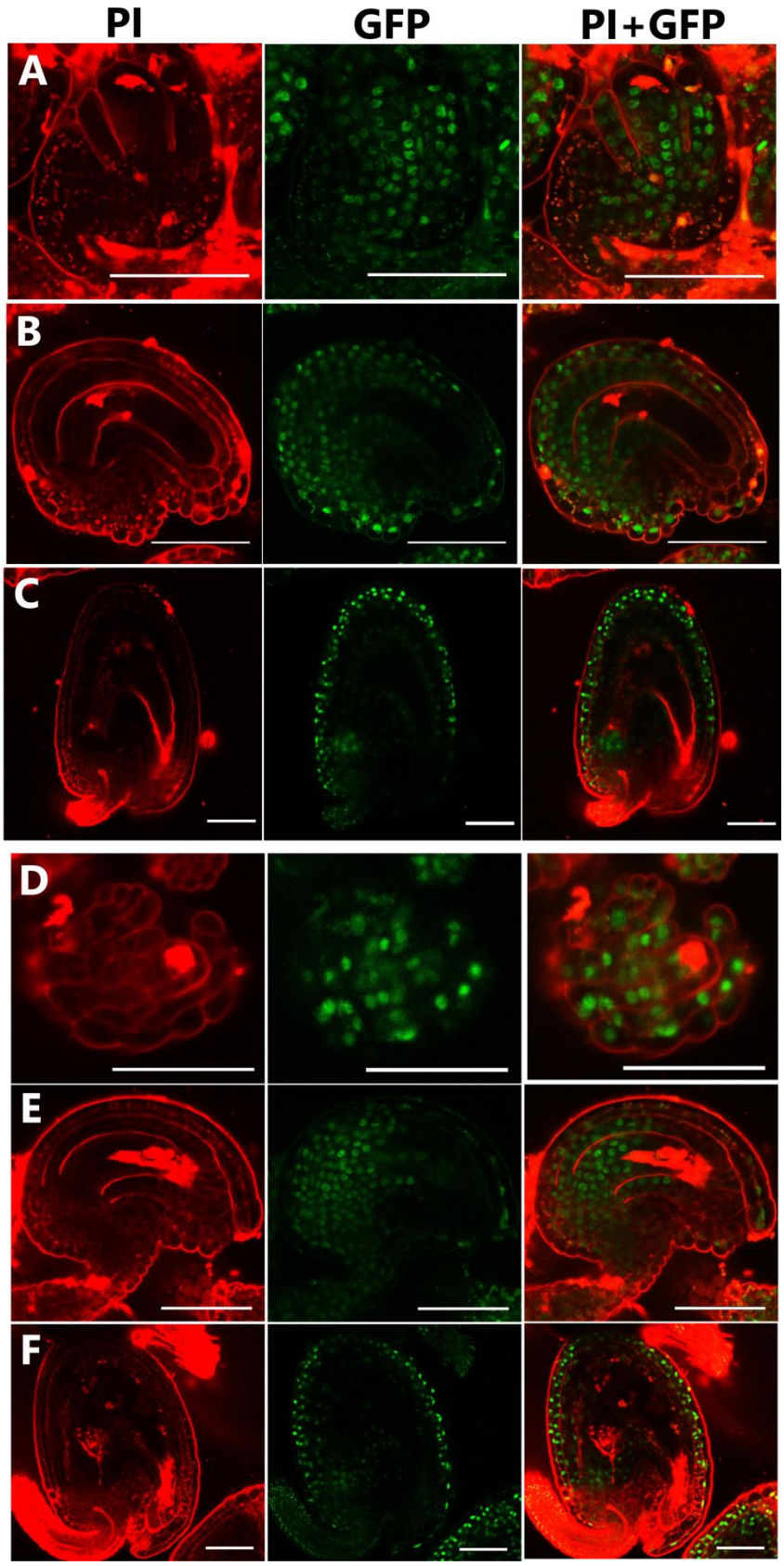

Figure 6 *SHP2* expression pattern in wild type (A-C) and *tt16* (D-F) ovules and seeds.
(A) to (C) Propidium iodide (PI), GFP and PI-GFP superimposed fluorescence images of *ProSHP2:gSHP2-GFP* stage 2-V ovule (A), stage 3-V ovule (B), and pre-globular embryo seed (C). (D) to (F) Propidium iodide (PI), GFP and PI-GFP superimposed fluorescence images of *ProSHP2:gSHP2-GFP*;*tt16* stage 2-V ovule (D), stage 3-V ovule (E), and pre-globular stage seed (F). Bars=50 µm.

SEP3 works as bridging protein to allow the formation of multimeric complexes with other MADS box transcription factors and regulate ovule development (Ehlers et al., 2016). *SEP3* has been shown to be expressed at high levels in all tissues of ovule primordia till stage 2-IV (Mandel and Yanofsky, 1998; Urbanus et al., 2009). To further characterize *SEP3* expression pattern at later stages, we used *SEP3* 1.5kb promoter and genomic sequence translationally fused to GFP (Urbanus et al., 2009) (*1.5ProSEP3:g SEP3-GFP*). We detected GFP florescence in the ii2 and oi1 integuments and a few cells in the distal region of ii1 integument, no GFP signal was detected in the nucellus of mature ovules and seeds 1 DAF (Fig.7).

Expression analyses of *SEP1* and *SEP2* genes, which show redundant function with *SEP3*, are currently underway.

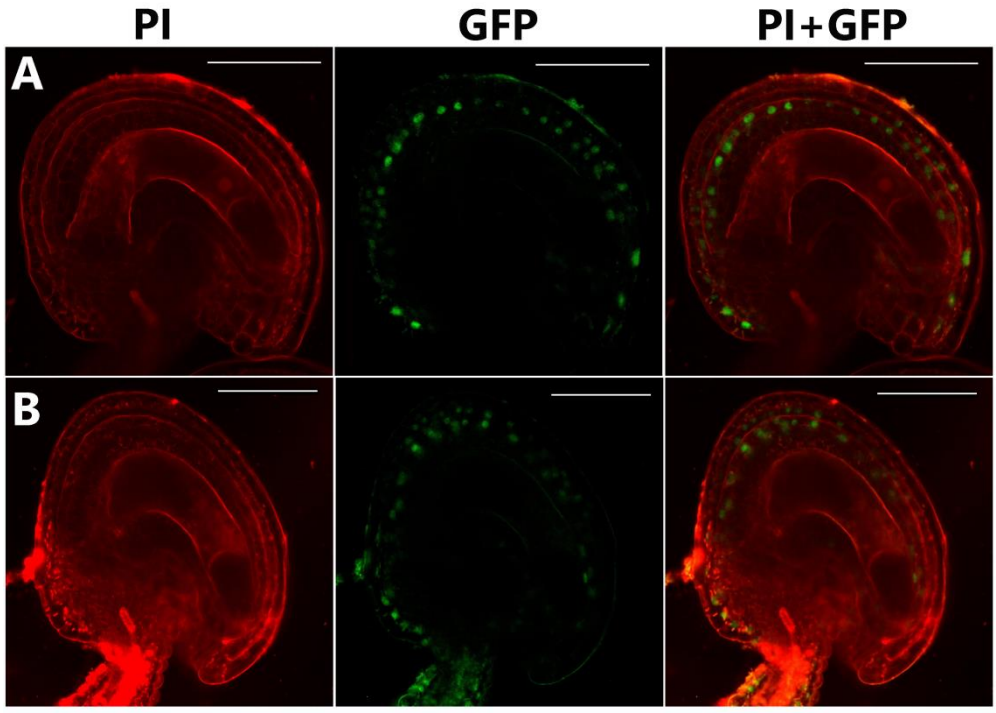

Figure 7 *SEP3* expression pattern in wild type ovules and seeds.
Propidium iodide (PI), GFP and PI-GFP superimposed fluorescence images of *ProSEP3:gSEP3-GFP* ovules at stage 4-I (A) and seeds at 1 DAF (B). Bars=50 µm.

shp1;shp2 double mutation suppresses *tt16* nucellus phenotype

It has been shown that *shp1* and *shp2* partially rescue the phenotype of *tt16* in seed coat development (Ehlers et al., 2016). To investigate the possible role of SHP1 and 2 in nucellus development and its genetic interactions with TT16, we imaged central longitudinal sections of wt, *tt16;goa*, *shp1;shp2*, and *shp1;shp2;tt16;goa* mutants seeds 6 DAF using the mPS-PI technique. As Xu et al. have previously described (Xu et al., 2016), a large part of the wild type distal nucellus degenerates during the first 6 DAF in wild type seeds (Fig 8A) whereas it grows bigger in *tt16;goa* seeds (Fig 8C). Similar to wild-type, the nucellus of *shp1;shp2* mutant seeds underwent elimination

of their distal region (Fig 8B). Interestingly, the nucellus of a number of *shp1;shp2;tt16;goa* seeds also appeared to display nucellus elimination as in wild type and *shp1;shp2* seeds (Fig 8D). To quantify such a phenotype we calculated the percentage of seeds at 6DAF showing a nucellus that crossed the distal line of the gynoapical seed coat (orange lines in Fig. 8) in seed longitudinal sectionsAll *shp1;shp2* seeds tested (n=30) displayed a nucellus well below the reference line, as observed in the wild type (Fig 8B). By contrast, 100% of *tt16;goa* seeds analyzed (n = 30) showed a nucellus above the reference line (Fig 8C). Finally, about 75% of *shp1;shp2;tt16;goa* seeds showed a nucellus below the reference line (Fig 8D), whereas the rest 25% of seeds above the reference line (Fig 8E) (n = 60).

Overall, these data indicate a genetic interaction between SHP1 and SHP2 and TT16. Whereas TT16 promotes transient nucellus elimination, SHP1 and SHP2 promote transient nucellus survival.

