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THE FEMALE GAMETOPHYTE: DEVELOPMENT AND FUNCTIONS

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Ai miei genitori,

"per aspera sic itur ad Astra"
[Seneca]

PART I

ABSTRACT

The plant life cycle alternates the diploid sporophyte and the haploid gametophyte. The female gametophyte of flowering plants develops within the ovule, a specialized structure within the ovary, which gives rise to the seed after fertilization.

Sexual reproduction in plants entails a series of developmental steps that culminate in the formation of the seed. The developing ovule protects the haploid female gametophyte, which is formed as the result of the megasporogenesis and megagametogenesis. Inside the female gametophyte, the two female gametic cells, the central and the egg cells, upon fertilization give rise to the seed endosperm and embryo respectively.

During my PhD, I dissect the genetic and molecular networks controlling female gametophyte formation and differentiation. I employed a yeast one-hybrid approach to identify *EC1.1* regulators; the *EC1* genes are specifically expressed in the female gamete and they are required for gamete fusion, therefore they are good candidates for clarify how gamete differentiation occurs in *Arabidopsis thaliana*. Among the transcription factors isolated, we focused on SUPPRESSOR OF FRIGIDA4 (SUF4). *In vivo* and *in vitro* evidences support SUF4 capacity to regulate *AtEC1.1*, furthermore *suf4* mutants show also a mild *ec1* phenotype.

Plant can produce progeny without sexual reproduction. One example is apomixis, where meiosis and fertilization of the egg by male gametes are by passed to result in the production of clonal progeny without a parental contribution. Apomixis is due to modifications of the sexual reproduction and it does not occur in the major crop species, but is found in many wild species like *Poa pratensis* and *Brachiaria brizantha.*

The idea of this work is to study genes involved in apomixis in apomictic plants, and then studies the function in the model organism *Arabidopsis thaliana*.

In *Poa pratensis* by the cDNA-AFLP technique several genes differentially expressed in apomictic and sexual genotypes have been isolated.

During my PhD I characterized the *Arabidopsis* homologue of *PpAPO1* (*Poa pratensis APOSTART 1*) that has been renamed *AtAPO1*.

Brachiaria brizantha is an important forage grass. The occurrence of both apomictic and sexual reproduction within *Brachiaria* makes it an interesting system for understanding the molecular pathways involved in both modes of reproduction.

INTRODUCTION

1. Overview

Reproduction enables an organism to multiple, thus ensuring its specie preservation. Asexual reproduction process is mainly finalised to dispersal, whilst sexual reproduction process adds the genome renewal and gamete-gamete interaction (Willemse, 2009; Kimble, 2011). Reproduction and dispersal are connected, indeed asexual reproduction and sexual reproduction shares several similarities however sexual reproduction relies on intense communication occurring between gametes, between organism and environment and also among organisms.

The remarkable evolutionary success of flowering plants, the second Darwin's "abominable mystery" (Crepet and Niklas, 2009), is caused by a confluence of features and it is undutiful that their reproductive strategies have contributed to their success.

Sexual reproduction requires the delivery of the sperm nuclei, via the pollen, to the embryo sac, where fertilization occurs and the new diploid sporophyte is formed.

The plant life cycle in the angiosperms is characterized by the alternation of generation between a diploid sporophyte and a haploid gametophyte. The sporophyte produces spores, which then develop into gametophytes. In contrast to lower plant species, in which the gametophyte is the dominant, free-living generation, gametophyte of angiosperms are smaller and less complex than the sporophyte and develop within specialized organs of the flower. Most of the plants are angiosperms, most shrubs and trees and grasses belong to this phylum.

All our food is derived, directly or not, from flowering plants; angiosperms are also sources of medicine, clothing and building materials.

Pollen matures within the anthers and is transported, often by insects, birds, or other animals, to the stigma of another flower. The relationship between plant and pollinator can be quite intricate (Feinsinger, 1987; Fontaine *et al*., 2005). Mutation in either partner can block reproduction.

Morphological innovations in reproductive strategies have played a fundamental role in the diversification of plants. The most successful strategy is the ability of developing seeds, which allowed plants to extensively colonize the Earth. The study of the seed conception is very important because it can be considered as a functional unit required for the protection and propagation of the offspring.

The first step in seed development is the formation of ovules. Consequences for reproduction and its regulation will be discussed.

2. Ovule, seed and fruit development in sexual plants

A strictly ordered and defined sequence of events is required to produce viable seeds in sexual species. Fertilisation results in seed formation and development, however fertilisation signals also induce and regulate ovary growth into fruit and fruit maturation. Indeed the parallel interdependent development of ovules and seeds is a highly complex process.

In angiosperms, seeds are the double fertilisation products, however several Anthophyta are able to produce seeds and fruits without meiosis and double fertilization. This process is named apomixis. The comprehension of apomictic seed development requires a deep knowledge of sexual reproduction (Koltunow and Grossniklaus, 2003), therefore before discuss apomixis, I will discuss ovule development and seed formation through sexual reproduction. I will

mainly present information obtained using the model plants *Arabidopsis thaliana*, *Poa pratensis* and *Brachiaria brizantha*.

2.1 Formation of angiosperm gametes

Angiosperms have a two-staged life cycle that alternates between a multicellular haploid organism, the gametophyte, and a multicellular diploid organism, the sporophyte: thus angiosperms are haplo-diplontic organisms (Drews and Yadegari, 2002).

Plant evolution is associated with several changes in the gametophytes, which have became reduced in size and complexity, in respect to sporophytes and, at least female gametophytes, became embedded within and physiologically dependent upon sporophytes.

Angiosperms are characterised by a heterosporous life cycle. The male gametophytes (pollen grains) develop within the anther and comprise two sperm cells encased in a vegetative cell (McCormick 1991, 2004). At maturity, the anther splits and pollen is released. Depending on the specie, pollen is then transferred to the pistil either by wind or an animal such as an insect or bird. The female gametophytes (also named embryo sacs or megagametophytes) develop within the ovules and exhibit a variety of forms.

Ovule primordia arise from the meristematic placental tissue and appear as a finger like protrusions. Along the proximal-distal axis of the developing ovule three elements can be distinguished: the funiculus, the chalaza, and the nucellus (Schneitz *et al*., 1995; Figure 1). **The funiculus** connects the ovule to the placenta and includes the vascular strand, which channels nutrients through the chalaza to the nucellus and the rest of the developing ovule. **The chalaza** originates the integuments that protect the developing gametophyte; while in **the nucellus**, the

megaspore mother cell, recognisable for its size, differentiates to form the embryo sac (Schneitz *et al.*, 1995; Gross-Hardt *et al.*, 2002).

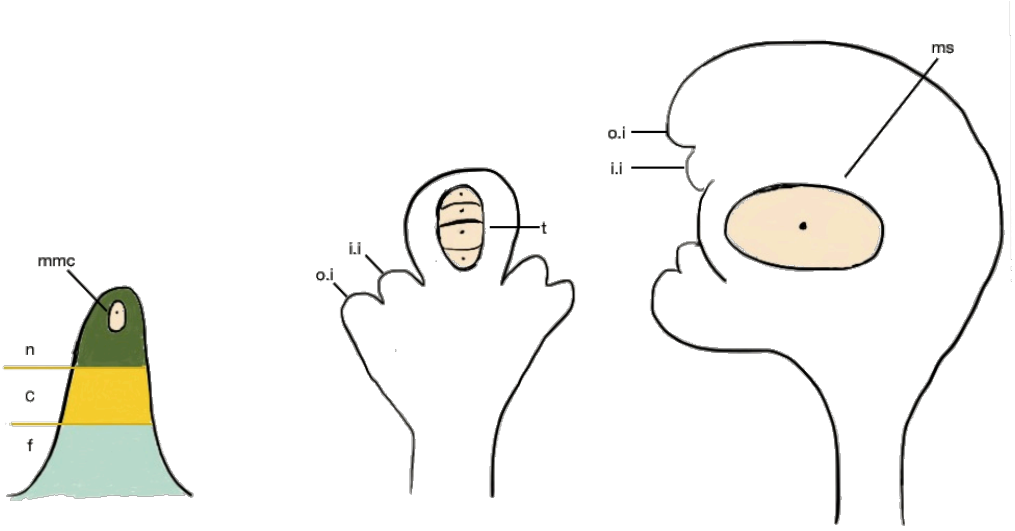

Figure 1. Ovule formation in *Arabidopsis thaliana* (adapted from Rita Groß-Hardt *et al.*, 2002). The ovule primordium is a finger-like structure that arises from the placenta. Along the proximal-distal axis of ovule primordial three domains can be distinguished (Schneitz *et al.*, 1995, 1999): the distal nucellus (n) that protects the megaspore mother cell (mmc), the central chalaza (c) and the proximal funiculus (f). The mmc divides meiotically to give rise to a tetrad (t) of haploid cells and simultaneously integuments grow to enclose the nucellus. The three distal cells of the tetrad die and the functional megaspore (ms) undergoes three rounds of mitotic division. Ii, inner integument; oi, outer integument.

The most common form of female gametophyte, found in approximately 70% of the species examined, is referred to as *Polygonum-type* (Palser, 1975): it is a seven-cell structure consisting of one egg cell, two synergid cells, one central cell and three antipodal cells (Yadegari and Drews, 2004) (Figure 2c). The model plant *Arabidopsis thaliana* develops a polygonum female gametophyte.

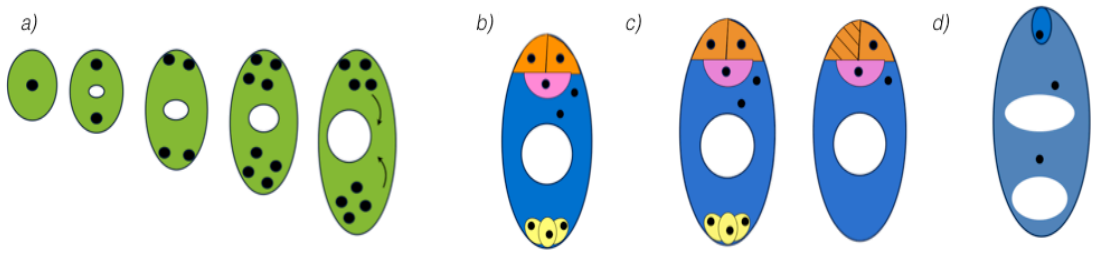

Figure 2. Schematic representation of the development of a *Polygonum-type* female gametophyte (adapted from Grimanelli *et al*., 2001). **a**, Three nuclear divisions occur in a syncytium to form an 8-nucleate female gametophyte. One nucleus from each pole migrates and will be enclosed by the central cell. **b**, Cellularization forms the typical 7-celled, 8-nucleate female gametophyte with two synergid cell (orange), one egg cell (pink), a bi-nucleate central cell (light blue) and the three antipodal cells (yellow). **c**, before fertilization the female gametophyte differentiates, the two polar nuclei fuse and one of the synergids degenerates as the pollen tube arrives. **d**, during double fertilization one sperm cell fuses with the egg cell to form the diploid zygote (blue), while the second sperm cell fertilizes the central cell to form the triploid endosperm (dark blue).

Female gametophyte formation is divided into two distinct phases referred to as megasporogenesis and megagametogenesis (Schneitz *et al*., 1995). During megasporogenesis, the diploid megaspore mother cell undergoes meiosis and gives rise to four haploid megaspores, three of which degenerate, whilst the persisting one, the functional megaspore, goes through three rounds of mitotic divisions to form a coenocytic eight nucleate embryo sac (Brukhin *et al*., 2005; Mansfield and Briarty, 1991; Mansfield *et al*., 1991) (Figure 2a, b).

Subsequently, nuclear migration, polar nuclei fusion and cellularization form a seven-celled embryo sac, consisting of three antipodal cells, one diploid central cell, two synergid cells and one egg cell. Mega-gametogenesis is divided in few steps named FG1-FG7 according to Christensen *et al*., 1997 (Figure 3A-G). The egg cell and the central cell are polarized and their nuclei lie very close to each other (Mansfield and Briarty, 1991). Furthermore, also the synergid cell cytoplasm appears highly polarised with a chalazally located vacuole, a centrally located nucleus and a highly specialised cell wall, the filiform

apparatus (Savidan *et al*., 2001). The regions between the egg, synergid and central cells lack the cell wall or the cell wall is discontinuous leading to direct contact among the plasma membranes of these cells (Mansfield and Briarty, 1991; Kasahara *et al*., 2005). This absence provides direct access of the sperm cells to the fertilization targets because the pollen tube releases its two sperm cells into one of the synergid (Sandaklie-Nikolova *et al*., 2007)(Figure 3).

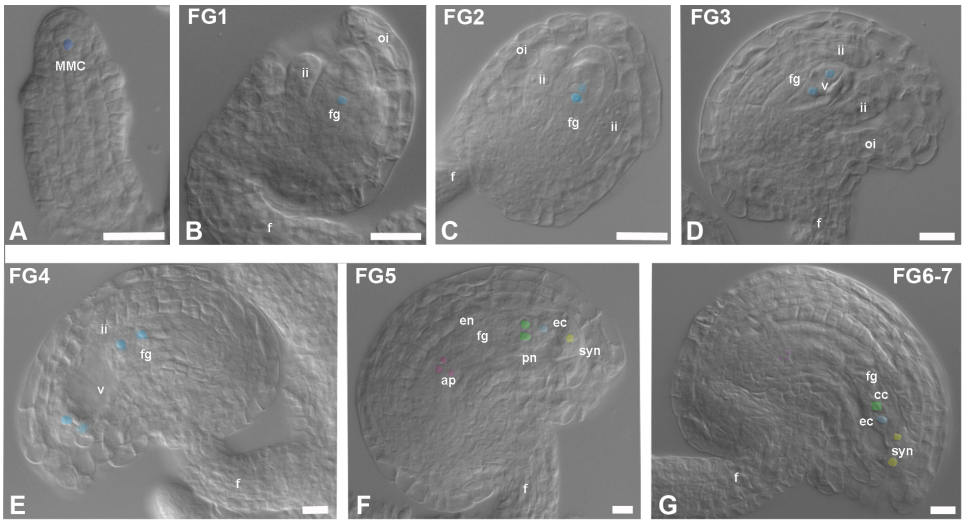

Figure 3. Wild-Type female gametophyte development in *Arabidopsis*, the FG stages are according to Christensen and collaborators (1997). (D) Wild-type embryo sac at FG3 stage, showing two nuclei separated by a vacuole. (E) Wild-type embryo sac at FG4 stage, showing two pairs of nuclei separated by a vacuole. (F) Wild-type embryo sac at FG5 stage, containing seven cells and eight nuclei. (G) Wild-type embryo sac at FG6-7 stage, containing seven cells and seven nuclei. Abbreviation: Ap, antipodal cell; Cc, central cell; Ec, egg cell; pole; Fg, female gametophyte; MMC, megaspore mother cell; Ii, inner integument; Oi, outer integument; Pn, polar nuclei; Syn, synergide; V, vacuole

2.2 Embryo sac: a case to study cell differentiation

The embryo sac is an ideal system to study cell differentiation due its low complexity.

Nevertheless, the molecular mechanisms governing cell identity in developing embryo sacs are largely unknown although several embryo sac defective mutants have been isolated and described (Christensen *et al*., 1997, 1998, 2002; Pagnussat *et al*., 2005, 2007, 2009; Gross-Hardt *et al*., 2007; Matias-Hernandez *et al*., 2010; Masiero *et al*., 2011).