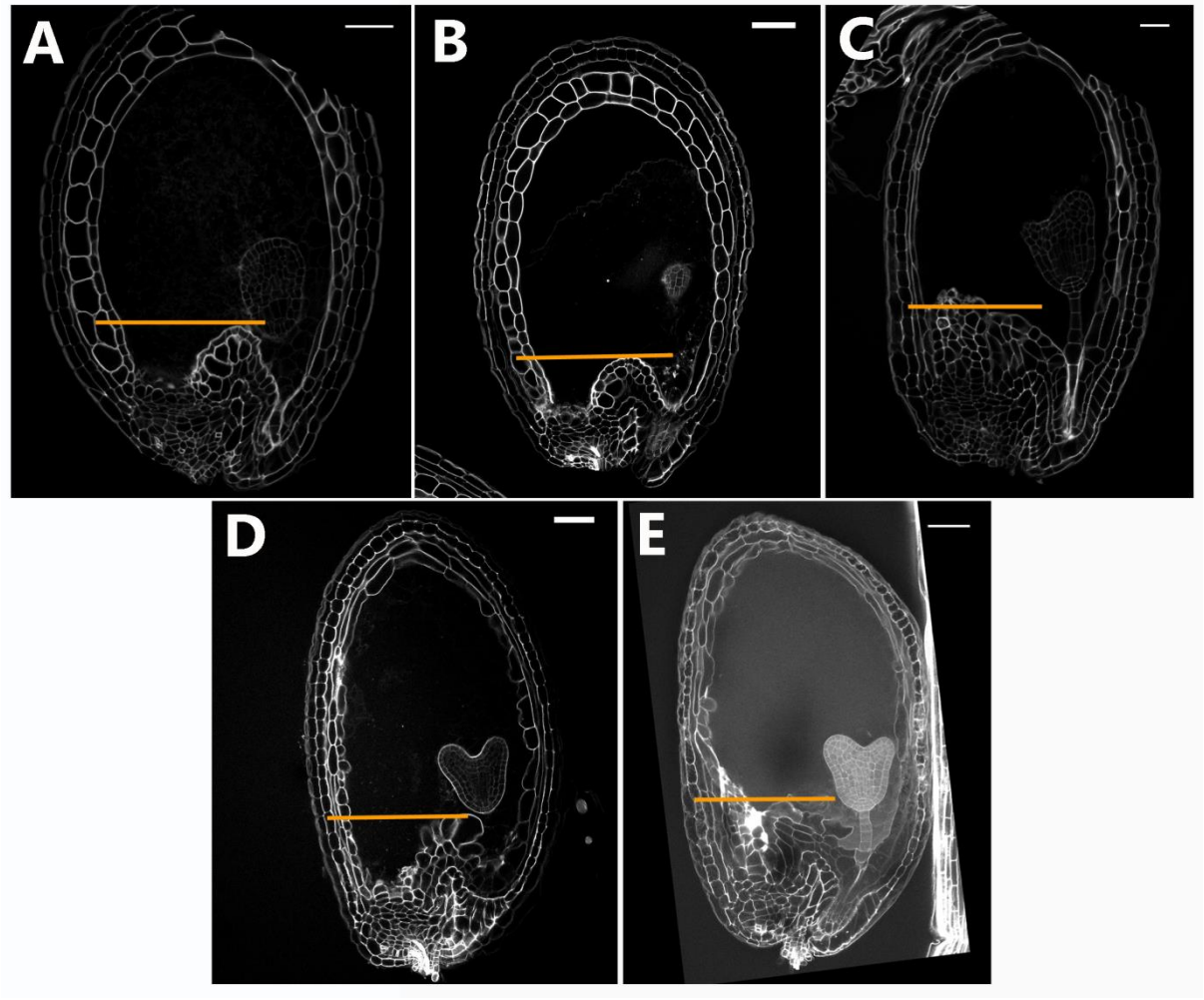

Figure 8 *shp1;shp2* rescued *tt16* nucellus phenotype.
(A) to (E) mPS-PI images of longitudinal sections of wt (A), *shp1;shp2* (B), *tt16;goa* (C), and *shp1;shp2;tt16;goa* (D and E) seeds at 6 DAF. Orange horizontal lines indicate the distal lines of the gynoapical seed coatused as reference. Bars=50 µm.

To test if the *shp1;shp2* mutation might lead to the elimination of the persistent nucellus, we

followed nucellus development in *shp1;shp2* seeds. A fraction of *shp1;shp2* appeared to have completely lost the persistent nucellus at torpedo embryo stage (Fig 9B), which suggests that SHP1 and SHP2 are important for the survival of the persistent.

Finally, we could not meaningfully analyse SEP function because of gene redundancy between *SEP1*, *2* and *3* and ovule-less phenotype of *sep1;sep2;sep3* triple mutant (Favaro et al., 2003).

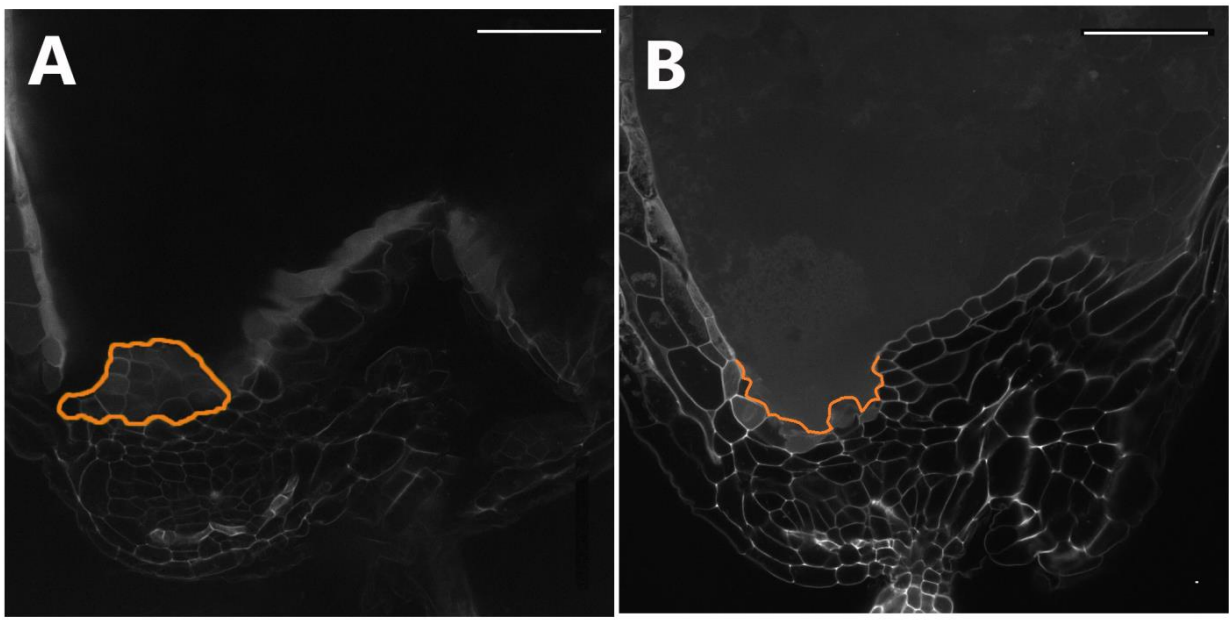

Figure 9 Nucellus elimination in *shp1;shp2 ;* seeds.
(A) and (B) mPS-PI images of longitudinal sections of *shp1;shp2* mutant seeds at late torpedo stages. Cell walls are stained with calcofluor (cyan). Orange lines highlight the nucellus. Bars=50 µm.

MATERIAL AND METHODS

Plant materials

All *Arabidopsis thaliana* plants are in the Columbia accession. The *tt16;goa* and *shp1;shp2* mutant lines have been previously described (Liljegren et al., 2000) (Prasad et al., 2010b). Days after flowering were counted starting from the emergence of the pistil from closed flowers; 0 DAF equals stage 3-V of ovule development (Schneitz et al., 1995c). Both DAF and embryo development have been used to determine seed developmental stages.

Cloning

SHP2 3kb promoter region and genomic sequence without the stop codon was amplified using the attB1-(5′CTCGTCTTTAGCACTGTGACGT3′) forward and attB2-(5′AACAAGTTGCAGAGGTGGTTG3’) reverse primers. AttB1 (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3′) and attB2 (5′-GGGGACCACTTTGTACAAGAAAGCTGGGTC- 3′) carry the Gateway recombination sites at the 5′-ends of the forward and reverse primers, respectively. The PCR products were amplified with

the high-fidelity Phusion DNA polymerase (Thermo Fisher Scientific), recombined into the *pDONR207* vector (BP Gateway reaction) according to the manufacturer's instructions (Thermo Fisher Scientific), and then sequenced. The PCR products cloned into the *pDONR207* vectors were then recombined into the *pMDC107* binary vector (Curtis and Grossniklaus, 2003), (LR Gateway reaction) according to the manufacturer's instructions (Thermo Fisher Scientific).

Transgenic plants (see chapter2)Pseudo-Schiff propidium iodide staining

This protocol allows the staining of cell walls of fixed plant material (Truernit et al., 2008a; Xu et al., 2016). Samples were mounted onto microscope slides and observed by confocal microscope (Leica SP8).

Calcofluor staining

For staining with calcofluorM2R white (fluorescent brightener 28; Sigma Aldrich), harvested septums were first immersed in a NaOH (0.2M), SDS (1%) solution at 37℃ for 2h. Samples were rinsed three times in water, and then transferred to12.5% bleach solution (1.25% active Cl^-) for 10 to15 min (until samples become white and transparent). Samples were rinsed three times in water, and then transferred to calcofluor solution (10 μg mL-1) at 4℃ overnight. Finally, the samples were mounted onto microscope slides with water and observed by confocal microscope (Leica SP8).

Microscopy

Stained samples were analyzed with Leica TCS-SP8 spectral confocal laser scanning microscope. The excitation wavelength for mPS-PI stained samples was 488 nm, and emission was collected at 520 to 720 nm (Truernit et al., 2008a). The excitation wavelength for calcofluor stained samples was 365 nm and emission was collected at 440 nm. Three-dimensional Z-stack confocal laser scanning microscope images of mPS-PI stained or calcofluor stained seeds were analyzed through the VolumeViewer plug-in of the ImageJ software. Mid-plane longitudinal sections were obtained. More than 30 independent seeds were analyzed for each genotype and time point.

DISCUSSION

***SHP2* and *TT16* regulate nucellus development**

TT16 in expressed in the persistent nucellus throughout ovule and seed development and promotes non-cell autonomously the elimination of the transient nucellus after fertilization (Xu et al., 2016). *GOA* is instead expressed in the transient nucellus and represses, redundantly with TT16, nucellus growth. Here, we showed that *SHP2* is expressed in both transient and persistent nucellus in ovules and seeds. The characterization of *SHP1* expression is currently underway. Furthermore, our genetic analyses demonstrate that *shp1;shp2* mutations suppress *tt16;goa* phenotype, thus

highlighting a genetic interaction among such MADS-box genes. While TT16 and GOA promote nucellus elimination, SHP1 and SHP2 promote nucellus survival. Finally, it has been observed that *SEP3* is expressed in the nucellus in ovule primordia (Mandel and Yanofsky, 1998; Urbanus et al., 2009). Our data indicate that SEP3 is not present in the nucellus across fertilization.