The developing female gametophyte is an highly polarised structure, and although controversial, it has been reported that in *Arabidopsis* developing female gametophytes an auxin gradient is formed through local auxin biosynthesis and contributes to cell fate determination (Pagnussat *et al*., 2009). According to Pagnussat and co-workers (2009), auxin concentration appears stronger at the micropylar pole and governs synergid cell fate, whereas weaker auxin activity correlates with antipodal cell fate. Continuous auxin production along the embryo sac destroys such gradient and conversely causes the conversion of antipodal cells into synergid ones (Pagnussat *et al*., 2007).

However the auxin controlled cell identity switch does not affect the nuclear positioning which acts as cell identity regulator too.

Indeed the *Arabidopsis* cell-cycle mutant *rbr1* (*retinoblastoma-related1*) and the maize mutant *ig1* (*indeterminate gametophyte1*) are characterised by female gametophytes containing extra-nummerary nuclei: extra synergid, egg and central cells are determined accordingly to their positions (Ebel *et al*., 2004; Guo *et al*., 2004).

Many other *Arabidopsis* mutants, such as *eostre*, *lachesis* (*lis*), *gametophytic factor1* (*gfa1*), *clotho* (*clo*), *atropus* (*ato*) and *verdandi* (*vdd*), show changes in cell identities inside the embryo sacs: in mutated

megagametophytes synergid cells express egg cell markers, whilst the antipodals show central cell-specific markers (Gross-Hardt *et al*., 2007; Pagnussat *et al*., 2007; Matias-Hernandez *et al*., 2010; Völz *et al*., 2012). Phenotypic analysis of female gametophyte mutants in *Arabidopsis* is facilitated by the fact that an *Arabidopsis* pistil contains a large number of ovules (50 to 60) and that the female gametophytes within these ovules develop synchronously (Drews *et al*., 1998; Christensen *et al*., 1997).

These genetic studies have been supported by molecular studies finalised to clarify the mechanisms controlling embryo sac cell differentiation. Differential gene expression between wild-type and female gametophyte defective mutants (Johnston *et al*., 2007; Jones-Rhoades *et al*., 2007; Steffen *et al*., 2007; Yu *et al*., 2005) and microarray expression analysis of *Arabidopsis* laser-dissected gametophytic cell types (Wuest *et al*., 2010) have been extensively employed together with exhaustive sequencing of EST egg cell libraries (Cordts *et al*., 2001; Le *et al*., 2005; Yang *et al*., 2006; Kumlehn *et al*., 2001; Sprunck *et al*., 2005). This approach allowed identifying a wheat gene cluster *TaECA1*-like (*Triticum aestivum ECA1-like*) specifically expressed in the egg cell and sharing high sequence similarity to the barley *ECA1* (Sprunck *et al*., 2005; Vrinten *et al*., 1999) and involved in cell signalling. *ECA* genes have been also identified in *Arabidopsis*; they form a wide gene family of 188 members. However a small subgroup, formed by just five genes, (named *EC1.1*, *EC1.2*, *EC1.3*, *EC1.4*, and *EC1.5*), is closely related to the *TaECA1*-like. The *AtEC1* genes are also exclusively detected in egg cells and are repressed by fertilisation events (Sprunck *et al*., 2012). Double and triple mutant plants developed normally, only the simultaneous silencing of all the *AtEC1* genes indicates that the secreted *EC1* is necessary for female and male gamete fusion (Sprunck *et al*., 2012).

In this thesis I will present my attempts to shed light into *Arabidopis* embryo sac formation and cell differentiation.

3. From ovule to seed

3.1 Integument formation

The seed coat or testa is the protective outer covering surrounding the plant embryo. The seed coat protects the embryo from mechanical damage and pathogen attack, maintains the dormant state dehydrated of the embryo until proper germination conditions and provides the means for initial water uptake. Integuments also protect the embryos from UV damage and provide dispersal mechanisms (Windosor *et al*., 2000). It has already been published that anthocyanins are known to be powerful antioxidants that also protect plants from UV damage (Tsoyi *et al*., 2008).

The *Arabidopsis* testa is the product of maternal parent and is formed from two integuments of epidermal origin that surround the mature ovule. The development of the integuments surrounding the *Arabidopsis* ovule has been well described (Gasser and Robinson-Beer, 1993; Schneitz *et al*., 1995). The two integuments are composed of several layers that provide an interface between embryo and the external environment during seed development, dormancy and germination (Haughn and Chaudhury, 2005). The integuments of a mature ovule at the time of anthesis consist of a two cell-layered outer integument and a mostly three cell-layered inner integument (Figure 4).

Differentiation of the seed coat from the ovule integuments includes important cellular changes and culminates in the death of the seed coat cells. The integuments are initiated at the base of the nucellus during megasporogenesis (Schneitz *et al*., 1995). At the micropyle end, an endothelium apparently does not develop.

The inner integument is of dermal origin, when the ovule

primordial arise form the placenta tissue. Cellular proliferation determines the formation of a ring-like belt that delimits the nucellus as the apical portion of the primordium. As the body of the ovule enlarges and the funiculus elongates, the ovule begins to exhibit the effect of asymmetric growth.

The outer integument is initiated through a series of similar cell divisions and it remains two-cell layered throughout seed development. At later stages of seed development, cells of the abaxial layer of the outer integument differentiate terminally into highly specialized seed coat cells that contain polysaccharide mucilage (Robinson-Beers *et al.*, 1992).

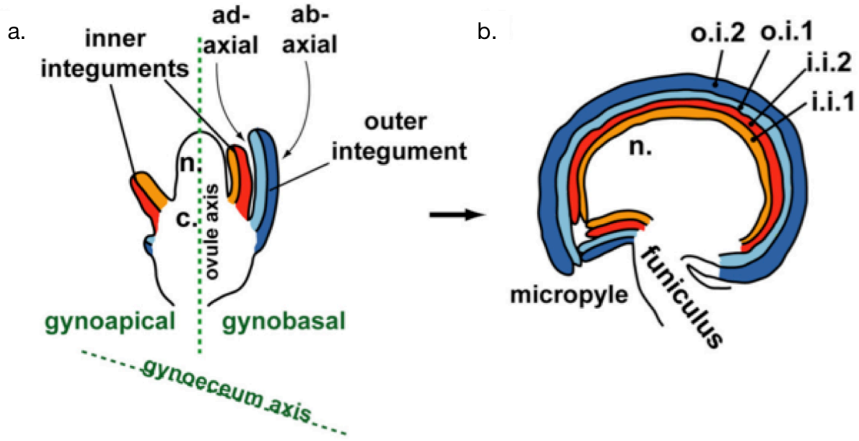

Figure 4. The development of ovule integuments in *Arabidopsis* (according to Truernit and Haseloff, 2008). **a**, two inner and one outer integument grow out from the chalaza (c) during early ovule development. b, ovule ate stage of fertilization: integuments have grown around nucellus (n), i.i.1: inner (adaxial) layer of inner integument, i.i.2: outer (abaxial) layer of inner integument, o.i.1: inner (adaxial) layer of outer integument, o.i.2: outer (abaxal) layer of outer integument.

The evolutionary origin of the integuments is still a matter of debate. The development of the *Arabidopsis* outer ovule integument involves the same basic processes required for the formation of other determinate lateral plant organs, such as leaves (Truernit and Haseloff, 2008).

3.2 The double fertilization

Pollination and subsequent double fertilization in the ovule are normally required for fruit and seed development in sexually reproducing plants.

Carpels are the female reproductive unit of flowers. They enclose the ovules and they provide and, eventually, select the appropriate (compatible) male gametophyte (Chapman and Goring, 2010). The carpel consists of a stigma, a style and an ovary joined to the floral receptacle by a short stem. One of the primary functions of the stigma is to provide sufficient water and nutrients for germination to pollen grains.

Nawaschin and Guignard simultaneously, more than a century ago, using *Liluim martagon* and *Lilium pyrenaicum* and *Fritillaria tenella*, described the double fertilization (Jensen, 1998).

Sexual reproduction is initiated when the male gametophyte is transferred from the anther to the stigma of the carpel; there the vegetative cell emits the pollen tube that grows through the carpel transmitting tissue to deliver the two sperm cells to the female gametophyte (Berger *et al*., 2008)(Figure 5).

Several mechanisms appear to be involved in pollen tube guidance, including chemo-attraction, mechanical guidance, adhesion, stimuli and competence control that enables the pollen tube to respond to the attraction signal (Dresselhaus and Franklin-Tong, 2013). Indeed the pollen tube follows chemotactic signals produced by the female gametophyte that drives it into the micropylar opening of the ovule (Dresselhaus and Marton, 2009; Woriedh *et al*., 2013). Nowadays, it is well accepted that female and male gametophytes actively communicate; moreover communication within the gametophyte, such

as the central cell and the synergid cell, seems critical for double fertilization.

Chemical visualization of LUREs is uncovering spatiotemporal dynamics of pollen tube attraction (Okuda *et al*., 2009; Kawashima and Berger, 2011). In *Torenia fournieri*, *LURE1* and *LURE2* encode cysteine-rich polypeptides that are secreted to the filiform apparatus of the syngergids cells (Punwani *et al*., 2007, Punwani and Drews, 2008; Okuda *et al*., 2009).

The cells of the female gametophyte control many steps of the fertilization process, including pollen tube guidance, fertilization, the induction of seed development and gametophytic maternal control.

Cell ablation studies prove that the synergid cells produce a guidance cue able to direct pollen tube growth into the ovule (Okuda *et al*., 2009) and one synergid burst is necessary for driving pollen tube attraction (Kanaoka *et al*., 2011).

After entering the embryo sac, the pollen tube ceases growth and discharges its contents into one of the two synergid cells. The synergid cell penetrated by the pollen tube undergoes cell death, finally, the sperm cells fuse with the egg cell and central forming the seed embryo and endosperm, respectively (Figure 5).

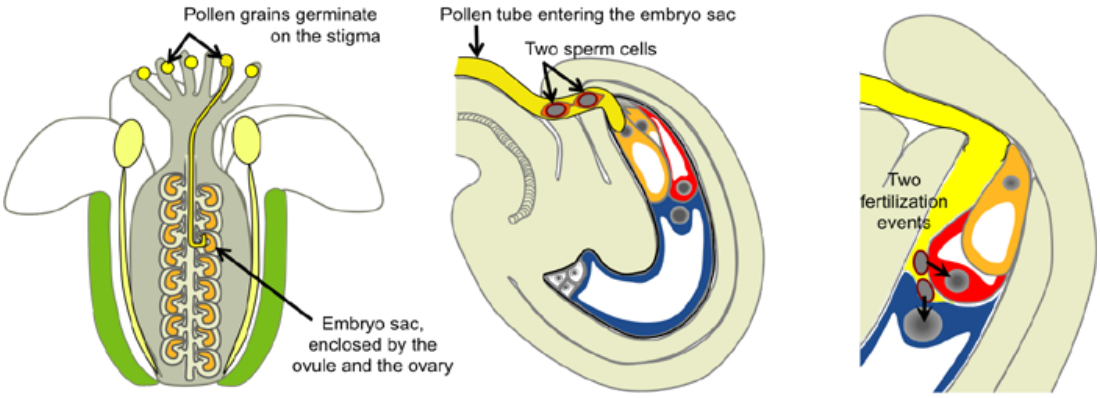

Figure 5. Fertilization in angiosperms is unique among all known organisms in that not one, but two, female reproductive cells are fertilized in a process called double fertilization. A complex mechanism involving two male gametes (sperm cells) and two female gametes (egg cell and central cell) results in two distinct fertilization products, the diploid embryo and the triploid endosperm. Both fertilization products are required to achieve successful seed development (according to Sprunck website).

Structural and genetic studies suggest that the synergid cells contain factors that control the arrest of pollen tube growth and the release of the pollen tube contents. In fact, sperm cell release requires communication between the pollen tube and the synergid; if this signalling process is disrupted, as in the *feronia* mutant, the pollen tube does not stop to grow and the sperm cells are not release (Huck *et al*., 2003; Escobar-Restrepo *et al*., 2007). In the absence of fusion with a sperm cell, the egg and central cell remain in a quiescent state and eventually degrade as the flower undergoes senescence (Koltunow and Grossniklaus, 2003).

Upon fertilization, the ovule is induced to develop into a seed.

Genetic and molecular studies indicate that central cell-expressed gene products control the activation of endosperm development.

The molecular processes by which the female gametophyte cells acquire their unique features and functions during cell differentiation are not fully understood. These processes probably involve distinct gene

expression profiles associated with each of the female gametophyte cell types. Thus, understanding how the female gametophyte cells become specified and acquire their unique features and functions requires mechanistic insight into the gene regulatory networks that control cell-specific gene expression during development of the female gametophyte.

A number of female gametophyte mutants have been identified in both *Arabidopsis* and maize indicating a requirement for haploid-expressed genes in megagametogenesis and female gametophyte function (Drews and Yadegari, 2002). Out of the 28,974 predicted open reading frames of *Arabidopsis thaliana*, a few thousand genes are predicted to be involved in embryo sac development. These genes can be grouped into two major classes: genes that are necessary during female gametogenesis and genes that impose maternal effects through the female gametophyte, and thus play essential roles for seed development (Bencivenga *et al*., 2011).

The onset of fruit development from the ovary, the so-called fruit set, occurs after fertilization of the ovules and it is coordinated by signals produced by the developing embryos, both pollination and seed derived signals are required for fruit initiation and subsequent development. It is known that fruit growth and shape depend on seed genotype and seed number (Sedgley and Griffin, 1989). Several evidences pinpoint that seed and fruit development are intimately connected and synchronized by phytohormone actions (Gillaspy *et al*., 1993).

However fruit formation can be uncoupled from fertilization and seed development as indicated by the existence of seedless mutant plants (tomato pat mutants) and seedless crops obtained by traditional breeding methods (grape, citrus, cucumber and watermelon) (Gourget,

et al., 2005; Voraquaux *et al*., 2000). A plant is seedless when its fruits are completely devoid of seeds, or contain a greatly reduced number of seeds or present aborted seeds. Parthenocarpic fruits develop without fertilisation.

Seedlessness is appreciated by consumers both in fruits for fresh consumption (e.g., grape, citrus, banana) as well as in conserved or processed fruits (e.g., frozen eggplants, tomato sauce). Seedlessness can contribute to increase the quality of the fruits when seeds are hard or have a bad taste. In the case of eggplant, the absence of seeds prevents browning and texture reduction of the pulp (Maestrelli *et al*., 2003) thus seed absence can increase the shelf life of the fruits allowing a better conservation.

4. Apomixis, an asexual method of reproduction through seeds

Many flowering plants can choose between no less than three fundamentally different modes of reproduction: (i) outcrossing sex; (ii) selfing sex; and (iii) asexuality (Richards 2003). Any reproductive process that does not involve meiosis or syngamy is said to be asexual, or vegetative. Syngamy means that such an event can occur in the sporophyte generation or the gametophyte stage. In botany, apomixis was defined by Winkler as the replacement of the normal sexual reproduction by asexual reproduction, without fertilization. Although it is sometimes referred to as a botanical curiosity, apomixes is far from rare, with a pattern of distribution that suggests that it has evolved several times during evolution. According to Richards (2003) apomixis occurs in ca. 60% of the British flora. Indeed It has been previously described in >400 flowering plant taxa and it is well represented among both monocotyledonous and eudicotyledonous angiosperms.

Several authors have noted a marked bias in the distribution of apomixis among angiosperms (Asker and Jerling, 1992; Mogie, 1992; Carman, 1997; Richards, 1997). Of the plants known to use gametophytic apomixis (Figure 1), 75% belong to three families, the Asteraceae, Rosaceae, and Poaceae, which collectively constitute only 10% of flowering plant species.

Self-pollination reduces genetic variability, however asexual reproduction results in the formation genetically individuals because only mitotic cell divisions occur. In the absence of meiosis, individuals that are highly adapted to a relatively unchanging environment persist for the same reasons that self-pollination is favoured. A better understanding of the molecular and genetic basis underlying plant reproductive development will transform current breeding strategies and seed production. Despite the relatively limited knowledge of the molecular mechanisms that control plant gametogenesis, some of the most spectacular advances in plant breeding and agriculture have come from a manipulation of the reproductive system.

The harnessing of apomixes, an asexual form of reproduction, has become an important goal of plant research. It results in the formation of progeny that are genetic clones of the maternal parent, a trait of major importance for agriculture (Barcaccia and Albertini, 2013).

As previously described, plants have evolved a characteristic life strategy with alternating generations, between a diploid and a haploid generation.

Figura 6. Dandelion dispersing seed. Weeds such as hawkweed and dandelions, can produce true seeds that are clones of themselves without sexual reproduction.

In apomitic plants, offspring are produced from an-unreduced cell that is either of sporophytic or gametophytic origin. Thus, the characteristic alternation of gametophytic and sporophytic generations of the plant life cycle is either bypassed, or occurs without the meiotic reduction of the somatic chromosome number (Koltunow and Grossniklaus, 2003).

The potential benefits of apomixes technology to agriculture are extensive and have been previously extolled. The main agronomic benefit that apomixes technology could deliver is the immediate fixation of any desired genotype and its indefinite propagation.

5. Modes of apomitic reproduction

There are two main modes of apomixes, depending on whether unreduced cells give rise to a megagametophyte (gametophytic apomixes) or directly to an embryo (sporophytic apomixes).

It can also be separated into three major elements required for visible seed set. These are:

- Absence or alteration of meiosis preventing reduction (apomeiosis),
- Activation of the egg cell to form an embryo in the absence of fertilization (parthenogenesis),
- Initiation of endosperm development (Spielman *et al*., 2003).

In gametophytic apomixis, embryogenesis begins from an unreduced gametophyte or originates either directly from nucellar cells or from a megaspore mother cell that undergoes aberrant meiosis resulting in the formation of two unreduced megaspores. Gametophytic mechanisms are further subdivided on the basis of cell types that will give rise to the unreduced embryo sac.

In **diplosporous** types, the gametophyte is derived from the megaspore mother cell and the megaspores result from an aberrant or modified meiosis that restore the genome of the mother. Alternatively, in **aposporous** pathways the megaspores are derived from somatic cells within the ovule that develop directly into a megagametophyte, bypassing meiosis. Diplosporous and aposporous embryo sacs may or may not resemble the reduced embryo sacs observed in related sexual plants (van Dijk and Bakx-Schotman, 2004).

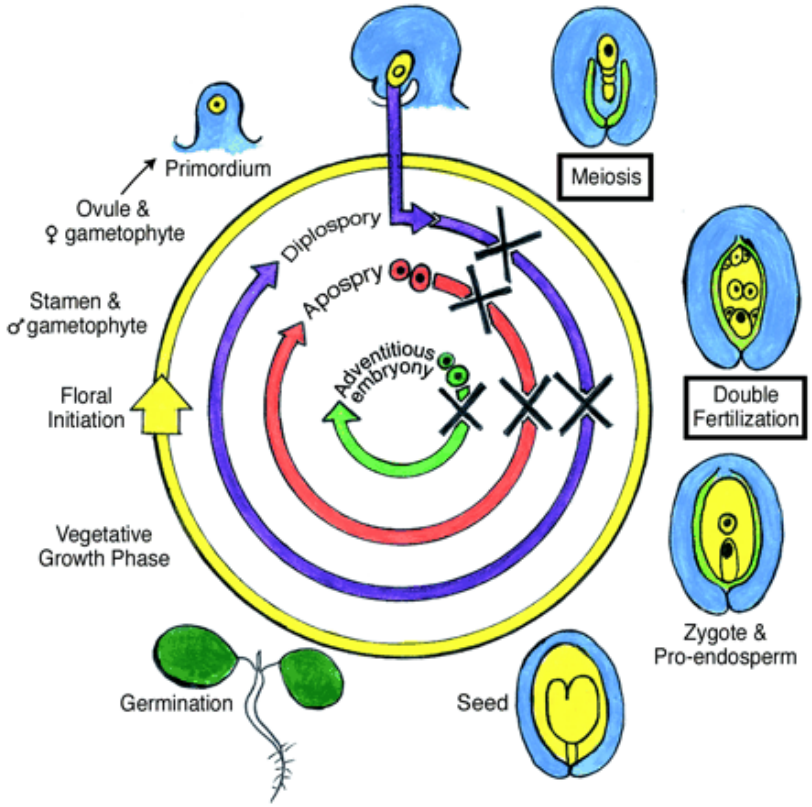

Figure 7. Initiation and Progression of Apomixis (according to Bicknell and Koltunow, 2004). The normally dominant vegetative phase of the life cycle is curtailed in this figure to emphasize the events of gametophyte formation; particularly the events in the ovule leading to sexually derived seeds (yellow). Diplospory (purple) and apospory (red) are termed gametophytic mechanisms because they initiate from a cell in the position of the MMC or from other ovule cells, respectively, that bypasses the events of meiosis and divides to mitotically to form an unreduced embryo sac. Adventitious embryony (green) is termed sporophytic apomixis because embryos form directly from nucellar or integument cells adjacent to a reduced embryo sac.

Embryogenesis occurs autonomously in both diplosporous and aposporous embryo sacs, and endosperm formation might be autonomous or might, more commonly, require fertilization (Grimanelli *et al*., 2001). In *Pennisetum* spp. and *Hieracium* spp. initiation of apospory leads to the abortion of the concurrent sexual process, whilst in *Brachiaria* spp. both sexual and aposporous embryo sacs coexist (Koltunow and Grossniklaus, 2003).

When the cell initiating apomixis directly develops into an embryo, this process is called adventitious embryony. In sporophytic apomixis,

an embryo forms directly from a nucellar or integumentary cell in the ovule. The embryo survival depends on successful fertilization of the adjacent meiotically derived embryo sac and on the ability of the adventitious embryo to grow sufficiently to gain access to the nutrient endosperm.

6. Apomixis and its potential in agriculture

The existence of apomixis in 40 plant families and the diversity of apomictic processes suggests that the routes that led to the evolution of apomixes may be as diverse, this hypothesis is also confirmed by the several cytological apomictic mechanism o far described.

Despite the occurrence of apomixis in over 400 species of angiosperms, it is found in only a few species of agricultural importance: several forages, apple, mango, and orchids (Wakana and Uemoto, 1987; Bashaw and Hanna, 1990; Naumova, 1992). For other major crops such as rice, wheat and barley, there are no apomictic wild relatives, which can be crossed with these important sexual crop varieties. Apomixis is an attractive trait for the enhancement of crop species because it mediates the formation of large genetically uniform populations; therefore this reproductive mechanism can perpetuate hybrid vigour through successive seed generations (Figure 7). Many agronomic advantages of apomixis can be envisioned: the rapid generation and multiplication of superior forms using germplasm collections; the reduction in cost and time of breeding and the avoidance of complications associated with sexual reproduction, such as pollinators and cross compatibility. Extensive efforts have been undertaken to introduce apomixis via back-crossing (Ozias-Akins and van Dijk, 2007).

For academic research groups, apomixis offers an ideal opportunity to study an efficient natural cloning system, and investigate a wide range of biological questions, from the molecular basis of genomic imprinting to the evolutionary role of sex. For farmers in the developed world, the greatest benefit is expected to be the economic production of new, advanced, high-yielding varieties for use in mechanized agricultural systems (Figure 7).

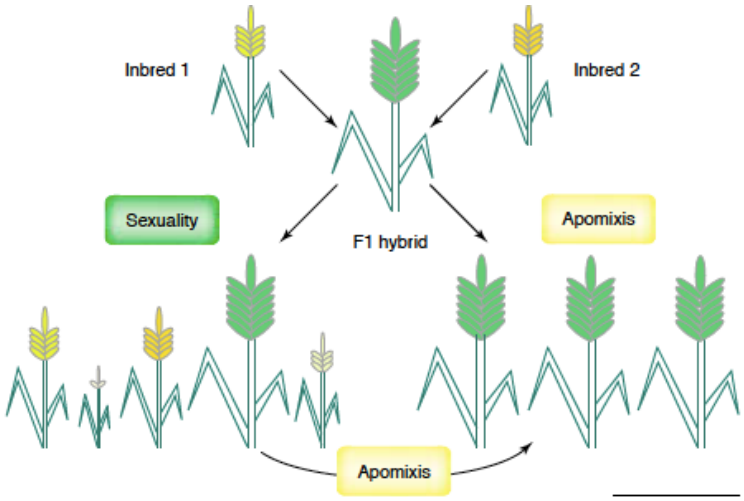

Figure 7. Apomixis is potentially a valuable means of crop improvement, one application being hybrid seed production (according to Grimanelli *et al.*, 2001).

However, apomixis is very poorly represented among crop species. The main exceptions to this appear to be tropical and subtropical fruit trees such as mango and *Citrus*, and tropical forage grasses such as *Panicum*, *Brachiaria* and *Pennisetum* (Sokolov *et al.*, 1998; Savidan, 2000, 2001).

Introgression approaches are crop-specific and have little generic applicability to developing conditional apomitic system that function across all crop species. Even if successful, it seems likely that introgression lines would provide limited flexibility in terms of pratical

capacity to manipulate apomixes in agricultural breeding systems. An alternative approach with more generic applicability and flexibility will be the engineering of conditional apomixes through biotechnology.

Current breeding efforts with apomictic crop species, such as the forage grasses *Brachiaria* (Figure 8) and *Panicum*, are complicated by the need to use complex breeding strategies to accommodate the inaccessibility of the female gamete to generate hybrid progeny (Valle and Miles, 2001).

Figure 8. Cultivated fields of *Brachiaria brizantha*

7. Genetic analysis of apomixis: few genes or many?

Defining the nature and genetic control of apomixis may be crucial for both understanding the trait itself and better illustrating the meaning of sexuality.

Apomixis may be variously influenced by environmental factors but it is generally accepted that apomictic behaviour is under strong genetic control (Albertini *et al*., 2005). A theory reveals that a delicate gene balance of recessive genes can regulate genetic control of apomixes and this might be disturbed after crosses. Today few dominant or codominant genes are responsible of the basic regulation, which allow a somatic nucellar cell to form an embryo sac without meiosis and an embryo to develop from an egg cell without fertilization (Albertini *et al*., 2005; Asker and Jerling, 1992; Koltunow *et al*., 1995).

The genes that control embryo cell formation, structure and embryo pattern formation are probably the same as those required for sexual embryo development even if the product of apomictic genes are proteins not produced in sexually reproducing plants or proteins that normally function to initiate events in sexual reproduction, but with altered activity.

It has been shown that specific genes are activated, modulated or silenced in the primary steps of plant reproduction to ensure that the embryo sacs develop from meiotic spores and/or apomitic cells (Rodrigues *et al*., 2008).

The genetic control of each apomixis component may be complex and involve more than one gene. Genetic analysis conducted in tetraploid *Panicum maximum*, have shown that apo-meiosis is inherited as a single dominant mendelian trait (Grimanelli *et al*., 2001). In the same experiments, apomeiosis and parthenogenesis were shown to co-

segregate strictly, suggesting that these two components rely on the same genetic control, or that parthenogenesis is a pleiotropic consequence of apomeiosis (Grimanelli *et al*., 2001).

Important model for studying apomixis is *Arabidopsis*. In *Arabidopsis*, analysis of meiotic mutations resembling the apomitic process has led to the isolation of a number of genes involved in early male and female sporogenesis (*NOZZLE, SPINDLY*), spontaneous induction of embryo production when overexpressed (*LEAFY COTYLEDON1*, *LEAFY COTYLEDON*2) or repressed.

Recently, careful staging of ovary development has led to the identification of differentially expressed transcripts in *Poa pratensis* (Albertini *et al*., 2004, 2005; Marconi *et al*., 2013). For seed production, endosperm formation is mandatory both in sexual and apomictic reproduction species. While endosperm development in sexual plants requires fertilization, it may proceed autonomously or require fertilization in apomicts (Eckardt, 2003).

Moreover, as it will be better describe later, in *Arabidopsis* there are proteins able to repress endosperm development in sexual plant in the absence of fertilization (FERTILIZATION-INDIPENDENT SEED1, FIS1, or MEDEA, FIS2 and FIS3 or FERTILIZATION INDIPENDENT ENDOSPERM, FIE), disruption of the corresponding genes will allow partial endosperm development in the absence of fertilization (Ohad *et al*., 1996; Luo *et al*., 2000).

Analysis of genes differentially expressed in apomictically and sexually reproducing genotypes should reveal differences in gene expression patterns. Comparative gene expression studies have been carried out during the early stages of apomictic and sexual embryo sac development. On the basis of available information apomixis do not result from the failure of a single reproductive pathway gene but rather

is caused by the silencing of the normal sexual reproductive pathway by a set of genes that act as a unit in polyploidy plants (Albertini *et al*., 2005).

8. Relationships between apomixes and sexual reproduction

In the past, apomixis and sexual reproduction were viewed as two distinct processes that have little in common. The pioneering studies of Nogler and Savidan (1982, 1984, 2000) proved that apomixis is under a tight genetic control, although genetic or environmental conditions may affect its penetrance.

Plants that reproduce by apomixis also retain the capacity to reproduce sexually to varying degrees. Sexual and apomictic reproductions appear independent, but they are not mutually exclusive. For example, in some apomictic, such as *Hieracium*, the sexual process ceases if apomixis initiates in the ovule, whereas in others, both processes occur side by side in a competitive manner (Tucker *et al*., 2003) (Figure 9).

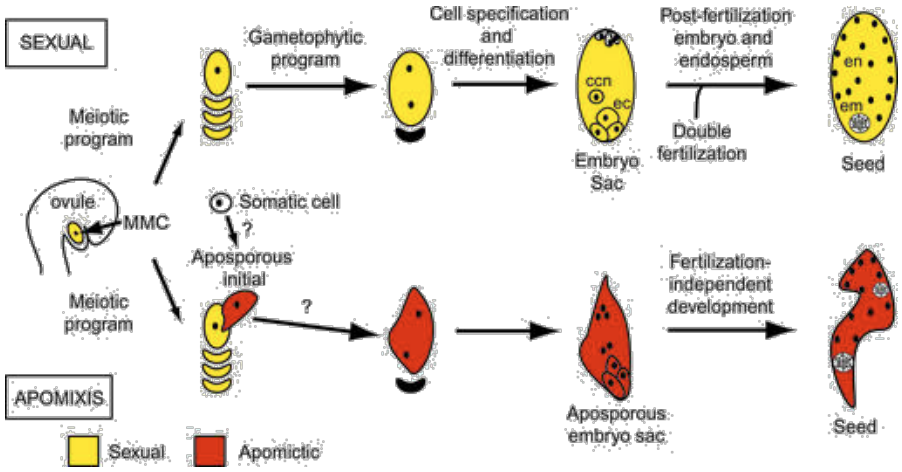

Figure 9. Mechanisms of Sexual and Apomictic Reproduction in *Hieracium*. Ccn, central cell nucleus; ec, egg cell; em, embryo; en, endosperm (Tucker *et al*., 2003).