Previous studies have revealed that MADS-domain proteins form dimers or higher-order protein complexes that are instrumental for function and specificity (Favaro et al., 2003; Honma and Goto, 2001; Theißen, 2001). For example, it has been shown that TT16-AG-SEP3 and TT16-STK-SEP3 protein complexes are involved in the determination of integument cell identity in the seed coat (De Folter et al., 2006; Kaufmann et al., 2005). Interestingly, it has also been shown that TT16, SEP3, SHP1 or SHP2 can form protein trimers but their biological function has not been elucidated (Kaufmann et al., 2005). Our analyses identified spatial-temporal overlap of such MADS-box gene expression as well as genetic interaction between TT16 and SHP1 and SHP2. We therefore, speculate that TT16 and SHP1 and 2 might time and regulate the extent of nucellus elimination by forming protein complexes. Besides, qRT-PCR has shown that *TT16* and *SHP1* or *SHP2* mutually repress each other expression directly or indirectly (Ehlers et al., 2016). We integrated all this information in a model that requires further testing for validation (Fig.10).

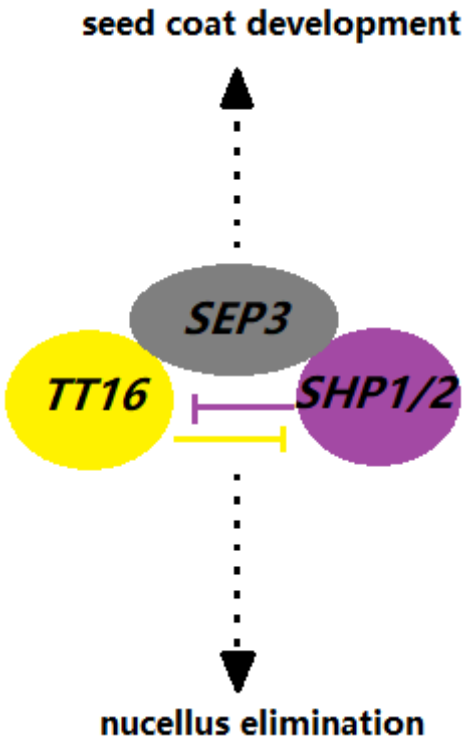

Figure 10 A model for TT16, SHP1, SHP2 and SEP3 function.
Full lines indicate genetic interactions whereas dotted line indicate functional relationships.

Perspectives

To better understanding the role of *SHP1/SHP2* and *TT16* in regulating the nucellus elimination pathway, we will analyse the expression of known TT16 downstream target genes in different mutant backgrounds. *HVA22d* has been shown to be regulated by TT16 and we therefore

introgressed the *ProHVA22d:gHVA22d-GUS* line into *shp1;shp2* and *shp1;shp2;tt16;goa* background, experiments are underway. Furthermore, we are cloning a marker line carrying the *SHP1* 3kb promoter region and genomic sequence, transcriptionally fused to *GFP* and we will observe the expression pattern of *SHP1* in the near future. We also plan to observe the expression pattern of *SEP1* and *SEP2* genes. To overcome the ovule-less phenotype of *sep1;sep2;sep3* plants, where flowers are converted into leaf-like or carpel-like structures, we plan on specifically expressing *SEP3* in flower meristems under the control of the *CLV1* promoter in a *SEP1/sep1;sep2;sep3* background. These line might bear wild type looking flowers that *sep1;sep2;sep3* ovules. We will also look into type I MADS box genes that are expressed in the nucellus such as *AGL57*, *AGL 59*, *AGL 64*, *AGL 100*, *AGL 77*. Overall, we hope to shed light on the role of MADS-Box transcription factors on nucellus evolution and development.

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ChapterV- *nucellus development in the Amaranthus perispermic seed*

INTRODUCTION

1 Perispermic seeds

A number of species of the *Amaranthaceae* family, such as *Chenopodium quinoa* and *Amaranthus hypochondriacus*, the nucellus (also referred to as perisperm) is the primary nutritive tissue of the seed (Lopez-Fernandez and Maldonado, 2013), alongside the embryo and a minute endosperm. Opposite to what observed in endospermic seeds, such as Arabidopsis, perispermic seeds, such as quinoa and amaranth, develop a large nucellus at the expense of the endosperm. Phylogenetic analyses showed that plants shifted several times between the endospermic and perispermic seed condition highlighting the antagonistic development of endosperm and nucellus as a defining mechanism for seed evolution. Nevertheless, little is known about the genetic bases underlying the perispermic seed architecture.

2 Amaranth as model perispermic seed plant

Quinoa and amaranth have raised the attention of agricultural ministries, food and health organization and agro-companies for their unique nutritional qualities and growth habit (Coimbra and Salema, 1994; Dakhili et al., 2019; Das, 2016). Both species have been domesticated in South America by pre-Columbian cultures (Coimbra and Salema, 1994; Lopez-Fernandez and Maldonado, 2013). Quinoa and amaranth produce perispermic seeds with an almost indistinguishable architecture. Nevertheless, the pericarp of quinoa sticks to the seed coat and need to be removed before human consumption as it is rich in saponins compounds with a bitter flavor (Ando et al., 2002; Brady et al., 2007; Chauhan et al., 1992; Comai et al., 2007; Dini et al., 2001; Farro, 2008; James, 2009; Jancurová et al., 2009; Koziol, 1990; Mastebroek et al., 2000; Spehar et al., 2007). This process is costly and can reduce the nutritional value of the seed. On the other hand, quinoa produces larger seeds due to its polyploid genome, a favorable agronomical trait. Efforts to produce polyploid amaranth are currently underway (Das, 2016; Misra et al., 1971; Pal and Khoshoo, 1968; Sun and Yue, 1993). The nutritional value of these pseudocereals is mostly connected to their protein content. Proteins are accumulated in the embryo (Ando et al., 2002; Gallardo et al., 1997; Oelke et al., 1992; Prego et al., 1998) and consist almost exclusively of globulins and albumins (Abugoch et al., 2009; Brinegar and Goundan, 1993; Brinegar et al., 1996; Marcone, 1999). The absence of prolamins, which is the main storage proteins of cereals such as wheat, makes quinoa and amaranth suitable for gluten-free diets (Das, 2016; Fairbanks et al., 1990; Thanapornpoonpong et al., 2008). Despite the protein content of these pseudocereals is comparable to that of cereals, their quality is superior. Protein nutritional quality is determined by the content of essential amino acids, which cannot be synthetized by the human body. Pseudocereals carry a complete and balanced set of amino acids rich in lysine, which is limiting in cereals (Das, 2016). Their amino acid profile matches the FAO/WHO suggested requirements for children (Das, 2016; James, 2009;

Miranda et al., 2010; Morales et al., 1988; Valcárcel-Yamani and Lannes, 2012; Wright et al., 2002). Furthermore, quinoa and amaranth proteins are highly bioavailable and their nutritional quality equals that of casein, the standard of nutritional excellence (Das, 2016). Amaranth and quinoa grains are considered nutraceutical food because of their carbohydrate content that has hypocholesterolemic and beneficial hypoglycemic effects and induce lowering of free fatty acids (Filho et al., 2017; Girija et al., 2011; Sangameswaran and Jayakar, 2008). The seed perisperm is responsible for the accumulation of carbohydrates, mainly in the form of starch (Coimbra and Salema, 1994; Lopez-Fernandez and Maldonado, 2013; Prego et al., 1998). The lipid content of quinoa and amaranth is more than double that of other cereals such as barley and wheat (Tang and Tsao, 2017). Pseudocereals have been indeed considered alternative oilseed crops as their fatty acid profile, with a high unsaturated/saturated ratio, is similar to that of soybean oil (Filho et al., 2017; Singhal and Kulkarni, 1988). Both pseudocereals are rich in vitamins, especially vitamin E that contributes to the stability and antioxidant properties of their oils, and minerals (Kozioł, 1992; Ruales and Nair, 1993; Tang and Tsao, 2017). Amaranth is also the plant with the highest content of squalene, a triterpene of high economic value for its anti-cancer properties and pharmaceutical and industrial use.

Whereas quinoa and amaranth are both promising crops, they are not equally feasible model organism for basic research. The genome of *A. hypochondriacus* is diploid and relatively small (466Mbp), only 3.4 times that of Arabidopsis. Its complete sequence is available at the online computational platform of the Joint Genome Institute. By contrast the genome of *C. quinoa* is allotetraploid and considerably larger (1.385Gbp) than *A. hypochondriacus*. Genome size and ploidy make *A. hypochondriacus* a better candidate for forward and reverse genetic analyses compared to *C. quinoa*. Furthermore, transgenic *A. hypochondriacus* plants have been obtained through *Agrobacterium* transformation and plant regeneration (Jofre-Garfias et al., 1997). We are currently growing *A. hypochondriacus* both *in vitro* and in soil. It is a robust plant that can be easily grown in a range of conditions (Brenner et al., 2000). All considered, we believe that *A. hypochondriacus* has the potential to become the mode plant for perispermic pseudocereals and is therefore the plant of choice for our study. Finally, the high degree of orthology between *A. hypochondriacus* and *A. hypochondriacus* genomes facilitate translational biology analyses between such species.

RESULTS

Nucellus development in *Amaranthus* seed

To characterize nucellus development in *Amaranthus* seeds, we adapted the Propidium-Pseudo-Schiff/Iodide (PS/PI) imaging technique (Xu et al., 2016). We followed nucellus patterning from ovule primordia till the torpedo embryo seed stage (Fig.1-4). Contrary to what previously

believed, we detected a domain of the amaranth nucellus that undergoes cell elimination after fertilization. We therefore divided the amaranth nucellus into two domains: the persistent nucellus, which is not eliminated, after fertilization, and the transient nucellus, which is consumed after fertilization (Fig.5). Similar to what observed in Arabidopsis, the transient nucellus of amaranth mature ovules is adjacent to the proximal side of the female gametophyte (Fig.5).