The coexistence of apomixis and sexual reproduction in an apomictic plant and the polyploidy nature of most apomicts complicate genetic analyses. There is a lack of data concerning the identity of cells that initiate apomixis, the molecular process that regulate it and the relationship between sexual and apomictic pathways (Tucker *et al*., 2003).

Some genes have been identified however comparative analyses in sexual and apomictic plants and their functional relevance to apomixis have not yet been determined (Tucker *et al*., 2003; Okada *et al*., 2013).

Genetic analyses of sexual plants have shown that the events leading to female gametophyte formation and seed development are both independent of and interdependent of the events and signals from surrounding sporophytic ovule tissues. Regarding apomixes, the ovule sporophytic signals governing the apomictic process are still elusive.

9. Apomictic model system

Very little information regarding apomictic genes and the molecular networks driving this peculiar reproductive mechanism are available.

To dissect this mechanism "special" model plant species have been identified. These plant species are relevant to agriculture systems and possess suitable features to explore apomixes and its molecular regulation (Koltunow *et al*., 1995).

In these plant species, apomixis is investigated at developmental, cell biological and molecular levels in comparison with sexual reproduction in a purely sexual sibling.

To facilitate a molecular study it is important that the model apomictic plant can be genetically transformed, permitting the introduction of marker genes and mutagenic sequences. It would be preferable for the model plant to have a small genome and ideally be already characterized.

Several model systems for apomixis have been previously proposed (Figure 10).

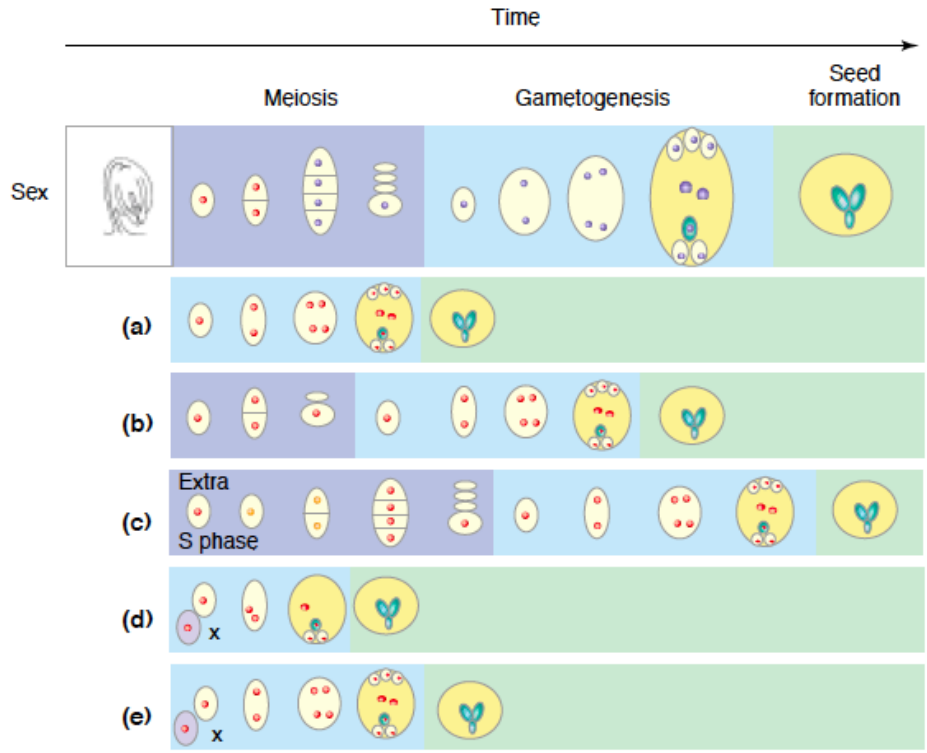

Figure 10. Many variations have been observed in both diplosporous and aposporous pathways (according to Grimanelli et al., 2001). In the *Antennaria* type of diplospory (a), the unreduced spore is formed without undergoing meiosis, whereas in th *Taraxacum* type, it results from the restitution of the nucleus at meiosis I (b). In the *Allium* type, meiosis is normal but preceded by an extra round of DNA replication before meiosis I (c). In the *Panicum* type, the megagametophyte is mature after only two mitoses and hence contains only four nuclei (d). In the *Hieracium* type, three mitoses occur, and the embryo sac contains eight nuclei, closely resembling the sexual one (e).

***Poa pratensis*.** Or Kentucky bluegrass (Figure 11) is a hardly, persistent, attractive forage and turf grass adapted to a wide range of soils and climate. Its mode of reproduction is extremely versatile and ranges from naturally obligate apomixis to complete sexuality. In *P. pratensis*, apospory involves the development of embryo sacs from somatic cells that differentiate into the nucellus. If unreduced polar nuclei positioned centrally within the embryo sac fuse with a sperm cell released from the pollen tube (pseudogamy), the unreduced egg can develop autonomously through parthenogenesis developing viable apomictic seeds (Albertini *et al*., 2004).

Because different plants may have contrasting modes of reproduction, *P. pratensis* is one the model species for investigating apomixis and its inheritance (Albertini *et al*., 2001). Sexually, it reproduces through out-crossing or selfing, whereas, apomictically, it is a pseudogamous aposporic parthenogenetic species.

Figure 11. Kentucky blue grass (*Poa pratensis* l.).

In *P. pratensis*, Albertini *et al*. (2004, 2005) isolated as many as 179 cDNAs differentially expressed between apomictic and sexual genotypes. Importantly, most of the transcripts were not specifically associated with apomictic or sexual genotypes. Indeed their expression is differentially modulated or quantitatively different; supporting the hypothesis that apomixis may result from a deregulated sexual pathway. In particular, *SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE* (*SERK*) and *APOSTART* (*APO*) were characterized in detail. All the data collected suggest that these gen products are involved in cell-to-cell signaling and hormone trafficking (Albertini *et al*., 2005).

APOSTART, in particular, is associated with apomixis and its transcript is detectable specifically in aposporic initials and embryo sacs. Functional characterization of the *Arabidopsis APOSTART1* gene

(*AtAPO1*) showed that it is expressed in mature embryo sacs and developing embryos (Albertini *et al*., 2004, 2005).

Brachiaria brizantha. Many genera of the *Poaceae* are economically important forage grasses and produce seeds through apomixis, an asexual mode of reproduction. An example is the genus *Brachiaria*, a native of Africa that is widely cultivated in South America (Figure 8), especially in Brazil (Duarte Silveira *et al*., 2008). Pseudogamy was detected in this species by observation that seed set is suppressed in plants that have had the stigmas excises from the flower.

In *Brachiaria*, megagametogenesis may follow two pathways, according to Valle and Savidan (1996). The first is sexual, where regular meiosis of the megaspore mother cell results in a tetrad of reduced cells.

One of these (chalazal surviving megaspore) undergoes three mitoses, resulting in a *Polygonum*-type reduced embryo sac (Singh *et al*., 2011). The second pathway is asexual, where the aposporic embryo sac develop from enlarged, unreduced nucellar cells after all four megaspores degenerate. Nucellar cells undergo two mitoses, producing four-nucleate (one egg cell, two synergids and one polar nuclei) *Panicum*-type embryo sacs (Koltunow, 1993; Kojima and Nagato, 1992).

Arabidopsis thaliana. The Model Plant (Arabidopsis genome 2000) has proposed as a versatile and convenient tool for to screen and to identify apomictic mutants. *Arabidopsis thaliana* is small in size (35-40cm) and has a rapid life cycle (5-6 weeks from seed to seed), which means that many plants can be screened after mutagenesis. *Arabidopsis* genome has been fully sequenced (2000), moreover the availability of several insertional populations greatly facilitate forward and reverse genetic studies (Koltunow, 1995).

Mutagenesis and screening for apomixis in the model sexual plant *A. thaliana* has been conducted in a more directed way. The *Arabidopsis* mutant *sporocyteless/nozzle* (*spl*) in unable to develop a functional MMC and shows defects in nucellar cell identity. Moreover, mutations in the *Arabidopsis* gene *WUSCHEL* (*WUS*), a regulator of stem cell identity in the shoot apical meristem, also result in defects in MMC specification (Groß-Hardt *et al*., 2002). Few *Arabidopsis* mutants have revealed that gametogenesis can be uncoupled from meiosis. For example, loss of certain *ARGONAUTE* genes and other genes in the small RNA pathway resulted in loss of restriction in gametic cell identity and fate in the ovule (Olmedo-Monfil *et al*. 2010, Barcaccia and Albertini, 2013).

Parallel mutant screens for apomixis enabled the identification of genes controlling the fertilization-independent initiation of seed development in *Arabidopsis*. These genes, called *FIS* genes, encode protein members of the Polycomb-related complex (Barcaccia and Albertini, 2013; Luo *et al*., 1999). The *fis* mutants are known to initiate endosperm development without fertilization (Kohler *et al*., 2003).

Nowadays, it is well known that the female gametophyte controls embryo and/or endosperm development at two different levels: (a) repression of embryo/endosperm development in the absence of fertilization through imprinting and (b) expression of factors that are required after fertilization. *FIE*, *MEDEA* (*MEA*) and *FIS2* repress endosperm development in the absence of fertilization. All *fis* mutations show aberrant embryo and endosperm development when fertilized but exhibit autonomous endosperm development if unfertilized (Chaudhury *et al*., 1998; Chaudhury *et al*., 2001; Drews and Yadegari, 2002; Kohler *et al*., 2003).

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AIM OF THE PROJECT

In angiosperms, the gametophytes are composed of few haploid cells that develop within the diploid sporophytic tissues of the flower sexual organs. The female gametophyte contains the egg cell and the central cell (the two female gametic cells), which upon fertilization give rise to the embryo and the endosperm respectively.

The establishment and maintenance of the four different cell types of the female gametophyte of higher plants as well as the pollen tube attraction and reception, the sperm cell release and the fusion of the gametes are essential for the double fertilization (Kägi and Gross-Hardt, 2010). During the fertilization process, the female gametophyte participates in directing first the pollen tube to the ovule and then the sperm cells to the egg and central cells. Mutants defective in almost all stages of female gametophyte development have been identified in *Arabidopsis thaliana*, allowing the functional analysis of genes required for this process (Drews and Yadegari, 2002).

Double fertilization begins when the pollen tube grows into one of the two synergid cells of the embryo sac, after penetrating the synergid, the apex of the pollen tube breaks open, releasing the two sperm nuclei and other contents into the synergid (Eckardt, 2007). As the synergid degenerates, it envelops the egg and endosperm cells, holding the two sperm nuclei close and the other expelled contents of the pollen tube. The egg cell then opens and engulfs the sperm cell, whose membrane breaks apart and allows the nucleus to move near the egg nucleus (Leshem *et al*., 2013). The nuclear envelopes then disintegrate, and the two nuclei combine to form the single diploid nucleus of the zygote. The other sperm cell fuses with the diploid central cell, forming a single triploid cell, the primary endosperm cell, which divides mitotically into the endosperm tissue.

The seed is the mature, fertilized ovule. The maternally derived

diploid cells of the ovule develop into the hard, water-resistant outer covering of the seed, called testa, or seed coat.

The diploid zygote develops into the embryo, and the triploid endosperm cells multiply and provide nutrition.

The endosperm may be consumed by the embryo, as in many legumes and in the model plant *Arabidopsis*, which use the cotyledons as a food source during germination. In other species the endosperm persists until germination, when it is used as a nutrient source.

My thesis has been finalized to uncover the genetic networks controlling female gametophyte formation and development; this topic has important applicative interests since ovule and female gametophyte are the maternal precursor of seeds and embryos (the new generation). In agriculture, seed is the material used for planting or regeneration purpose.

For most of human history, seeds have been regarded as sacred: seeds provide the all-important link from last year's harvest to this year's crop, on which our life has depended since the neolithic revolution. The understanding of seeds and their management that made agriculture possible generates the economic basis for modern human societies.

Seed is one of the key factors of crop productivity. Therefore, a comprehension of the mechanisms underlying seed formation in cultivated plants is crucial for the quantitative and qualitative progress of agricultural production.

Many plants are able to produce seeds asexually. In this process, known as apomixis, female gametes develop without meiosis (or with abnormal meiosis) and embryos develop without fertilization. Apomixis occurs in many wild species and in a few agronomically important species such as citrus and mango, but not in any of the major cereal

crops. Because it offers the promise of the fixation and indefinite propagation of a desired genotype, there is a great deal of interest in engineering this ability to produce clonal seeds into crops, especially cereals (Spillane *et al*., 2001).

Although it is a complex process, apomixis often is inherited as a simple Mendelian trait, which may suggest that it is controlled by relatively few "master" regulatory genes. It is thought that apomixis may have evolved (probably multiple times) through modifications of the normal sexual reproduction pathway, rather than constituting a novel pathway distinct from sexual reproduction (Grimanelli *et al*., 2001; Koltunow and Grossniklaus, 2003).

One of the aims of my PhD was to characterize genes involved in apomixis in apomictic plants that can be genetically transformed, permitting the introduction of marker genes and mutagenize sequences. For this analysis I took advantage of the model system *Arabidopsis thaliana*.

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PART II

- submitted -

Arabidopsis thaliana SUF4 (SUPPRESSOR OF FRIGIDA 4) CONTROLS EGG CELL 1 EXPRESSION.

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Arabidopsis thaliana SUF4 (SUPPRESSOR OF FRIGIDA 4) CONTROLS *EGG CELL 1* EXPRESSION.

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Key words: yeast one-hybrid, egg cell, *EC1* genes, SUF4, transcription factors, MOM1

Summary

The *EC1* gene family of *Arabidopsis thaliana* comprises five members that are specifically expressed in the egg cell and act redundantly during double fertilization, where they were found to be essential for the gamete-fusion events.

To dissect the molecular network controlling *EC1* gene expression, we have used the *EC1.1* promoter as bait in yeast one-hybrid screenings. As a result we isolated SUF4 (SUPPRESSOR OF FRIGIDA 4), a C_2H_2 transcription factor known to be involved in flowering time regulation. Here we show that SUF4 binds to all five *EC1* promoters *in vitro* and that it is able to regulate *EC1.1* and *EC1.2* expression *in vivo*. Moreover, all five *EC1* genes are down regulated in *suf4-1* ovules as shown by quantitative RT-PCR. To identify more gene products able to regulate *EC1* expression together with SUF4 we performed co-expression studies that led to the identification of *MOM1 (Morpheus Molecule 1)*, a component of a silencing mechanism independent of DNA methylation marks. In *mom1* ovules both *SUF4* and *EC1* genes are significantly down regulated, while *pEC1.1::GUS* shows ectopic expression in young carpels and placentas, suggesting that MOM1 contributes to the regulation of *SUF4* and *EC1* gene expression.

Introduction

The female gametophyte (embryo sac) of flowering plants originates from a specialised cell, the megaspore mother cell (mmc). During meiosis four haploid megaspores are formed (Wilson and Yang, 2004; Drews and Koltunow, 2011), three of which degenerate whilst the chalazal megaspore undergoes three mitotic divisions and gives rise to the embryo sac. 80% of angiosperms (including the model plant *Arabidopsis thaliana*) produce a Polygonum-type female gametophyte, a seven-celled/eight-nucleate embryo sac (Yadegari and Drews, 2004). The development of the female gametophyte of *Arabidopsis thaliana* is a morphologically well-described multistep process (from FG1 to FG7) also known as megagametogenesis (Christensen *et al*., 1997). The mature embryo sac is formed by three antipodal cells at the chalazal pole, two medial polar nuclei and one egg cell surrounded by two synergids (Mansfield *et al*., 1991). In *Arabidopsis*, the two polar nuclei are fused and form the diploid nucleus of the central cell.

The embryo sac is an interesting system for studying cell differentiation due its low complexity (Sprunck and Gross-Hardt, 2011). The molecular mechanisms regulating the establishment of cell identity in developing embryo sacs are largely unknown although several embryo sac defective mutants have been isolated and described (Christensen *et al*., 1997; Pagnussat *et al*., 2005; Pagnussat *et al*., 2007; Gross-Hardt *et al*., 2007; Matias-Hernandez *et al*., 2010; Masiero *et al*., 2011). The *Arabidopsis* cell-cycle mutant *rbr1* (*retinoblastoma-related 1*) and the maize mutant *ig1* (*indeterminate gametophyte1*) are characterised by female gametophytes containing extra nuclei. Notably, the extra synergid, egg and central cells present develop according to their position within the female

gametophyte (Ebel *et al.*, 2004; Guo *et al.*, 2004), indicating that positional information is involved in the cell specification process.

Many other mutants such as *lachesis (lis), gametophytic factor1 (gfa1), clotho (clo), atropos (ato)* and *eostre* show changes in female gametophyte cell identities. In *lis, clo* and *ato* the synergid cells and the central cell express egg cell markers, while the antipodal cells express central cell markers. LIS, CLO and ATO share high levels of similarity with human and yeast splicing factors (Gross-Hardt *et al.*, 2007; Völz *et al.*, 2012). The *eostre* mutant is caused by misexpression of *BLH1*, a BEL1-like homeodomain transcription factor. This results in defects in nuclear migration, and synergid cells are converted into egg cells (Pagnussat *et al.*, 2007).

A number of molecular approaches have also been employed to investigate the mechanisms controlling embryo sac cell differentiation, such as differential gene expression analyses between wild-type and female gametophyte defective mutants (Yu *et al.*, 2005; Johnston *et al.*, 2007; Jones-Rhoades *et al.*, 2007; Steffen *et al.*, 2007), microarray expression analysis of *Arabidopsis* laser-dissected female gametophytic cells (Wuest *et al.*, 2010), or exhaustive sequencing of expressed sequence tags (EST) from the cDNAs of isolated female gametophytic cells (Cordts *et al.*, 2001; Kumlehn *et al.*, 2001; Le *et al.*, 2005; Sprunck *et al.*, 2005; Yang *et al.*, 2006; Koszegi *et al.*, 2011) Isolation of wheat egg cells and subsequent EST analyses resulted in the identification of the large, egg cell-specific transcript cluster *TaEC-1* (Sprunck *et al.*, 2005) that shares sequence similarity to the barley *Early Culture Abundant (ECA1*) transcripts present in early androgenetic microspore cultures (Vrinten *et al.*, 1999). *TaEC-1* transcripts encode small proteins having six conserved cysteine residues and a putative secretion signal sequence. Five *EC-1* related genes are present in the genome of *Arabidopsis thaliana,*

namely *EC1.1*, *EC1.2*, *EC1.3*, *EC1.4* and *EC1.*5, and they are exclusively expressed in egg cells. Simultaneous silencing of all five *AtEC1* genes results in defective double fertilisation, suggesting that the secreted EC1 proteins are necessary for the two gamete fusion events (Sprunck et al., 2012).

We have been able to shed light on *EC1* gene regulation. Using the yeast one-hybrid approach we have identified putative *EC1.1* transcriptional regulators, among them the C_2H_2 zinc finger transcription factor SUPPRESSOR OF FRIGIDA4 (SUF4; Kim and Michaels, 2006; Kim *et al*., 2006). Sequence prediction reveals the presence of a nuclear localisation signal in the C-terminus and a proline-rich domain most probably involved in protein/protein interactions. *In vivo* and *in vitro* evidence indicate that SUF4 is able to regulate all five *AtEC1* genes. Furthermore, *suf4* mutants show a mild *ec1* phenotype. Bioinformatic approaches have revealed that *SUF4* is co-expressed with *MOM1* (*Morpheus' Molecule 1*, Amedeo *et al*., 2000). In the current work we show that *SUF4* is down-regulated in *mom1* mutant pistils and that the five *EC1* genes are partially down-regulated, while GUS under the control of the *EC1.1* promoter is ectopically expressed at stage 11 in the carpel leaves of *mom1-3* plants. Taken together our data suggest that MOM1 participates in restricting *EC1.1* expression in the female gamete cells, while SUF4 promotes *EC1* transcription in the egg cell.

Results

SUF4 positively regulates *EC1.1*

A 463 bp promoter region of the *EC1.1* gene has been shown to be responsible for the specificity of expression of this gene in the *Arabidopsis* egg cell (Ingouff *et al.*, 2009; Sprunck *et al.*, 2012). To dissect the molecular network controlling egg cell fate, we employed the *EC1.1* promoter as bait in yeast one-hybrid screenings to isolate interacting transcription factors. The 463 bp *EC1.1* regulatory region was divided in two fragments, which were integrated into the MATα yeast strain Y187, and this was subsequently mated with yeast strain AH109 previously transformed with a normalized total plant *Arabidopsis* cDNA library (Cuaiser, 2004; Costa *et al.*, 2013; H. Sommer and S. Masiero, unpublished). 7 to 7.5 million diploid clones were analysed in each single screening. 31 positive clones matched a total of nine different proteins (see supplementary Table S1), and all these clones were able to grow on media lacking histidine and leucine and supplemented with 20 mM 3-AT (3-Amino-1, 2, 4-triazole, a HIS3 competitive inhibitor). Notably, one of the transcription factors identified was the C_2H_2 zinc finger protein SUF4 (Figure 1 a, b; Kim and Michaels, 2006). SUF4 is able to bind the most proximal fragment of the *EC1.1* promoter (from -245 bp to -1 bp before the ATG; Figure 1c) when used as bait. To confirm this protein-DNA interaction, full length *SUF4* cDNA was cloned into pGADT7 and used to re-transform the yeast strain containing the proximal region of the *EC1.1* promoter. *HIS3* reporter gene activation confirmed that SUF4 is able to bind to the *EC1.1* promoter in yeast (Figure 1a, b).

SUF4 is expressed in developing female gametophytes

The expression pattern of *SUF4* has already been investigated by RT-PCR analyses and by *pSUF4::SUF4-GUS* fusion (Kim and Michaels, 2006). The chimeric SUF4-GUS protein has been detected in roots, developing ovules and seeds. We therefore used the *pSUF4::SUF-GUS* line previously described (Kim and Michaels, 2006) to investigate in detail the presence of SUF4-GUS during embryo sac formation. GUS activity driven by the genomic *pSUF4::SUF4* locus was not detected in ovule primordia, or the diploid megaspore mother cell, nor during meiosis (Figure 2a, b). However, SUF4-GUS has been detected in developing female gametophytes immediately after meiosis (Figure 2c). SUF4-GUS is present in the nucleus of the functional megaspore and GUS activity persists during megagametogenesis (Figure 2d-g). In the seven-celled embryo sac (FG6, ovules at stage 3-V; developmental stages according to Schneitz *et al*., 1995), SUF4-GUS is detectable in all the eight nuclei, including the two polar nuclei of the central cell and the egg cell nucleus (Figure 2f, in the insert a closer view of the two synergid and the egg cells). At late stage 3-VI (FG7) SUF4-GUS is no longer detected in the egg cell nucleus and GUS activity also disappears from the antipodal cell nuclei (Figure 2g).

SUF4 regulates *EC1* gene expression

To confirm that SUF4 is able to control *EC1.1* expression, transgenic plants homozygous for *pEC1.1::GUS* (Figure 1d) were crossed with homozygous *suf4-1 suf4-1* plants. This line contains a previously introgressed functional *FRIGIDA (FRI)* allele (Micheals *et al*., 2004). We checked the parental *pEC1.1::GUS* marker line in which we analysed 455

ovules from 9 pistils collected 24 hours after emasculation and GUS activity was detected in 443 (97.36%) of them (Figure 1d). All the F1 plants showed a late flowering phenotype due to FRI. The F1 progeny plants were used to perform GUS assays on mature pistils collected 24 hours after emasculation. The ratios expected for marker gene expression in the female gametes of heterozygous plants would be 50% (Yadegari and Drews, 2004). However, if SUF4 were to be a positive regulator of *EC1.1* we would expect a reduction of GUS activity in egg cells from 50% to 25%. We analysed a total of 1392 ovules and detected enzyme activity in only 356 egg cells (25.6%; Table 1), no activity being detected in the remainder (Figure 1c). To verify whether SUF4 controls the expression of other members of the *AtEC1* family we also crossed *suf4-1 suf4-1* with a plant homozygous for the *pEC1.2::GUS* insertion. Like *pEC1*.1 the p*EC1.2* promoter, is able to drive egg cell-specific reporter gene expression (Sprunck *et al*., 2012). In the F1 developing carpels 301 female gametophytes (24.8%) were GUS positive out of the 1210 analysed (Table 1).

We also analysed GUS activity in the F2 segregating population of *SUF4 suf4-1/pEC1.1::GUS*. Five plants were selected that were homozygous for *suf4-1* and positive for *pEC1.1::GUS*. About 300 ovules were examined in each plant and none showed GUS activity. Coherently, plants genotyped as *SUF4 SUF4/pEC1.1::GUS* showed GUS activity.

To infer the genotypes of the F2 lines analysed, 60 offspring seeds were sown for each line and selected by BASTA spraying. All the seedlings of two lines survived BASTA application, clearly indicating that the progenitor plants were homozygous for the *pEC1.1::GUS* insertion. In the hypothetical case that SUF4 does not participate in *EC1* activation, we should have detected GUS activity in around 97% of the egg cells, and that was not the case. Moreover, three offspring lines were found to

be sensitive to BASTA selection, mortality being 16 seedlings out 58, 19 out of 61 and 22 out of 61. These segregation data indicate that the *pEC1.1::GUS* insertion was hemizygous in these three lines.
In four *suf4-1 suf4-1/pEC1.2::GUS* plants GUS activity was not observed in mature embryo sacs (in total about 700 ovules were analysed). A very weak and diffuse GUS signal was only noted when the assay reaction was left for more than 12 hours (data not shown).
Taken together our *in vivo* segregation data indicate that both the *EC1.1* and *EC1.2* promoters are inactive in *suf4-1* egg cells, suggesting that SUF4 promotes *EC1.1* and *EC1.2* transcription.
To confirm the role of SUF4 with respect to *EC1* regulation, real time PCR analyses were also performed with cDNAs generated from *suf4-1 suf4-1* and wild type (*SUF4 SUF4*) carpels using primers specific for *EC1.1*, *EC1.2*, *EC1.3*, *EC1.4* and *EC1.5*. The real time experiments showed all five *EC1* genes to be significantly down-regulated in *suf4-1* mutant carpels, therefore SUF4 positively regulates the expression of all five *EC1* genes (Figure 1f).

SUF4 binds the *EC1* promoters *in vitro*

Recombinant SUF4, expressed in *E. coli* either as 6xHIS-SUF4-STREPII or as a 6xHIS-MBP-SUF4 fusion, was purified and used for *in vitro* DNA binding assays. We performed electrophoretic mobility shift assays (EMSAs) to confirm the SUF4-*EC1.1* promoter interaction and investigate the binding of SUF4 to the promoter fragments of all five members of the *EC1* gene family. A 108 bp radiolabelled *EC1.1* promoter fragment resembling part of the promoter proximal fragment that was used in the yeast one-hybrid screening showed significant binding to increasing amounts of purified 6xHIS-SUF4-STREPII (Figure 3a).

Competition experiments with non-radiolabelled probe verified that SUF4 binds specifically to the *EC1.1* promoter fragment (Figure 3b).
Using 6xHIS-MBP-SUF4, and comparing it to the control fusion protein 6xHIS-MBP, we showed that MBP-tagged SUF4 is able to specifically bind the radiolabelled fragments of all five *EC1* promoters (Figure 3c). This confirms our RT-PCR data of *EC1* down-regulation observed in *suf4-1* mutants (Figure 1f). The gel retardation assays, together with the yeast data, clearly prove that SUF4 is able to bind to *EC1* promoters, while the quantitative RT-PCR data of reduced *EC1* expression and the loss of *EC1.1* and *EC1.2* promoter activities in the *suf4-1* mutant suggest that this interaction is necessary to promote *EC1.1* transcription in egg cells.

suf4-1 shows a weak *ec1* phenotype

Our data reveal that SUF4 positively regulates the expression of all five *EC1* genes. It has been shown that simultaneous down-regulation of *EC1.2* and *EC1.3* by RNA interference in the homozygous triple mutant *ec1.1*/*ec1.4*/*ec1.5* (termed *ec1-RNAi*; Sprunck *et al*., 2012) severely affects double fertilisation since the sperm cells delivered into mutant ovules do not fuse either with the mutated egg cell or with the central cell. The failure of gamete fusion results in polytubey, multiple sperm delivery, and a significantly reduced seed set (Sprunck *et al*., 2012). We therefore investigated the seed set in siliques of homozygous *suf4-1* plants but did not observe any obvious number of undeveloped seeds (Figure S1a), which is in agreement with the fact that the five *EC1* genes are only partially down-regulated in the *suf4-1* mutant (Figure 1f). In order to explain the residual *EC1* messengers observed in *suf4-1* pistils, we checked *SUF4* expression in homozygous *suf4-1* plants. We could show

that the T-DNA inserted in *suf4-1* does not provoke a complete knockout but only a knock-down as revealed by both RT-PCR and real time RT-PCR performed with primers located upstream and downstream of the T-DNA inserted in *SUF4* (Figure S1b, c).

To investigate sperm cell behaviour during double fertilisation in *suf4-1* plants, we emasculated the pistils of both wild type and homozygous *suf4-1* plants and pollinated them with the sperm cell marker line HTR10-mRFP1 (Ingouff *et al.*, 2007) in which sperm nuclei emit a strong red fluorescence. Using this marker line successful plasmogamy and on-going karyogamy of male and female gametes are recognizable by the spatial separation of the sperm nuclei and the decondensation of sperm chromatin, respectively. Notably, at 18 to 20 hours after pollination (HAP) we detected a significant portion of *suf4-1* ovules (23%; n = 232 ovules) exhibiting either non-fused sperm cells (Figure 4a), sperm cells delayed in fusion (Figure 4b), or multiple sperm cell delivery despite successful gamete fusion (Figure 4c). None of these categories were observed in the wild type ovules (Figure 4d). The observed category of fertilised *suf4-1* ovules containing a second unfused sperm pair indicates that the gamete fusion event was delayed and therefore polytubey was not prevented like in wild type ovules. It is known that a second pollen tube is attracted and able to deliver another pair of sperm cells into the same embryo sac when gamete fusion fails (Sprunck *et al.*, 2012; Kasahara *et al.*, 2012; Beale *et al.*, 2012). Delayed gamete fusion events are also supported by the observation that 3% of *suf4-1* ovules analysed at 18-20 HAP displayed sperm nuclei during gamete fusion or karyogamy. By contrast, all wild type ovules were successfully fertilised by that time (Figure 4d), which is in agreement with observations that gamete fusion takes place at 6-9 HAP in the wild type (Faure *et al.*, 2002; Ingouff *et al.*, 2007; Sprunck *et al.*, 2012).