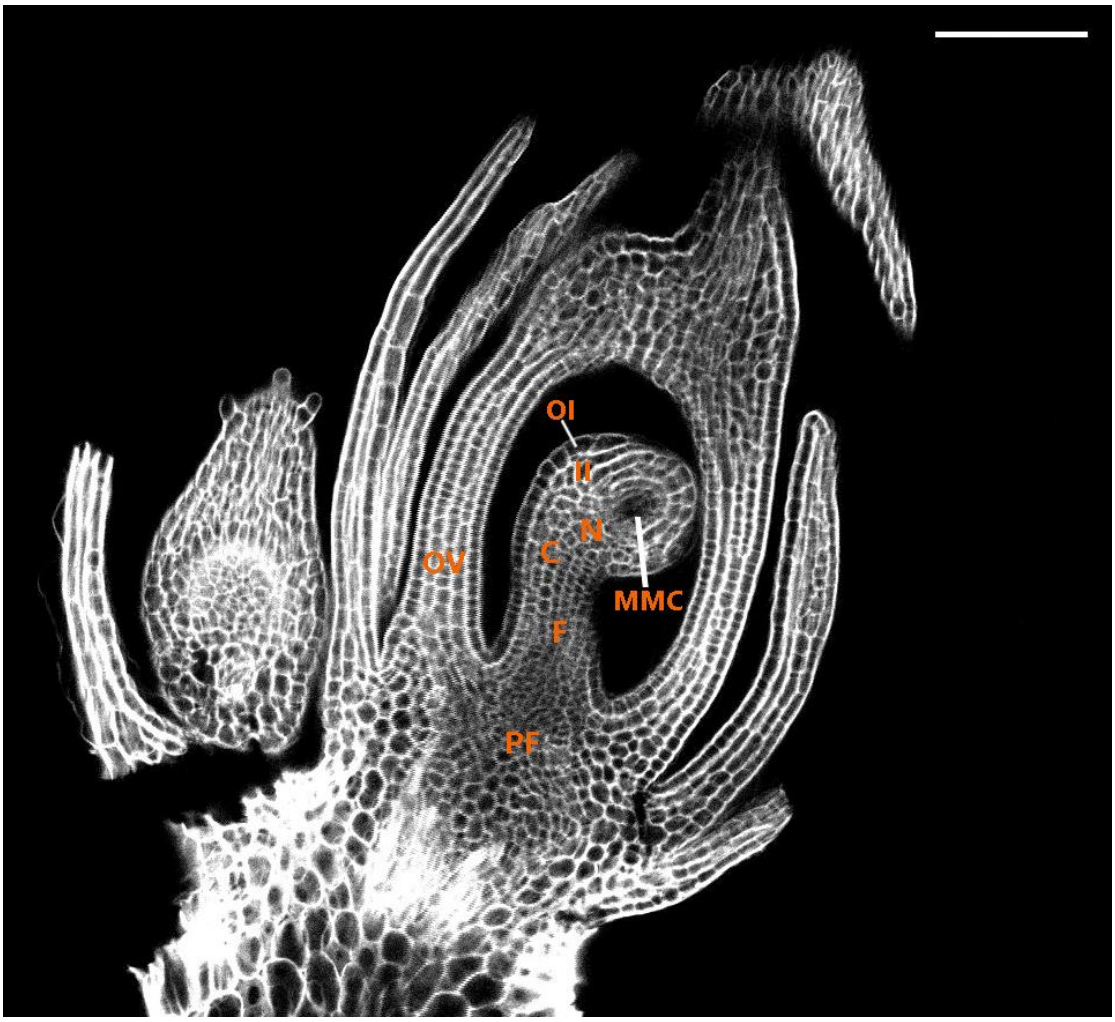

Figure1. Longitudinal section of an *Amaranthus hypochondriacus* pistillate flower (PF) imaged by mPS-PI. Ovary (OV), inner integuments (II), outer integuments (OI), megaspore mother cell (MMC), nucellus (N), chalaza (C) and funiculus (F). Scale bar = 100 μm.

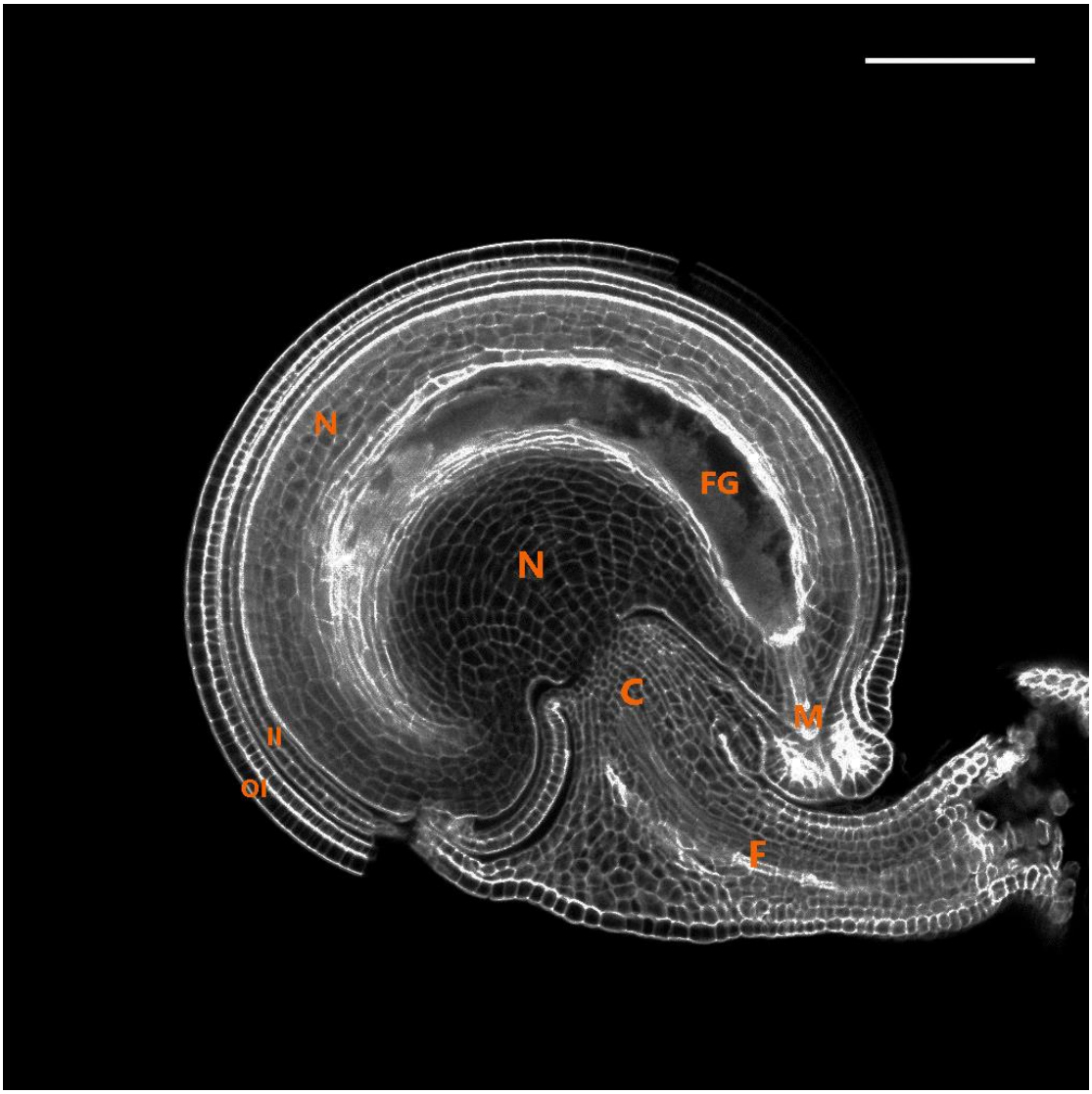

Figure2. Longitudinal section of a mature ovule of *Amaranthus hypochondriacus* imaged by mPS-PI. inner integuments (II), outer integuments (OI), nucellus (N), female gametophyte (FG), chalaza (C), micropyle (M) and funicle (F). Scale bar = 100 μm.

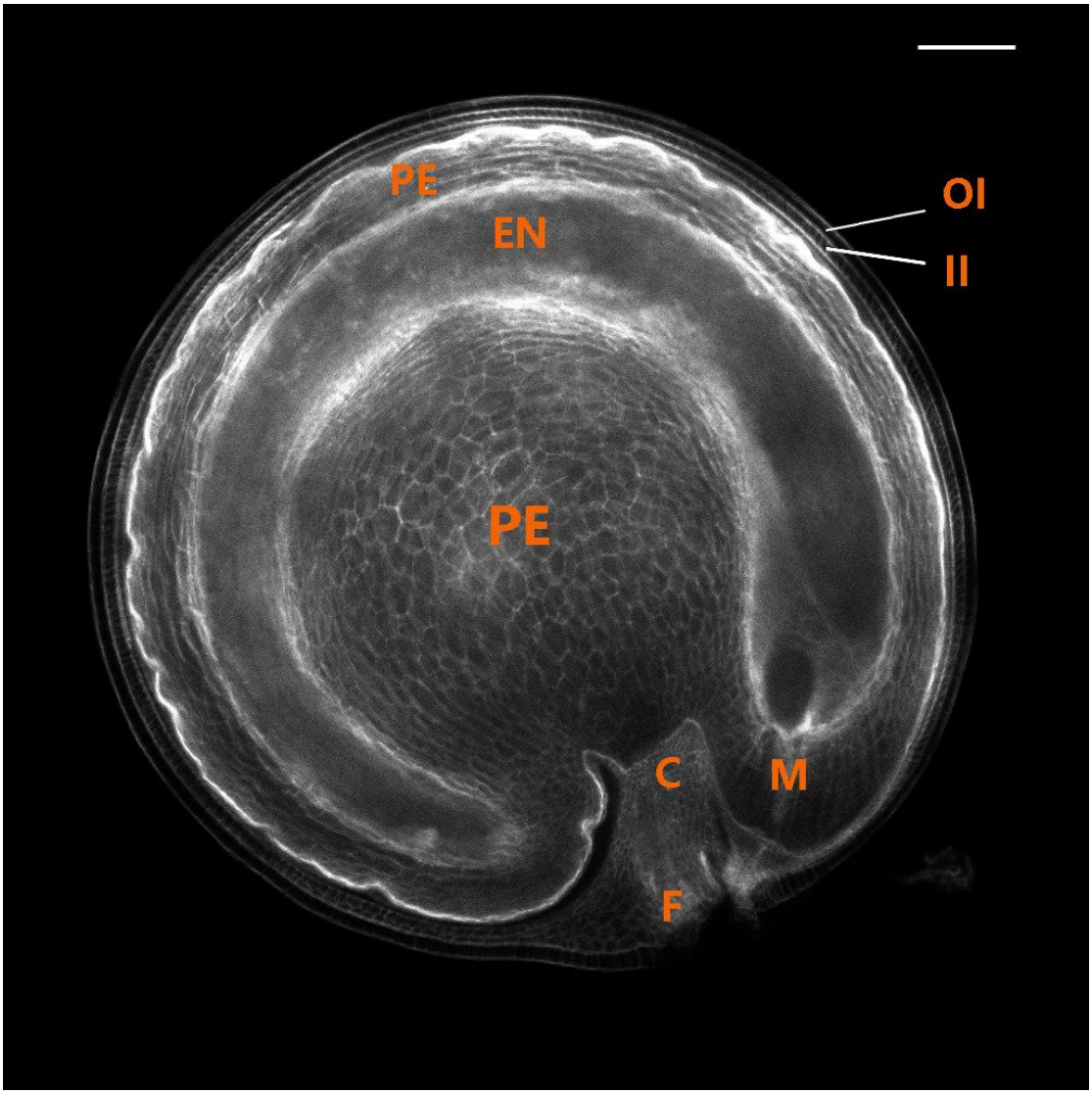

Figure3. Longitudinal section of a young *Amaranthus hypochondriacus* seed imaged by mPS-PI. Inner integuments (II), outer integuments (OI), perisperm (PE), endosperm (EN), chalaza (C), micropyle (M) and funicle (F). Scale bar = 100 μm.

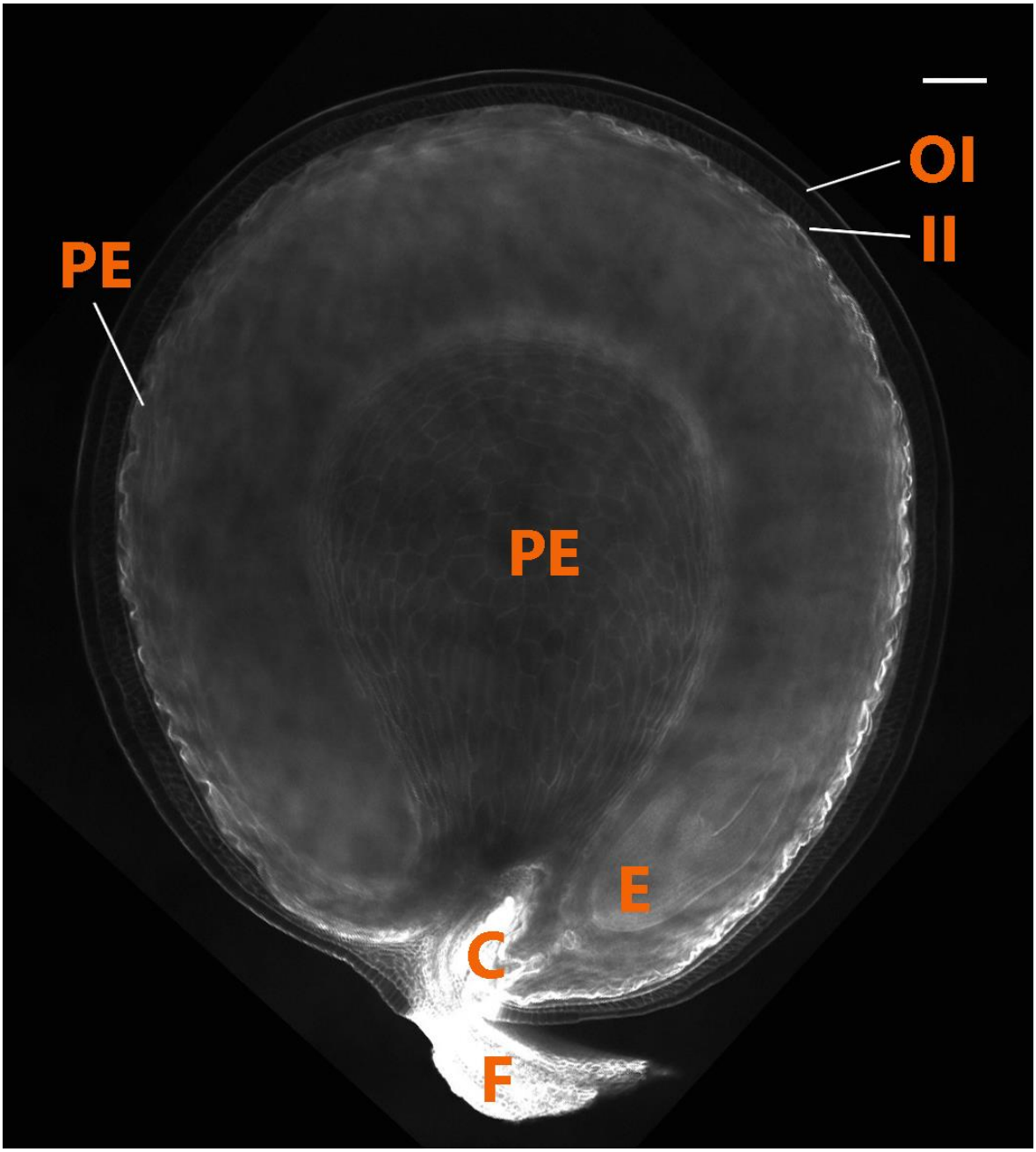

Figure4. Longitudinal section of a torpedo embryo stage *Amaranthus hypochondriacus* seed imaged by mPS-PI. Inner integuments (II), outer integuments (OI), perisperm (PE), endosperm (EN), embryo (E), chalaza (C), micropyle (M) and funicle (F). Scale bar = 100 μm.

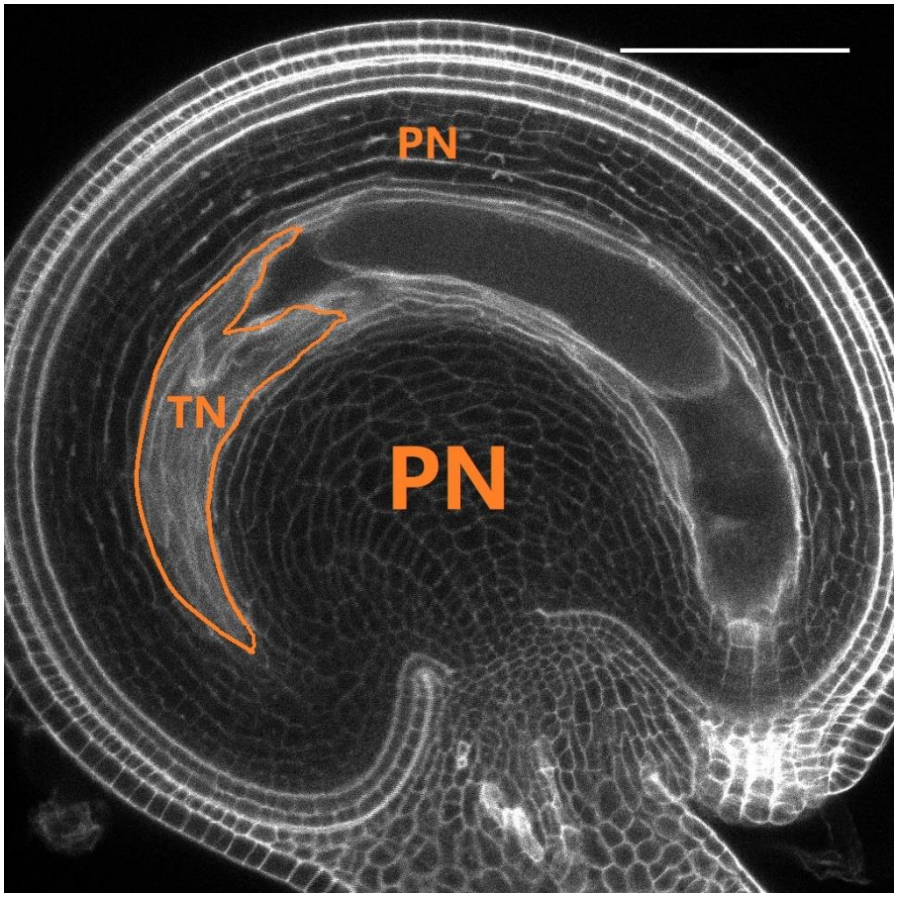

Figure5. Longitudinal section of *Amaranthus hypochondriacus* mature ovule imaged by mPS-PI. Transient Nucellus (TN); Persistent Nucellus (PN). Scale bar = 100 μm.

The Amaranthus TT16 gene

Our team characterized the role of the TT16 transcription factor in promoting nucellus elimination in Arabidopsis. Arabidopsis *tt16* mutant seeds mimic perispermic seeds as they develop a large nucellus, at the expense of the endosperm. It has been shown that forms in nature evolve largely by altering the expression of functionally conserved proteins (Carroll, 2008). To test if different *TT16* expression patterns in Arabidopsis and amaranth might account for different seed architectures, we cloned *TT16* orthologue gene in amaranth and studied its expression pattern by RNA *in situ* hybridization analyses. We detected *TT16* expression in the cell layer of the persistent nucellus surrounding the female gametophyte and the transient nucellus and in the endothelium, the innermost seed coat layer of the inner integument, of amaranth seeds right after fertilization (Fig. 6A). Older amaranth seeds displayed a similar *TT16* expression pattern in the nucellus, whereas we did not detect signal in the endothelium (Fig. 6B). We used *TT16* sense probe as a negative control (Fig. 6D). As a positive control, we instead hybridized seed sections with a *HISTONE4* (*HIS4*) antisense probe and observed its characteristic patchy expression pattern in actively dividing cells (Fig. 6C) (Blein et al., 2008).