Taken together, the observed delayed gamete fusion phenotype in *suf4-1* ovules and the lack of undeveloped seeds in *suf4-1* siliques, suggests that the down-regulation of *SUF4* and in turn the down-regulation of *EC1* gene expression, impairs efficient double fertilisation but does not abolish it.

MOM1 participates with SUF4 in regulating the *AtEC1* genes

A coordinated behaviour of gene expression across a variety of experimental conditions prelude to a functional relationship among genes (Aoki *et al*., 2007), therefore to identify other gene products that may be involved along with SUF4 in regulating *EC1* gene expression we performed correlation analyses using around 1700 microarray measurements (as described in Menges *et al*., 2008). *MORPHEUS' MOLECULE1 (MOM1)* was one of the genes showing a strong correlation value with *SUF4*.

Real-time PCR indicated *MOM1* to be widely expressed since its transcript is detectable in siliques, leaves and inflorescences (supplementary Figure S2a). To study the expression pattern of *MOM1* in more detail during ovule development, we transformed *Arabidopsis Col-0* plants with a construct in which the bacterial ß-Glucuronidase (GUS) gene is under the control of the *MOM1* putative promoter (*pMOM1::GUS*). In transgenic *pMOM1::GUS* plants GUS activity was found in the ovule placental tissue when ovule primordia arise (Figure 5a), but no GUS signal was recorded in ovule primordia at stages 1-II and 2-1 (Figure 5a, b). GUS enzymatic activity was detected in developing ovules since stage 2-III (Figure 6c), in particular the *MOM1* promoter is active in the funiculus and in chalaza. The GUS signal also persists in mature ovules at stage 3-VI (Figure 5d).

Real-time PCR analyses showed that *SUF4* is down-regulated in *mom1* plants (supplementary Figure S2b) and, as expected, the expression of the five *EC1* genes is also down-regulated in the *mom1* mutant background (Figure 5g).

To investigate the activity of the *EC1.1* promoter in *mom1-3*, we crossed homozygous *pEC1.1::GUS* and *mom1-3* individuals. In the F2 segregating population we analysed homozygous *mom1-3* plants containing the *pEC1.1::GUS* insertion. Interestingly GUS activity was detected in the carpel leaves of *mom1-3* flowers at developmental stage 11 (Figure 5e; Smyth *et al*., 1990). The GUS reporter activity recorded in the carpel leaves was also confirmed by real time and RT-PCR analyses which proved that in *mom1-3* flowers (stage-11) *EC1.1* is transcribed, whilst in *Col-0* flowers at the same developmental stage the *EC1.1* messenger is not detected (Figure 5h). As a control we analysed *EC1.1* expression in flowers at developmental stage 12 (Smyth *et al*., 1990), and as expected *EC1.1* transcription is lower in *mom1-3* flowers compared to those of wild type.

In *mom1-3* mutant plants homozygous for the *pEC1.1::GUS* insertion, GUS enzymatic activity was detected in 68 to 73% of egg cells analysed, in total we have analysed at least three carpels per plants collected by four independent individuals. Coherently in *mom1-3 mom1-3* plants hemizygous for the *pEC1.1::GUS* insertion, GUS activity was detected in a range from 25 till 37% of the analysed egg cells. Again we analysed at least three carpels per plants. The genotypes of these plants were determined as previously described, thus evaluating the capacity of the offspring seedlings to survive BASTA selection.

Discussion

The yeast one-hybrid trap: a sensitive tool to dissect molecular networks

In this work we present attempts to shed light on embryo sac cell differentiation. Using the yeast one-hybrid technique, we aimed to identify transcription factors driving female gamete differentiation. Whilst this tool to detect protein-DNA interactions *in vivo* permits prey proteins to acquire their native configuration (Lopato *et al*., 2006) and is simple, rapid and sensitive (Reece-Hoyes *et al*., 2011), it does nevertheless suffer certain limitations such as its inability to identify those transcription factors that are unable to bind the target DNA only if not post-translationally modified or if they comprise elements of higher order complexes (Deplancke *et al*. 2006).

It is quite common to perform yeast one-hybrid screenings using multiple copies of small bait elements, such as *cis*-regulatory motifs (Tran *et al*., 2004; Lopato *et al*., 2006). However we preferred to split the *EC1.1* promoter into just two fragments since this facilitates the interaction of transcription factors with the *EC1.1* regulatory sequences even without a detailed knowledge of the key *cis*-regulatory elements. Indeed, quite large promoter fragments have already been used as baits in yeast one-hybrid screenings (Roccaro *et al*., 2005; Gaudinier *et al*., 2011). One potential difficulty with this approach is the presence of several *cis*-regulatory elements that might be bound by yeast DNA binding proteins activating the transcription of the reporter gene even without any prey GAL4AD chimeric protein. Nevertheless, we did not experience self-activation for either of the two *EC1.1* bait fragments.

To uncover the molecular mechanisms controlling cell differentiation we chose *EC1.1* since its expression pattern is very specific and it has been extensively used as a developmental marker to evaluate female gamete development (Ingouff *et al*., 2009; Matias-Hernades *et al*., 2010). *EC1.1* and all the *Arabidopsis EC1* genes are exclusively expressed in egg cells (Sprunck *et al*., 2012; Figure 1e and S3). This trait is evolutionarily conserved since all the EC1 *orthologs* identified to date display strong expression in the egg cell (Sprunck *et al*., 2005; Ohnishi *et al*. 2011).

We used the *EC1.1* promoter as bait to screen an *Arabidopsis* total plant cDNA library. To reduce the prevalence of abundant transcripts the cDNA library has been normalised, thus enriching for rare transcripts to facilitate their assessment. This normalisation was extremely important since our interest was to identify those transcription factors responsible for *EC1.1* regulation, and transcription factors are often encoded by rare transcripts (Lopato *et al*., 2006).

The yeast one-hybrid tool revealed very powerful to identify a regulator of the *Arabidopsis EC1* genes. Gel retardation assays subsequently confirmed the ability of SUF4 to bind the promoters of all five *AtEC1* genes. These data suggest that SUF4 is a master regulator of the *EC1* genes in egg cells. We can also infer that the *Arabidopsis EC1* genes most probably share a common regulatory mechanism, strengthening their functional redundancy (Sprunck *et al*, 2012).

SUF4 contributes in gamete differentiation

The interaction between SUF4 and *pEC1.1* has also been confirmed *in planta* by genetic evidence. *suf4-1* mutants have been crossed with *pEC1.1::GUS* and *pEC1.2::GUS* marker lines, the GUS activity driven by these two egg cell specific promoters *pEC1.1* and *pEC1.2* being abolished

in *suf4-1* egg cells (figure 1c). These data corroborate the conclusion that SUF4 activates *pEC1.1* and *pEC1.2*, whilst gel mobility assays and yeast one-hybrid data indicate that SUF4 binds to the regulatory regions under examination.

The expression pattern of SUF4 in embryo sacs is also consistent with the occurrence of this protein/DNA interaction. The presence of SUF4 has been determined analysing transgenic plants carrying the *pSUF4::SUF4-GUS* insert. The chimeric protein is nuclear localised (Kim and Micheals, 2006; Figure 2c-g) and it is biologically active since it is able to rescue the early flowering phenotype of *suf4-1 suf4-1 FRI FRI* plants (Kim and Michaels, 2006). SUF4 is expressed in developing female gametophytes soon after meiosis and this persists throughout megagametogenesis. GUS activity, driven by the SUF4 genomic locus, is detected in all female gametophyte nuclei (Figure 2c-g). In stage FG6 embryo sacs (Christensen *et al*., 1997), SUF4 is present in the egg cell nucleus anticipating *EC1* activation. At FG7 the SUF4 signal disappears from the egg cell whilst it persists in the synergids and in the central cells (Christensen *et al*., 1997). The disappearance of SUF4 represents a specific marker for this developmental stage.

SUF4 is a flowering time gene

Our data indicate that SUF4 exerts direct positive regulation on *EC1* genes only in the female gamete. However SUF4 has been isolated in a secondary genetic screening aimed at identifying the genetic loci able to suppress the late flowering phenotype of Col-FRIGIDA. FRIGIDA (FRI) and FLOWERING LOCUS C (FLC) (Gazzani *et al*., 2003; Michaels *et al*., 2003) are the two flowering time master regulators. FRI promotes FLC expression, which is repressed by vernalization (Michaels and Amasino,

2001). This suppression is accomplished through a series of histone modifications caused by the chromatin remodelling complexes, among them the H3K27 methyltransferase Poly-comb Repression Complex 2 (PRC2) (Wood *et al*., 2006; De Lucia *et al*., 2008).

SUF4, a C_2H_2-type zinc-finger protein, was recently characterised as an interacting partner of FRI (Kim *et al*., 2006). Indeed, SUF4 binds to the FLC promoter and recruits the FRI- complex (FRI-C) to activate *FLC* (Choi *et al*., 2011). Besides SUF4, the FRI-C is formed by FLX (FLC EXPRESSOR), FES1 (FRI ESSENTIAL 1) and FRL1 (FRIGIDA LIKE 1) (Geraldo *et al*., 2009; Choi *et al*., 2011; Ding *et al*., 2013). *FLC* regulation is quite complex and involves the *Arabidopsis* homologs of the members of the yeast RNA polymerase II-associated factor 1 (PAF1) complex, EARLY FLOWERING 7 (ELF7) - also known as VERNALIZATION INDEPENDENCE 2 (VIP2), ELF8 (VIP6), VIP4 and VIP5 (He *et al*., 2004; Oh *et al*., 2004; Kim *et al*., 2005). Unlike SUF4, *FLC* is not expressed in developing embryo sacs before gamete formation, although *FLC* is expressed in developing embryos, to prevent flowering, but its precocious reactivation does not depend on FRI and SUF4 activities (Choi *et al*., 2009).

SUF4 binds the *FLC* promoter recognising an A/T rich consensus sequence (5'-CCAAATTTTAAGTTT-3') (Choi *et al*., 2011), however we have not been able to recognise this domain in the *EC1* promoters. It is well accepted that transcription factors show sequence specific binding and that interacting proteins can modulate this property. Indeed, SUF4 can interact with several proteins besides FRI-C, for instance the complex SUF4/LD (SUF4/ LUMINIDEPENDS) is formed in the absence of FRI and supresses SUF4 activity (*i.e.* FLC promoter binding) (Kim *et al*., 2006).

Apart from being a C2H2 protein, SUF4 also contains a BED domain.

The name BED domain originates from the *Drosophila* proteins *BEAF* and *DREF* (Aravind, 2000). Interestingly, the human ZBED1-6 (Zinc BED, Mokhonov *et al*., 2012) proteins act as transcriptional regulators by modifying local chromatin structure upon binding to GC-rich sequences.

We speculate that SUF4 regulates the EC1 genes by acting together with other as yet unidentified transcription factors. *EC1* regulation is not achieved through a SUF4-FRI dimer since the *EC1.1* expression pattern, either by *in situ* either by analysing transgenic plants (*pEC1.1::GUS*, *pEC1.1::GFP*; Sprunck *et al*., 2012), has been studied using the *Arabidopsis* strains Columbia (*Col-0*): *Col-0* lacks FRI activity because of a small deletion at the *FRI* locus (Lempe *et al*., 2005).

SUF4 binds the EC1 promoter

The interaction between SUF4 and *pEC1.1* has been confirmed *in planta* also by genetic evidences. *suf4-1* mutants have been crossed with *pEC1.1::GUS* and *pEC1.2::GUS* marker lines, the GUS activity driven by these two egg cell specific promoters *pEC1.1* and *pEC1.2* was abolished in *suf4-1* egg cells (Figure e). These data corroborate the conclusion that SUF4 activates *pEC1.1* and *pEC1.2*, whilst gel mobility assays and yeast one-hybrid data indicate that SUF4 binds directly the regulative regions under examination.

Also SUF4 expression pattern in embryo sacs supports this protein/DNA interaction.

More genetic evidences confirm that SUF4 participates to regulate the *EC1* genes. Indeed *suf4* mutant plants show a weak *ec1* phenotype: in *suf4-1* siliques seed set is normal, fertilisation events occur although in delay in respects to the wild type control plants.

The *ec1-RNAi* ovules properly attract the pollen tubes, sperm cells are realised but gamete fusion (plasmogamy) is prevented. The *ec1-RNAi* phenotype suggests that the egg cell plays a key role in blocking polytubey (Beale *et al*., 2012; Sprunck *et al*., 2102), since in 45% of the *ec1-RNAi* ovules examined polytubey occurs. This is quick pollen repulsion after sperm delivery and gamete fusion whilst the degeneration of the persisting synergid represents a later slow block (Beale *et al*., 2012; Kasahara *et al*., 2012).

Once the pollen tube bursts, the two sperm cells are pushed at the chalazal pole of the degenerated synergid cell, here they arrest their movement for few minutes, afterwards their migration re-start: such feature label the plasmogamy. In *suf4-1* egg cells 3% at 18-20 HAP have just completed plasmogamy (Figure 4b) whilst in 19% of the ovules the fluorescent signal of the sperm cells is still localised at the chalazal pole of the degenerated synergid cell (Figure 4a). In *suf4-1* fertilisation is only delayed and not fully prevented. By using RT and Q-RT PCR we could detect normal *SUF4* messenger since the T-DNA element responsible for *suf4-1* lies in an intron and it is spliced out (Wang *et al*., 2008).

The observed phenotype of delayed sperm cell fusion resembles the phenotype of ovules ectopically expressing the EC1-interacting phosphatase subunit PP2A B' in the synergid cells, most likely as a result of decreased amount of EC1 proteins (Rademacher and Sprunck, ms in preparation).

All together these information suggest EC1 must accumulate to a threshold level to drive plasmogamy, moreover SUF4 is not the unique *EC1* regulator. Indeed SUF4-GUS is not detected in the egg cell of FG7 embryo sacs (Figure 2g); therefore we cannot exclude that after FG6 *EC1* might be activated in delay by a not identified positive regulator.