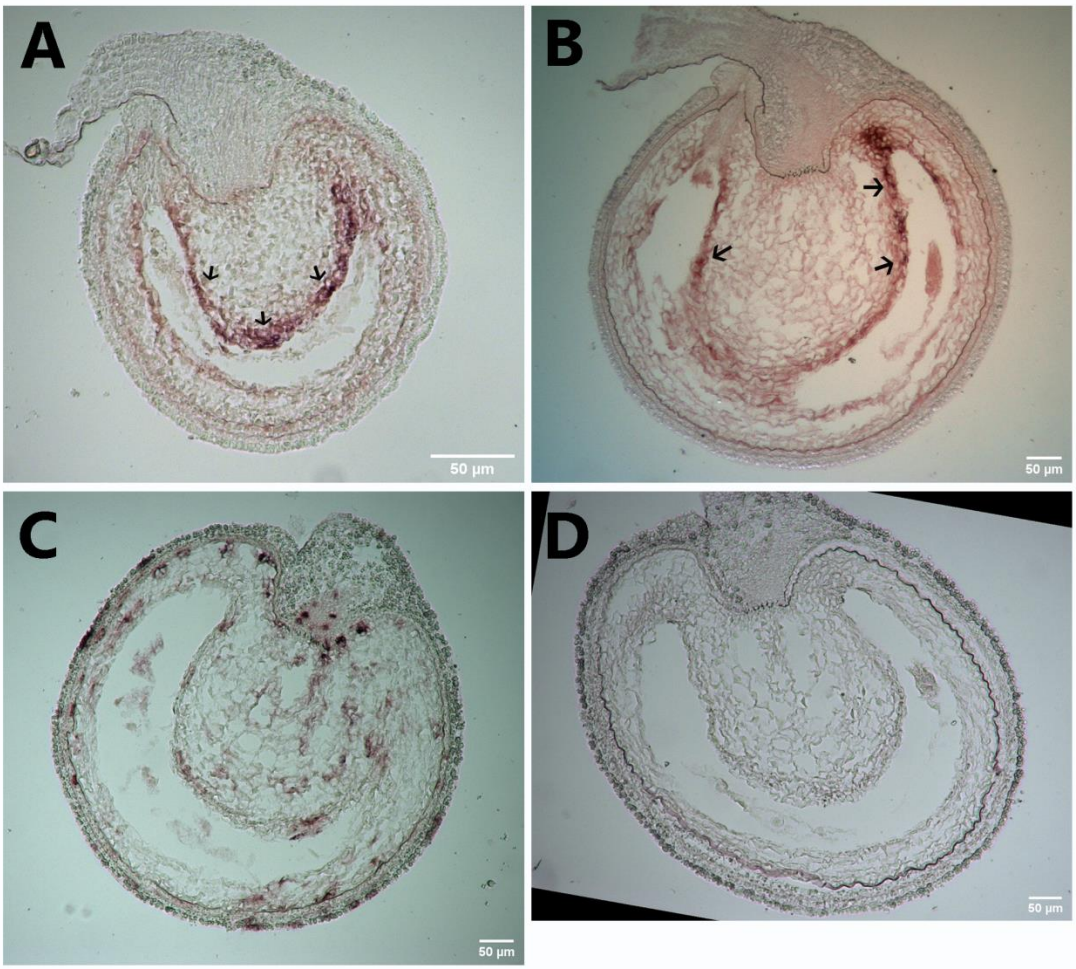

Figure6. Amaranthus *TT16* gene is expressed in nucellus cell layer surrounding the female gametophyte. *Amaranthus TT16* expression was detected by RNA *in situ* hybridization with a *TT16* antisense probe on *Amaranthus hypochondriacus* seed longitudinal sections (A and B). Black arrows point to the expression signal in the outermost cell layer of the perisperm. *HIS4* expression was detected with a *HIS4* antisense probe on *Amaranthus hypochondriacus* seed longitudinal sections (C). No *Amaranthus TT16* expression was detected with a *TT16* sense probe on *Amaranthus hypochondriacus* seed longitudinal sections (D). Scale bars = 50 µm.

Alternatively, perispermic and endospermic seed structures might have evolved due to the functional divergence of the TT16 protein. To test this hypothesis, we cloned the whole genome sequence and seven splicing forms (two of which corresponding to the only two known Arabidopsis *TT16* splicing forms) of *Amaranthus hyoochondriacus TT16* gene (Fig. 7). We performed domain and motif amalyses on all the *TT16* splicing forms of *Amaranthus hyoochondriacus* and *Arabidopsis thaliana* by using the SMART (http://smart.embl-heidelberg.de/) domain analysis tool. MADS domain was found at N-terminal end of all splicing forms in both *Amaranthus hyoochondriacus* and *Arabidopsis thaliana* (Fig.7). Beside the MADS domain, the K coiled-coil domain, known to drive protein-protein interaction (Davies et al., 1996; Fan et al., 1997), was found in three splicing forms (Ah TT16-I, Ah TT16-II, Ah TT16-III) of *Amaranthus hyoochondriacus* and two splicing forms of *Arabidopsis thaliana TT16* gene. To study if Amaranthus and Arabidopsis TT16 proteins share the same function we performed complementation analyses in the Arabidopsis *tt16* mutant background. We cloned all amaranth *TT16* splicing forms and genomic sequences

downstream the *TT1* promoter region, which drives expression specifically in the inner integument. We could not express them in the nucellus because, to date, we do not have a promoter specific for the nucellus and Arabidopsis *TT16* promoter alone is not sufficient to drive expression in the nucellus as it requires *TT16* genomic sequence. Nevertheless, the laboratory already demonstrated that TT16 works non-cell autonomously and *TT16* expression under the control of the *TT1* promoter complemented all known Arabidopsis *tt16* phenotypes. We already obtained several complementation lines and their analysis is underway.

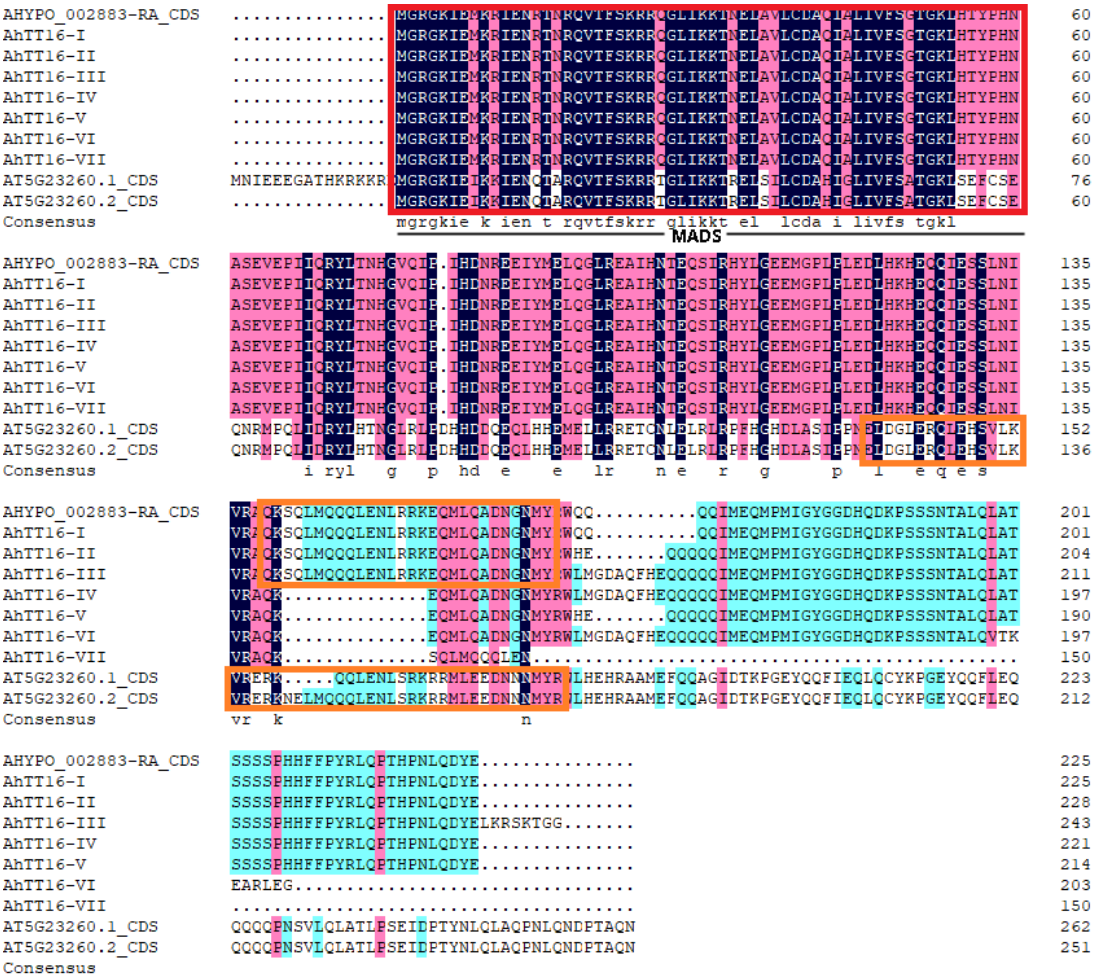

Figure7 Protein sequence alignment and domain analysis of Arabidopsis and *Amaranthus hyoochondriacus TT16* splicing forms. At5G23260.1 and At5G23260.2 are from Arabidopsis and AhTT16-I to AhTT16-VII are from *Amaranthus hyoochondriacus*. AHYPO_002883RA is the only amaranth splicing form annotated in Phytozome. The alignment is made with the DNA MAN software. Red and orange squares indicate MADS domain and K domain, respectively.

MATERIAL AND METHODS

Material

Amaranthus hyoochondriacus seeds were obtained by the USDA amaranth germplasm station, Nebraska, USA. at greenhouse of INRAE Versailles. All *Arabidopsis thaliana* plants are in the Columbia accession. The *tt16-1* mutant comes from Prasad (Prasad et al., 2010). *pDONR221-gAtTT16*; *pDONR221-At5G23260.1* and *pDONR221-At5G23260.2* plasmids were obtained from Wenjia Xu, *pGUCB2-ProTT1* modified binary vector were obtained from Olivier Coen.

RNA extraction and Reverse transcription

RNA extraction and reverse transcription were performed as described above (see chapter 2).

Cloning and construction

Amaranthus hyoochondriacus TT16 splicing forms were amplified using the attB1-(5′ATGGGAAGAGGAAAGATAGAAATG3′) forward and attB2-(5′ CAAGACTGGAGGGAGCTCAG 3') reverse primers. attB1 (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3′) and attB2 (5′-GGGGACCACTTTGTACAAGAAAGCTGGGTC-3′) carry the Gateway recombination sites at the 5′-ends of the forward and reverse primers, respectively. The PCR products were amplified with the high-fidelity Phusion DNA polymerase (Thermo Fisher Scientific), recombined into the *pDONR221* vector (BP Gateway reaction) according to the manufacturer's instructions (Thermo Fisher Scientific), and then sequenced. The PCR products cloned into the *pDONR221* vectors were then recombined into the *pGUCB2-ProTT1* modified binary vector (LR Gateway reaction), according to the manufacturer's instructions (Thermo Fisher Scientific).

Arabidopsis transformation

Arabidopsis transformation was performed as described above (see chapter2).

Pseudo-Schiff propidium iodide staining

Pseudo-Schiff propidium iodide staining was performed as described above (see chapter4).

RNA *in situ* hybridization

RNA *in situ* hybridization analyses were conducted as previously shown (Xu et al., 2016).

Primers used for probes:

Name	Sequences	Probes
TT16as F	5'-GAAGCAATTCACAACACTGAGC	**TT16as**
TT16as R	5'-TGTAATACGACTCACTATAGGGCATTAGGATGAGTGGGTTGAAGG	
TT16s F	5'-TGTAATACGACTCACTATAGGGCGAAGCAATTCACAACACTGAGC	**TT16s**
TT16s R	5'- ATTAGGATGAGTGGGTTGAAGG	
HIS4as F	5'-GGAAGAGGAAAGGGAGGAAA	**HIS4as**
HIS4as R	5'-TGTAATACGACTCACTATAGGGCCCACCAAAACCATAAAGAGTACG	

DISCUSSION

Arabidopsis and amaranth display opposite seed architectures. Whereas Arabidopsis seeds eliminate most of the nucellus tissue in favor of the endosperm, amaranth seeds undergo nucellus growth alongside a minute endosperm.

We found out that a domain of the amaranth nucellus also undergoes cell elimination, thus showing that both Arabidopsis and amaranth have transient and persistent nucellus domains. These data indicate that the basic tissue organization of endospermic and perispermic seeds is more similar than previously thought. Our discovery suggests that Arabidopsis and amaranth seed structures might be driven by different cell fates of the persistent nucellus more than a change in the nucellus elimination program.

Angiosperms shifted several times between the endospermic and perispermic seed condition, thus suggesting that a relatively simple genetic mechanism is underlying these different seed structures. During my thesis I challenged the hypothesis that a change in *TT16* expression or function might be responsible for the evolution of endospermic and perispermic seed structures. Amaranth seeds express *TT16* in the nucellus cells surrounding the female gametophyte and the transient nucellus, similar to what observed in Arabidopsis. These data suggest that TT16 might be responsible for the elimination of the transient nucellus also in amaranth seeds. Further confirmation will come from the complementation analyses. If the amaranth TT16 will complement the Arabidopsis *tt16* nucellus phenotype, we will be able to speculate that TT16 function in Arabidopsis and amaranth is conserved. To fully address this question, the team has created an amaranth EMS mutant library and it is currently looking for *tt16* mutant lines by TILLING.

The amaranth *TT16* is not expressed in the entire persistent nucellus domain, in contrast to what observed in Arabidopsis. Therefore, TT16 exclusion from most of the persistent nucellus might explain its different cell fate, when compared to Arabidopsis. This hypothesis could be tested by ectopically expressing *TT16* in the perisperm of amaranth seeds. To address this question, the group is working on a transformation protocol for amaranth.

Finally, we demonstrated that the SHP transcription factors promotes nucellus growth in Arabidopsis. Therefore, it would be interesting to study SHP expression and function in amaranth following the same steps used for TT16 analysis.

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GENERAL CONCLUSION

My work has shed light on some of the genetic and physiological mechanisms underlying the development of the nucellus in Arabidopsis and amaranth seeds, which have opposite architectures. The Arabidopsis nucellus is almost completely eliminated to make space to the endosperm whereas the amaranth nucellus grows to become the main storage tissue.

In Arabidopsis the distal nucellus (transient nucellus) is eliminated by the endosperm whereas a few cell layers of the proximal domain of the nucellus (persistent nucellus) persist. Whereas we had a general understanding of the nucellus elimination process, driven by the action of the MADS domain transcription factor TRANSPARENT TESTA 16 (TT16), we did not know 1) its physiological implications and 2) mechanisms of containment.

Here, I show that the nucellus is capable of receiving nutrients as it is symplastically connected to the chalaza, the nutrient unloading zone. The transient nucellus accumulates starch and releases it in the apoplastic space during its elimination. This result indicates that the process of nucellus elimination is not triggered by nutrient starvation and highlights a novel mechanism of sharing resources. By contrast, the transient nucellus does not accumulate starch and, together with the chalaza, contributes to the apoplastic transport of sugars to the fertilization products by expressing the *SWEET4* and *SWEET10* sugar facilitator genes, respectively.

I bring evidence for a symplastic block between transient and persistent nucellus that might explain why the cell elimination process arrests. Furthermore, I was able to identify the *SHATTERPROOF* (*SHP*) MADS box genes as genetic suppressors of the *tt16* mutation. This result indicates that the survival of the nucellus is also regulated at the genetic level.

Finally, I shifted my attention toward the amaranth seed that is characterized by a large nucellus tissue. Compared to what previously thought, I identified a domain of the amaranth nucellus tissue that is eliminated. Interestingly, this transient nucellus domain is surrounded by a layer of persistent nucellus cells expressing the amaranth gene orthologous to *TT16*. This results indicate that the nucellus elimination mechanism identified in Arabidopsis might be conserved in Amaranth but limited only two a small region of the nucellus.

GENERAL DISCUSSION

In the literature, the study of nucellus development is considerably behind other seed tissues. More attention has been given to the study of the fertilization products, embryo and endosperm, which constitute a unique biological model. Furthermore, perispermic seed plant models, such as quinoa or amaranth, which display a prominent nucellus, have emerged only recently.

The study of nutrient transport in cereals has advanced the understanding of the physiological role of the nucellus. In most cereals, the nucellus plays a dominant or sole role in transferring nutrients to the endosperm and develop into specialized structures, such as the nucellus projection. By contrast, in Arabidopsis the seed coat was thought to be the only tissue dedicated to nutrient transport. The discovery of a persistent nucellus domain in Arabidopsis made us postulate the hypothesis that the nucellus might play a role too. Our results show that the Arabidopsis seed evolved three paths for sugar transport, one for sucrose through the seed coat, one for hexoses through the persistent nucellus and chalaza, and one based on the release of starch in the apoplastic space through the elimination of the distal nucellus. We speculate that the persistent nucellus offers a shorter path toward the endosperm and that is why it might move hexoses, known to be more reactive compared to sucrose. Nevertheless, other hypotheses are possible. For example, hexoses are known to work as developmental signals as well. Therefore the nucellus might also play a role in signaling, a scenario that should be investigated further. We observed a delay in embryo development in the chalaza and nucellus *sweet* mutants, compared to the wild type, which could be interpreted as lack of nutrient export. Alternatively, it might be interpreted as a disruption of a signaling pathway between embryo and maternal tissues, yet to be discovered. A comparable hexose transport pathway has been shown in the rice nucellar projection, which also rely on SWEETs for sucrose export and invertases for its conversion into hexoses. In rice, seed coat and endosperm are separated by one nucellus cell layer, the nucellus epidermis, which might be the functional analogue in sugar transport of the Arabidopsis seed coat. Finally, the accumulation of starch by the transient nucellus was also observed in cereals, where evidence of hydrolytic activity has been found. A similar mechanism might be put in place also in Arabidopsis to allow the recycling of such starch. Unclear is instead the advantage, if any, behind the accumulation of starch in the transient nucellus, since its elimination rapidly follows. We speculate that it might be an evolutionary relic, if the perispermic seed architecture will appear to have evolved before the endospermic, a topic still debated. Our results brought also evidence of co-regulation of the tissue and nutrient partitioning processes by TT16. On the one hand, TT16 promotes the elimination of the transient nucellus and the release of starch in the apoplastic space. On the other hand, it negatively regulates *SWEET10* expression, possibly favoring the nucellus SWEET4 pathway of sugar transport. This opens a novel perspective in the field that should be further explored. In the past, nutrient and tissue partitioning have been analyzed independently but they are clearly linked processes. In the

future, the focus should be put in better understanding such regulation. Furthermore, it would be interesting to address the co-regulation, if any, of different sugar pathways. Are the seed coat and nucellus pathways independent? Is TT16 regulating the seed coat pathway as well? Finally, the same approach should be used to analyze the transport of other nutrients such as amino acids.

The mechanism that leads to the elimination of the nucellus is still unknown and clearly differs from other known ones as it involves the complete degradation of the cell wall. The analysis of cell wall components as well as genes known to underlie modifications of the cells wall in different domains of the nucellus might be the key to study this process in the future. I did not directly study the physical mechanism behind cell elimination but shed more light on its genetic regulation. The role of the TT16 transcription factor in promoting nucellus elimination had been shown in Arabidopsis and rice. The discovery of the SHPs proteins as putatively responsible for the survival of the persistent nucellus domain opens to novel interpretations. SHP and TT16 were shown to physically interact through the action of the SEPALLATA3 (SEP3) MADS domain transcription factor. Nevertheless, I showed that these transcription factors are co-expressed in the nucellus only during early ovule development. The protein complex might, therefore, specify the cell fate of different domains of the nucellus early on in development. Alternatively, the absence of SEP3 in the nucellus of the Arabidopsis seed might allow TT16 and SHPs to independently specify different nucellus domains. To complicate the interpretation of the data is the non-cell autonomous effect of TT16 that promotes the elimination of the transient nucellus from the persistent nucellus. Preliminary transcriptional data do not show a drastic effect of TT16 or SHPs on each other expression pattern, but we cannot exclude that even a lower decrease in the expression level might affect the stoichiometry of transcriptional complexes. Our discovery of a genetic control for cell survival is also important to understand how the mechanism of cell elimination is contained. We can suppose that the breakage of a cell might be a toxic event and affect neighboring tissues. By contrast, the seed coat but also the proximal domain of the nucellus survives to such an event. The elimination of the nucellus has been shown to spread centripetally, from distal to proximal, suggesting that the diffusion of a death signal or toxic material might spread the elimination process in the transient nucellus. The clear-cut transition from transient to persistent nucellus domains called instead for the presence of a physical barrier. Our finding of a symplastic block between the two domains of the nucellus appear to confirm such an hypothesis. More biochemical and genetic evidences are necessary to confirm our model. We are planning to analyze the function of callose synthases putatively expressed in the nucellus. Furthermore, we wonder if SHP regulates the formation of the above-mentioned symplastic block. The analysis of symplastic connections in the *shp1;2* mutant background will further challenge our model. The nucellus is also known to be isolated from the neighboring seed coat cell by a cutin apoplastic barrier. The analyses of mutant defective in the formation of such cuticle layers will help us understand if apoplastic barriers might

also have a role in the process of containing the cell elimination process.

Finally, my study of the amaranth seed is the first attempt to understand nucellus development in perispermic seeds. The finding of a transient nucellus domain and the expression of *TT16* in the surrounding nucellus cells of the amaranth seed seem to indicate that there is a higher level of conservation between endospermic and perispermic seeds. Our complementation analyses using the Amaranth *TT16* gene in the Arabidopsis *tt16* mutant will help us understand if TT16 function is also conserved between these two species but the analysis of an amaranth *tt16* mutant is necessary to finally prove this hypothesis. The absence of *TT16* expression in certain domains of the amaranth nucellus might explain their different cell fate. Alternatively, the analysis of SHP genes in amaranth seeds might be a novel path of investigation to follow. The creation of an amaranth mutant library, currently underway, to be screen for mutants defective in nucellus growth will be necessary to better understand the process. Such a forward genetic approach has the potential to reveal novel genes and help us test novel hypotheses also in Arabidopsis. Finally, our approach to the analysis of nutrient partitioning in Arabidopsis should be applied also in Amaranth to understand how nutrients flow in a perispermic seed. Amaranth seeds are economically valued because of their content in protein, similar to that of animal sources. It would therefore be especially relevant to study the transport of amino acids through the nucellus in perispermic seeds and be confronted to that of endospermic ones.