SUF4 and MOM1 act together in *EC1* gene regulation

The rapid generation of genome-wide gene expression data allows the creation of gene co-expression networks by examining the co-expression patterns of genes over a large number of experimental conditions. We used publicly available sets of microarray data obtained in a wide range of different stress and developmental conditions to investigate the co-expression of genes with *SUF4*, with the idea to identify other genes, which products collaborate with SUF4 to achieve an optimal *EC1* spatiotemporal regulation. Our analysis pinpointed that *MOM1* and *SUF4* are co-expressed, with a good Pearson coefficient. In agreement with such observation *EC1* genes are down regulated in *mom1* mutants.
As shown by real time RT-PCR analyses (Supplementary Figure S2) MOM1 acts on *SUF4* expression consequently affecting the *AtEC1* gene expression levels. Nevertheless MOM1 action on *EC1.1* is also SUF4-indipendent as suggested by GUS analyses in *SUF4 SUF4 mom1-3 mom1-3* or *suf4-1 suf4-1 mom1-3 mom-3* plants. In both lines, regardless *SUF4* genotype, the GUS activity driven by the *EC1.1* promoter is not anymore restricted to the egg cells (Supplementary Figure S3). GUS activity is transiently detected also in the carpel leaves. These data are corroborated by real time RT- PCR analyses finalised to monitor *EC1.1* expression level. The transient *EC1.1* miss-regulation does not cause any developmental defects, this was expected since transgenic plants over-expressing *EC1.1* (*p35S::EC1.1*) looks (Sprunck *et al*., 2012)
EC1.1 miss-expression in carpel leaves caused by *MOM1* is SUF4 independent, however MOM1 down regulates *SUF4*, therefore we think that the poorest *EC1* messenger accumulation in *mom1-3* egg cells is caused by to the lower SUF4 production.
MOM1 is a big protein nuclear localised containing an incomplete and

highly degenerate helicase domain related to a similar domain found in CHD3 chromatin-remodeling factors. The nuclear localization signal (NLS) and a short fragment of MOM1 of less than 200 amino acids (1663 to 1859) containing a conserved plant-specific motif of 82 amino acids (1734 to 1815), named Conserved MOM1 Motif 2 (CMM2), is required for TGS activity.

It is not clear how MOM1 acts on transcriptional gene silencing, indeed in *mom1* mutant poor alteration in heterochromatin state are recorded, indeed MOM1 does not affects DNA and histone methylation (Vaillant *et al*., 2006). Recently it has been shown that MOM1 promotes gene silencing together with the RNA polymerase V (Yokthongwattana *et al*., 2009; Wierzbicki, 2010).

Definitely a full comprehension of MOM1 molecular action will allow to shed light also into its role in egg cell differentiation and will clarify the its relation with SUF4.

Experimental procedures

Yeast One-Hybrid Experiments

The *EC1.1* regulatory region of 463 bp, known to be sufficient to drive egg cell-specific marker gene expression (Ingouff et al., 2009), was amplified as two distinct fragments of 220 and 264 bp using the primer pairs Atp1681/Atp1682 and Atp1684/Atp1685 (see supplementary table S1). The amplified fragments were digested with *Eco*RI and *Xba*I and cloned into the pHISi vector (Clontech, Palo Alto, CA). The two bait plasmids were linearised with *Xho*I (pHISi) and used to transform yeast strain Y187. A whole normalised total plant cDNA library (Sommer and Masiero, unpublished), which was cloned in pGADT7-rec and

introduced into yeast strain AH109. The library has been generated putting together pools of cDNA obtained by rosette leaves collected at different days after germination (DAG, Boyes *et al*., 2001), by flowers from stage 1 till 10 and rom stage 11 till stage 13, whole inflorescences, siliques till 5 DAP, siliques 6-11 DAP and 12-16 DAP, developing roots. Such expression library was mated with the modified Y187 strains (containing the *EC1.1* regulative regions) as described in the Clontech user manual PT4085-1. The diploids were selected on medium lacking Leu and His and supplemented with 35 mM 3-amino 1,2,4-triazol (3-AT).
Plasmids were extracted from positive colonies and retransformed into Y187 to discover and discard false positives.

Plant materials

Wild-type *Arabidopsis thaliana* seeds (*Col-0*) were obtained from European Arabidopsis Stock Centre (NASC). *suf4-1 suf4-1 FRI FRI and pSUF4:SUF4::GUS* seeds were donated by S.D. Michaels, *mom1-3* mutants by J. Paszkowski. Plants were grown under long-day conditions (14 h light/10 h dark) at 22°C. Seeds were surface-sterilized, chilled at 4°C for 3 days, and then germinated and grown on plant growth medium Murashige and Skoog (1962) supplemented with 0.5% sucrose under the same photoperiod conditions as soil-grown plants.

Plasmid construction

Cloning was done using the Gateway® system (Invitrogen, http://www.invitrogen.com/). Phusion® High-Fidelity DNA Polymerase (Finnzymes, http://www.finnzymes.fi/) was used for the

amplification reactions. For the *pMOM1::GUS* fusion a 1.1 kb genomic region upstream of the *MOM1* ATG start codon, corresponding to the *MOM1* putative promoter was amplified by PCR using Atp3015 and Atp3016 primers. The PCR product was cloned in the pBGWFS7 vector (Karimi *et al.*, 2002). The construct was verified by sequencing and used to transform the wild type (*Col-0*) (Clough and Bent, 1998). All the information about the pBGW vectors are available at http://www.psb.ugent.be/gateway.

Determination of genotypes by PCR

Genomic DNA was extracted from plants as previously described (Masiero *et al*., 2004). The *SUF4* wild-type allele was amplified using Atp2279 and Atp2569 primers, while for *suf4-1* Atp2569 and Atp2562 were used. *MOM1* was amplified with AtP2948 and Atp2949 while *mom1-3* with Atp2948 and Atp2950 primers. Plants expressing *pEC1.1::GUS* were followed with primers Atp1576 and Atp1684 (lying on the GUS gene) and Atp1684 with Atp1576 (*pEC1*). *pEC1.2::GUS* were followed with Atp1756 and Atp1682.

Whole-mount preparation, GUS assays and fluorescence microscopy

Pistils and developing siliques were dissected under a Leica stereomicroscope MZ6, and images were acquired with IM50. The GUS staining assays were conducted as previously described by Colombo et al. (2008). Samples were observed using a Zeiss Axiophot D1 microscope (http://www.zeiss.com/) equipped with differential interface contrast (DIC) optics. Images were recorded with an Axiocam MRc5 camera (Zeiss) using the Axiovision program (version 4.1).

For phenotypic analysis of *suf4-1* ovules, stage 12 flowers (Smyth *et al.*, 1990) were emasculated and pollinated with pollen of stage 13 flowers of the sperm cell marker line *HTR10-mRFP1* two days later (Ingouff *et al.*, 2007). After 18-20 hours after pollination (HAP) the developing siliques were removed from the flowers. For fluorescence microscopy, one silique was then transferred into 50 mM sodium phosphate buffer pH 7.5, the placenta was separated lengthwise into two halves and analysed at the Nikon ECLIPSE TE2000-S microscope with the filter set F36-506 (575/15 nm excitation; BP 624/40) for mRFP1 detection.

Quantitative RT PCR and RT-PCR analyses

To determine *EC1* gene expression levels in *suf4-1* and *mom1-5* plants, stage 12 flowers were emasculated and pistils were collected the day after. Messenger RNA extraction was performed using Dynabeads® mRNA DIRECT™ Micro Kit following the supplier's instructions. Total RNA was extracted using NucleoSpin RNA Plant KIT (Macherey-Nagel). PolyA or total RNA was reversely transcribed using the ImProm-II Reverse Transcription System (Promega). The cDNAs were standardized relative to *UBIQUITIN 10 (UBI10*, AT4G05320) and *ACTIN 8 (ACT8*, AT1G49240). Transcripts, and gene expression analysis was performed using the iQ5 Multi Color Real-Time PCR detection system (Bio-Rad) with a SYBR Green PCR Master Mix (Bio-Rad). Baseline and threshold levels were set according to the manufacturer's instructions. The primers used are listed in supplementary table S1.
To evaluate *SUF4* expression in *suf4-1* plants, stage 12 flowers were emasculated and 5 pistils were collected two days later. mRNA extraction and cDNA synthesis was performed as described previously (Gebert *et al.*, 2008). Primers for *ACTIN3* (At3g53750) were used as

control for successful cDNA synthesis (AtpSS1 and AtpSS2). For simultaneous amplification of the three *SUF4* splicing variants *SUF4.1*, *SUF4.2*, and *SUF4.3*, the primers AtpSS3and AtpSS4 were used.

Purification of recombinant SUF4 and Electrophoretic Mobility Shift Assays (EMSAs)

Expression vectors for recombinant protein expression in *E. coli* were cloned using the GATEWAY® system (Invitrogen). The coding sequence of *SUF4* was amplified by PCR from inflorescence cDNA using the primer pair AtP3041and AtP3042, and cloned into pENTR/D-TOPO. LR-Clonase reactions were performed using the *SUF4* entry vector and the destination vector pET-53-DEST® (Novagen) and pDEST-HisMBP (Nallamsetty et al., 2005), respectively. The resulting expression vectors were used to express a 6xHis-SUF4-StrepII fusion protein and a 6xHIS-MBP-SUF4 fusion protein, respectively. After expressing 6xHis-SUF4-StrepII in *E.coli* Rosetta™(DE3) (Novagen) the soluble fraction of the crude extract was purified by Immobilized Metal Ion Affinity Chromatography (IMAC) under native conditions using Ni-NTA-Agarose (Qiagen) and gravity flow columns, following the manufacturer´s instructions. The 6xHis-MBP and 6xHis-MBP-SUF4 recombinant proteins were expressed in *E.coli* BL21-Codon Plus (DE3)-RIPL cells (Stratagene) and purified under native conditions using TALON® Metal Affinity Resin (Clontech Laboratories Inc.). The *EC1* promoter fragments were amplified with terminal *Xba*I restriction sites via PCR using Taq Polymerase (Fermentas). The purified promoter fragments were digested with XbaI and radio-labelled using Klenow enzyme (Fermentas) and [a-32P]ddATP. Unincorporated [α-32P]dATP

was removed by spin-column chromatography (Illustra ProbeQuant G-50 Micro columns; GE Healthcare).

For the EMSAs the radio-labelled promoter fragments (10 or 18 ng) were incubated with different amounts of SUF4 (10 to 400 ng) in 1x EMSA-buffer (10 mM Tris-HCl, pH 7.5; 100 mM KCl; 1 mM EDTA; 0.1 mg/ml BSA; 100 μM $ZnCl_2$; 6% glycerol; 1 mM DTT) in a 20 μl reaction for 1h at 4°C. Afterwards, the reactions were separated on a 5% polyacrylamide gel in TAE buffer (40 mM Tris and 2.5 mM EDTA, pH 7.8) at 10 V/cm gel length for 1h. For the competitor assays, the respective unlabelled probe was added in excess (50x and 100x) to the binding mixture. Gel images were obtained using autoradiography (Cyclone Phosphoimager A431201, Packard Inc.).

Correlation analysis

The computation of the Pearson correlation coefficient and the microarray dataset employed were as described previously (Menges *et al*., 2008; Berri *et al*., 2009).

Acknowledgments

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Captions

Figure 1.

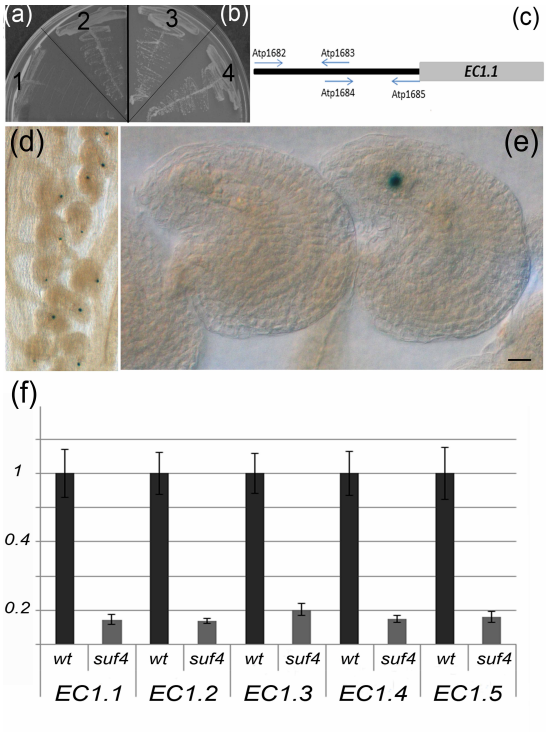

Figure 1. SUF4 regulates *EC1.1* in yeast and *in planta*.

(**a-b**) Yeast one-hybrid analysis of interactions between SUF4 and *pEC1.1*. SUF4 was isolated in a yeast one-hybrid screen aiming to identify those proteins able to bind the *EC1.1* promoter. The full length *SUF4* cDNA was cloned into the yeast expression vector pGADT7 (Clontech) and used for transformation of a modified yeast strain carrying the reporter gene *HIS3* under control of the *EC1.1* promoter. The *EC1.1* promoter was divided in two bait fragments (see experimental procedures). To detect interactions, transformed yeast strains with the proximal fragment of the EC1.1 promoter 1 were grown on either permissive –His –Leu (a) or selective –His-Leu with 5 mM 3-AT (b) medium. 1 and 4 pGADT7 without any insert as negative control, and 2 and 3 pGAD-SUF4 on selctive medium (1-2) and permissive medium (3-4).

(**c**) Schematic representation of the *EC1.1* locus. The regulative region is 463 bases long. Arrows indicate the four primers used for the two yeast one-hybrid bait construction.

(**d**) GUS staining on the homozygous *pEC1.1::GUS* plants crossed with *suf4-1*, all the egg cells are stained indicating that the *pEC1.1:GUS* insertion is homozygous.

(e) SUF4 is important for *EC1.1* promoter activity also *in planta*. Homozygous *suf4-1*

mutants were crossed with homozygous *pEC1.1:GUS* plants. In the F1 carpels only 25% instead of the expected 50% of egg cells were GUS positive, indicating that *pEC1.1:GUS* activation relies on SUF4.

(f) All the five *EC1* genes are down regulated in *suf4-1* mutant pistils as indicated by quantitative real time PCR. To normalise the expression level we used *Ubiquitin10*. The *EC1* down regulation in *suf4-1* and *mom1* mutants has been also demonstrated employing *Actin8* for normalization (data not shown). The expression of each *EC1* gene has been calibrated to 1 in wild-type pistils.

Scale bar: 20 μm.

Figure 2.

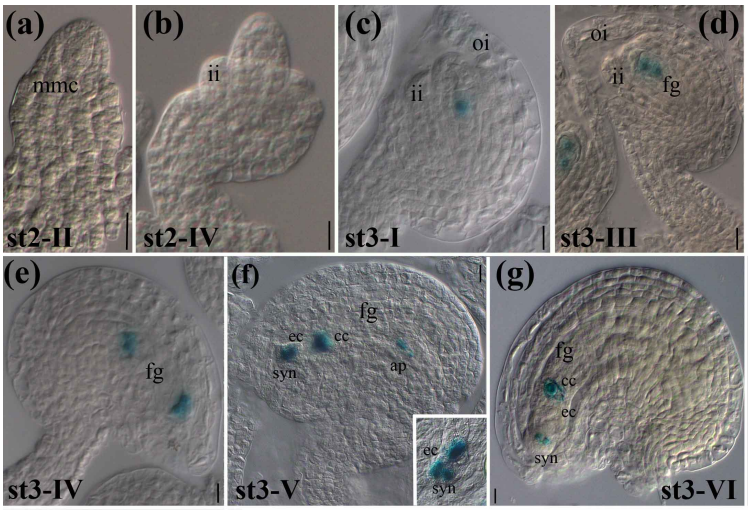

Figure 2. SUF4 is expressed in developing female gametophyte.

To investigate the SUF4 expression pattern during ovule development we used the *pSUF4::SUF4-GUS* reporter construct described by Kim and Michaels (2006). Ovule stages are classified according to Schneitz *et al*. (1995).