Résumé en français

Le partitionnement tissulaire est une force motrice qui façonne la structure des graines lors de leur développement. La contribution relative de chaque tissu à la masse finale de la graine varie considérablement selon les espèces et sous-tend différentes stratégies de stockage des réserves. Le partitionnement tissulaire s'effectue au travers de programmes d'élimination cellulaire qui régulent la dégénérescente d'un tissu en faveur d'un autre. Le nucelle, tissu maternel, joue un rôle déterminant dans la définition de la structure de la graine au côté des produits de la fertilisation. Chez les Gymnospermes, la plus grande partie du nucelle est éliminée et remplacée par le gamétophyte femelle, principal tissu de réserve. Ce dernier est à son tour consumé par l'embryon, unique produit de la fertilisation, lors de son développement. Chez les Angiospermes, les graines se répartissent en trois types d'architectures selon la part relative du nucelle et des produits de fertilisation, endosperme et embryon. Chez les graines de type endospermique (i.e. céréales, Arabidopsis), l'endosperme entoure l'embryon et joue un rôle important dans le stockage des nutriments. Au contraire, l'endosperme chez les graines non-endospermique (*i.e.* la plupart des légumineuses) est complètement consumé par l'embryon, qui devient le tissu primaire de stockage. Enfin, les graines périspermiques (*i.e.* pseudo-céréales telles que l'amarante et le quinoa) ont un large périsperme, tissu provenant du nucelle, ainsi qu'un endosperme réduit. L'origine endospermique ou périspermique des graines des angiospermes est encore en débat. En effet, les angiospermes primitives possèdent soit un large nucelle, soit un endosperme, comme compartiment primaire de stockage. De plus, les plantes ont changé plusieurs fois de stratégies entre développement endospermique et périspermique, mettant en lumière ce mécanisme développemental antagoniste de l'endosperme et du nucelle dans l'évolution des graines.

Durant ma thèse, le rôle du nucelle lors du partitionnement tissulaire et nutritif a été étudié dans la graine d'Arabidopsis. Le laboratoire avait précédemment démontré que le facteur de transcription à MADS-box TRANSPARENT TESTA 16 (TT16) promeut l'élimination de la région distale du nucelle, appelée nucelle transitoire, en réponse à un signal provenant de l'endosperme. Nous avons caractérisé deux suppresseurs du mutant *tt16*, les gènes à MADS-box *SHATTERPROOF 1* (*SHP1*) et *SHP2*, qui promeuvent le maintien du nucelle. SHPs et TT16 répriment mutuellement leurs expressions. Cependant, nous avons pu détecter une superposition des expressions des gènes à MADS-box *SHP*s, *TT16* et *SEPALLATA3* (*SEP3*), dans la région proximale du nucelle, appelée nucelle persistant du fait de son absence de dégénérescence. Il a été montré que SEP3 établit un pont lors des interactions physiques entre TT16 et SHPs, suggérant ainsi que l'élimination du nucelle est coordonnée par la formation de complexes protéiques impliquant TT16 et SHPs. De plus, nous avons identifié une nouvelle voie de transport des sucres passant par le nucelle dans la graine d'Arabidopsis. Nous avons montré que le nucelle est connecté

symplastiquement à la chalaze, la zone de de déchargement des nutriments. Le nucelle fonctionne à la fois comme puits pour les sucres et source au côté du tégument de la graine. Le nucelle transitoire accumule de l'amidon très tôt après la fécondation et le restitue dans l'espace apoplastique lors de son élimination. Le nucelle persistant et la chalaze, au contraire, exportent les sucres vers l'endosperme grâce aux facilitateurs de sucres SWEET4 et SWEET10, processus partiellement régulé par TT16. De façon intéressante, nous avons découvert que les nucelles, persistant et transitoire, étaient déconnectés symplastiquement. Nous avons détecté assez peu de plasmodesmes dans le nucelle transitoire et leurs formes rappellent celles de plasmodesmes bloqués par l'accumulation de callose. Nous émettons l'hypothèse que le développement d'une barrière symplastique entre les nucelles persistant et transitoire pourrait empêcher la propagation du processus d'élimination cellulaire, qui en effet n'affecte que la région distale du nucelle. Enfin, nous avons caractérisé le développement du nucelle dans la graine d'Amaranthe, qui, au contraire de la graine d'Arabidopsis, est très important et lui confère le statut de tissu primaire de réserve. Pour tester l'hypothèse selon laquelle le facteur de transcription TT16 pourrait être responsable de l'évolution de l'architectures des graines, nous avons cloné le gène *TT16* orthologue chez l'Amaranthe et déterminé son profil expression et sa fonction. En conclusion, ce travail présente les mécanismes de régulation qui coordonnent les processus de partitionnement des tissus et des nutriments dans les graines.

Titre : Contrôle génétique et moléculaire du développement de la graine et de l' accumulation des réserves : le rôle du nucelle

Mots clés : nucelle, régulation transcriptionnelle, répartition des réserves, développement de la graine

Résumé : L'architecture de la graine est dictée par des processus de partitionnement de tissus, déterminant le ratio volumique entre les tissus maternels et les tissus zygotiques, et par des processus de partitionnement nutritif régulant l'allocation des nutriments entre ces tissus. Chez les angiospermes, le développement précoce de la graine se caractérise par un développement antagoniste, pour la mise en place de tissus puits pour les sucres, entre le nucelle, un tissu maternel, et l'endosperme, issu de la fécondation. Un tel processus témoigne de l'évolution des angiospermes et contribue aux plus anciennes architectures de graines

Durant ma thèse, le rôle du nucelle lors du partitionnement tissulaire et nutritif a été étudié dans la graine d'Arabidopsis. Le laboratoire avait précédemment démontré que le facteur de transcription à MADS-box TRANSPARENT TESTA 16 (TT16) promeut l'élimination de la région distale du nucelle, appelée nucelle transitoire, en réponse à un signal provenant de l'endosperme. Nous avons caractérisé deux suppresseurs du mutant *tt16*, les gènes à MADS-box *SHATTERPROOF 1* (*SHP1*) et *SHP2*, qui promeuvent le maintien du nucelle. SHPs et TT16 répriment mutuellement leurs expressions. Cependant, nous avons pu détecter une superposition des expressions des gènes à MADS-box *SHP*s, *TT16* et *SEPALLATA3* (*SEP3*), dans la région proximale du nucelle, appelée nucelle persistant du fait de son absence de dégénérescence. Il a été montré que SEP3 établit un pont lors des interactions physiques entre TT16 et SHPs, suggérant ainsi que l'élimination du nucelle est coordonnée par la formation de complexes protéiques impliquant TT16 et SHPs. De plus, nous avons identifié une nouvelle voie de transport des sucres passant par le nucelle dans la graine d'Arabidopsis. Nous avons montré que le nucelle est connecté symplastiquement à la chalaze, la zone de de déchargement des nutriments. Le nucelle fonctionne à la fois comme puits pour les sucres et source au côté du tégument de la graine. Le nucelle transitoire accumule de l'amidon très tôt après la fécondation et le restitue dans l'espace apoplastique lors de son élimination. Le nucelle persistant et la chalaze, au contraire, exportent les sucres vers l'endosperme grâce aux facilitateurs de sucres SWEET4 et SWEET10, processus partiellement régulé par TT16. De façon intéressante, nous avons découvert que les nucelles, persistant et transitoire, étaient déconnectés symplastiquement. Nous avons détecté assez peu de plasmodesmes dans le nucelle transitoire et leurs formes rappellent celles de plasmodesmes bloqués par l'accumulation de callose. Nous émettons l'hypothèse que le développement d'une barrière symplastique entre les nucelles persistant et transitoire pourrait empêcher la propagation du processus d'élimination cellulaire, qui en effet n'affecte que la région distale du nucelle. Enfin, nous avons caractérisé le développement du nucelle dans la graine d'Amaranthe, qui, au contraire de la graine d'Arabidopsis, est très important et lui confère le statut de tissu primaire de réserve. Pour tester l'hypothèse selon laquelle le facteur de transcription TT16 pourrait être responsable de l'évolution de l'architectures des graines, nous avons cloné le gène *TT16* orthologue chez l'Amaranthe et déterminé son profil expression et sa fonction. En conclusion, ce travail présente les mécanismes de régulation qui coordonnent les processus de partitionnement des tissus et des nutriments dans les graines.

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ÉCOLE DOCTORALE
Sciences du végétal : du gène à l'écosystème (SEVE)

Title : Genetic and molecular control of seed tissue and nutrient partitioning: the role of the nucellus

Keywords : nucellus, transcriptional regulation, reserves allocation, seed development

Abstract : The architecture of the seed is shaped by the processes of tissue partitioning, which determines the volume ratio of maternal and zygotic tissues, and nutrient partitioning, which regulates nutrient distribution among tissues. In angiosperms, early seed development is characterized by the antagonistic development of the nucellus maternal tissue and the endosperm fertilization product to become the main sugar sink. Such a process marked the evolution of angiosperms and outlined the most ancient seed architectures.

During my thesis, the role of the nucellus in Arabidopsis seed tissue and nutrient partitioning was investigated. The laboratory had previously demonstrated that the MADS-box transcription factor TRANSPARENT TESTA 16 (TT16) promotes the elimination of the distal region of the nucellus, the so-called transient nucellus, in response to an endosperm signal. Here, we characterized two genetic suppressors of the *tt16* mutant, the *SHATTER PROOF 1* (*SHP1*) and *SHP2* MADS-box genes, which promote the survival of the nucellus. SHPs and TT16 mutually repress each other expression. Nevertheless, we detected expression overlap of *SHPs*, *TT16*, and *SEPALLATA3* (*SEP3*) MADS-box genes in the proximal region of the nucellus, referred to as persistent nucellus as it does not degenerate. SEP3 has been shown to bridge TT16 and SHPs physical interaction, thus suggesting that nucellus elimination is coordinated by the formation of TT16 and SHPs protein complexes. Furthermore, we identified a novel path of sugar transport through the nucellus in the Arabidopsis seed. We showed that the nucellus is symplastically connected to the chalaza, the seed nutrient unloading zone, and works both as sugar sink and source alongside the seed coat. The transient nucellus accumulates starch early on after fertilization and releases it in the apoplastic space during its elimination. By contrast, the persistent nucellus and the chalaza export sugars toward the endosperm through the SWEET4 and SWEET10 sugar facilitators, a process that is partially regulated by TT16. Interestingly, we discovered that persistent and transient nucellus are symplastically disconnected. We detected relatively few plasmodesmata in the transient nucellus and their shape was reminiscent of plasmodesmata blocked by the accumulation of callose. We hypothesize that the development of a symplastic barrier between persistent and transient nucellus might prevent the spreading of the cell elimination process, which indeed affects only the distal region of the nucellus. Finally, we characterized nucellus development in the *Amaranthus* seed that, opposite to Arabidopsis, grows a large nucellus as primary storage tissue. To challenge the hypothesis that the TT16 transcription factor might be responsible for the evolution of such different seed architectures, we cloned *TT16* orthologous gene in *Amaranthus* and characterized its expression pattern and function. Overall, this work gives an insight in the regulatory mechanisms that coordinate the processes of nutrient and tissue partitioning in seeds.

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