(**a**) SUF4-GUS is neither expressed in the mmc (megaspore mother cell) nor in the tetrad of megaspores (**b**). SUF4-GUS is detected in developing ovules from stage 3-I on. SUF4-GUS activity is first detected in the nucleus of the newly formed haploid female gametophyte (c). SUF4-GUS expression persists in the developing embryo sac until stage 3-VI (see 4d to e). In the seven-celled embryo sac (stage 3-V), SUF4-GUS is detected in all the seven nuclei (4f). In mature ovules at stage 3-VI, SUF4-GUS is not expressed any more in the egg cell (4g).

ap, antipodal cells; cc, central cell; ec, egg cell; fg, female gametophyte; ii, inner

integument; mmc, megaspore mother cell; oi, outer integument; syn, synergid cells
Scale bars: 20 μm

Figure 3.

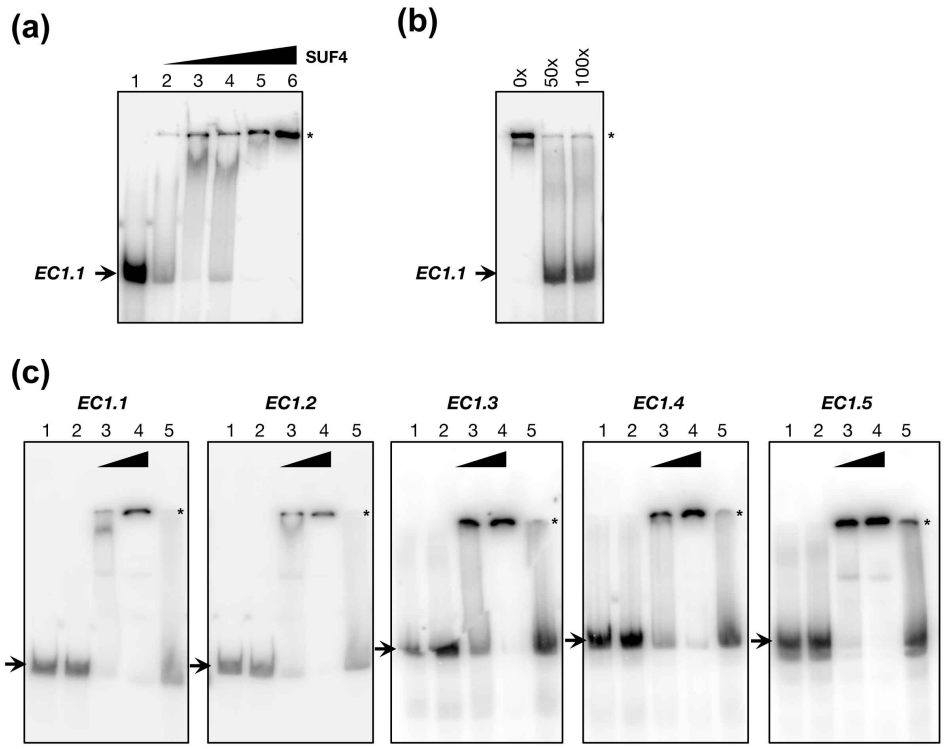

Figure 3. SUF4 binds to all five Arabidopsis *EC1* promoters.
(**a**) EMSA analyses confirmed the interaction between SUF4 and the promoter sequence of *EC1.1*, which was used as bait in the yeast one-hybrid screening. EMSA without (1) and with 10 (2), 50 (3), 100 (4), 200 (5), and 400 ng (6) of recombinant 6xHIS-SUF4-STREPII, added to a radiolabelled 108 bp fragment of the *EC1.1* promoter. (b) EMSA with the 50-fold (50x) and 100-fold (100x) excess of unlabelled promoter fragments as a cold competitor, added to the reaction mix with 200 ng of 6xHIS-SUF4-STREPII, compared to a control reaction without cold competitor (0x). (c) 50 and 150 ng of recombinant 6xHIS-MBP-SUF4, or 150 ng of 6xHIS-MBP as control, were mixed with 10 ng of labelled promoter fragments of *EC1.1* (108 bp), *EC1.2* (115 bp), *EC1.3* (167 bp), *EC1.4* (199 bp) and *EC1.5* (189 bp). In addition, the 100-fold excess of the respective unlabelled promoter fragments were added as cold competitor. (1) labelled promoter fragment only; (2) labelled promoter fragment with 150 ng 6xHIS-MBP tag

only; (3) labelled promoter fragment with 50 ng 6xHIS-MBP-SUF4; (4) labelled promoter fragment with 150 ng MBP-SUF4; (5) labelled promoter fragment with 150 ng MBP-SUF4 and 100-fold excess of cold competitor.

Figure 4.

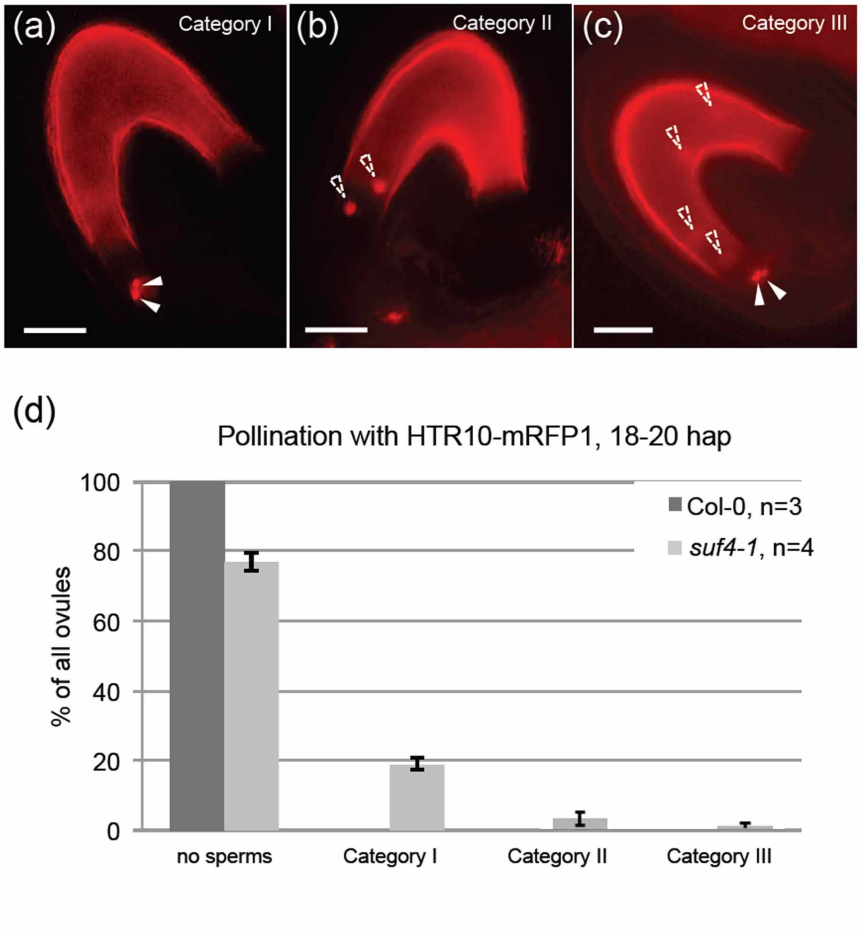

Figure 4. *suf4-1* plants show a weak *ec1* phenotype.

suf4-1 flowers were emasculated, pollinated with the sperm cell marker line HTR10-mRFP1 and analysed by fluorescence microscopy 18-20 hours later.

(a) Category I: one pair of non-fused sperm cells (arrowheads). (b) Category II: one non-fused pair (arrowheads) and a second pair of decondensed sperm nuclei recognizable by HTR10-mRFP1 derived fluorescence in endosperm nuclei (dashed arrowheads). (c) Category III: decondensed sperm nuclei are visible (dashed arrowheads). Bars = 20 μm. (d) Quantification of categories shown in (b-e). n = number of pistils (*Col-0*: 167 ovules, *suf4-1*: 232 ovules); error bars = SEM

Figure 5.

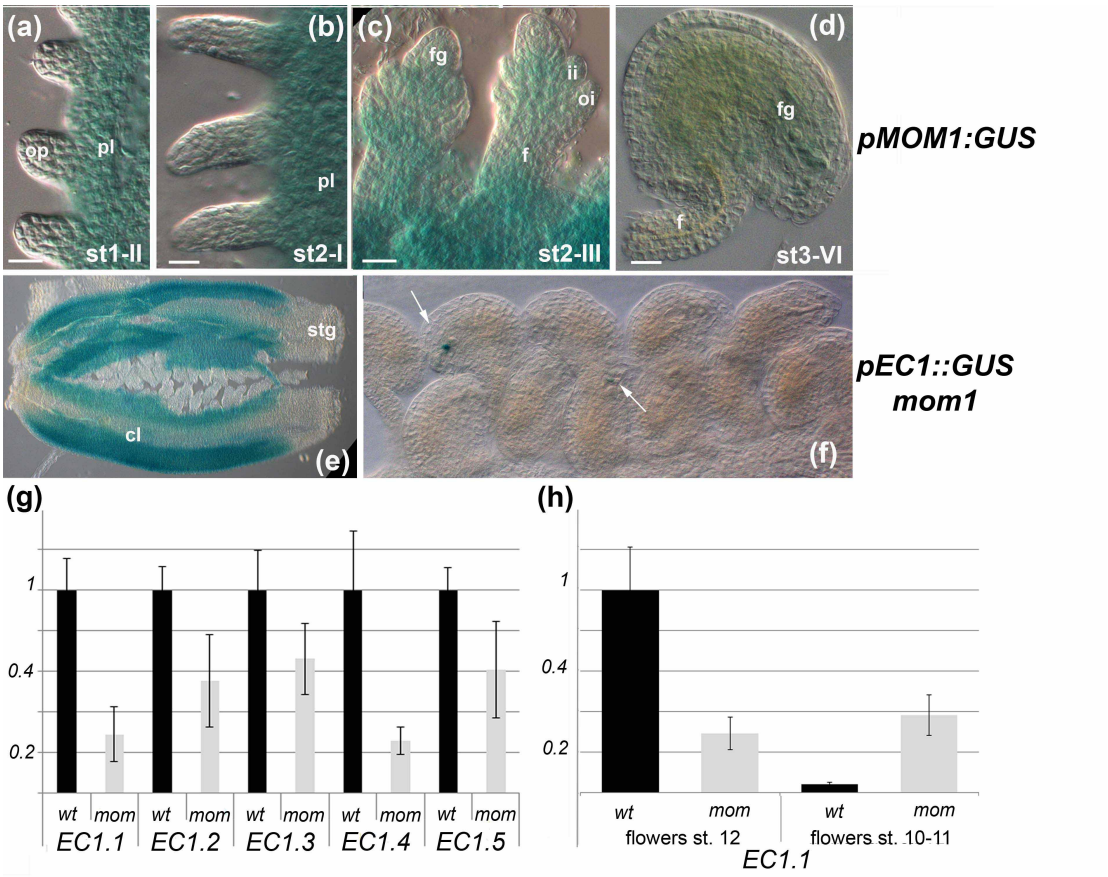

Figure 5. *MOM1* promoter is active in developing ovules and MOM1 participates to regulate *AtEC1.1*.

To clarify *MOM1* spatial and temporal expression pattern, we generated and analysed *pMOM::GUS* transgenic plants. *MOM1* promoter is active in developing carpels, GUS activity is detected in the placenta when ovule primordia appear (a) and at developmental stage 2-I (b). In ovule stage 2-II, the enzymatic activity is detected also in the funiculus, in the chalaza and in the integument primordia (c). *pMOM1::GUS* is still active in fully developed ovules (stage 3-VI, d).

MOM1 participates to regulate AtEC1.1. In *mom1-3* mutants, *pEC1.1::GUS* is expressed transiently also in the carpel leaves and in the placenta (e), moreover *mom1-3 mom1-3* plants carrying a *pEC1.1:GUS*, the enzymatic activity of the GUS reporter gene is detected only in 25-36%% of the analysed egg cells (arrows) and not as expected in the 50%.

In *mom1-3* mutant pistils all the five *AtEC1* genes are down regulated (g). Real time PCR analyses confirm that *EC1.1* is transiently expressed in mom1-5 flowers at stage

10-11 (flower stages have been determined following Smyth *et al.*, 1990), whilst no expression is detected in wild type flowers, as expected *EC1.1* is down regulated in *mom1*-3 flowers at stage 12.

cl, carpel leaves; f, funiculus; fg, female gametophyte; ii, inner integument; oi, outer integument op, ovule primordium; pl, placenta; stg: stigma

Scale bars: 20 μm

Table 1.

Genotypes	Ovule						
	Ob. GUS+	Ob. GUS-	Tot.	Ex. GUS+	Ex. GUS-	χ^2 value*	p value
SUF suf4-1 EC1.1::GUS +/-	356	1036	1392	348	1044	0.24	0.5<P<0.75
SUF suf4-1 EC1.2::GUS +/-	301	924	12010	302.5	907.5	0.307	0.5<P<0.75

Table 1. GUS activity in egg cells. GUS analyses conducted on mature ovules of F1, obtained by crossing *suf4-1* mutants with *pEC1.1::GUS* or *pEC1.2::GUS* homozygous lines. Note that the χ^2 statistics indicate that SUF4 regulates *pEC1.1::GUS*, as well as *pEC1.2::GUS*.

GUS+, GUS positive ovules; GUS-, GUS negative ovules.

Supplementary information

Figure S1.

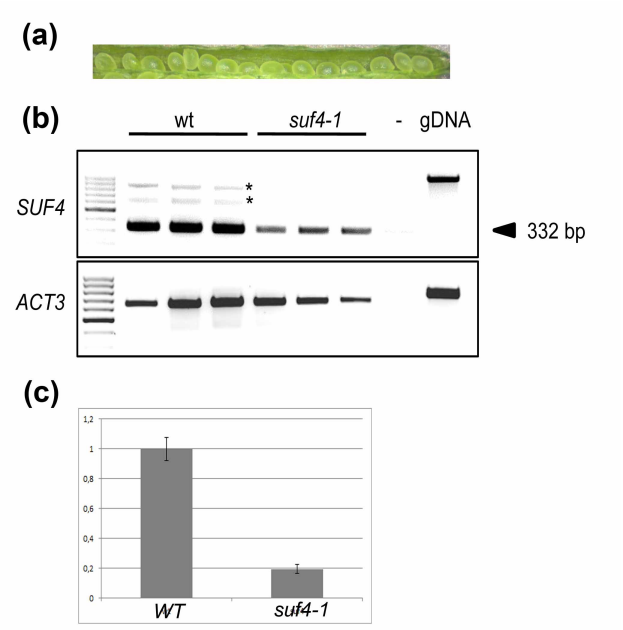

Figure S1. *Suf4-1* is not a full knock out mutant.
(a) *suf4-1* siliques show no significantly reduced seed set. (b) RT-PCR analyses show that *suf4-1* is not a full knock out mutant. The primer pair used to detect *SUF4* transcripts was designed to amplify all three splicing variants described for *SUF4* (Kim et al., 2006). In the wild type control, three distinct fragments are amplified, of which the lowermost represents the 332 bp amplicon of *SUF4.1*, which was shown to be a functional splicing variant (Kim *et al*., 2006). The two alternative splicing variants *SUF4.2* and *SUF4.3* (asterisks) result in fragments of 495 and 851 bp, respectively. The transcript variant *SUF4.1* is also detectable in pistils of homozygous *suf4-1* plants, indicating that the *suf4-1* mutant is still able to produce functional *SUF4* transcripts. (c) The presence of residual *SUF4.1* transcript was confirmed by quantitative real time PCR analyses.

Figure S2.

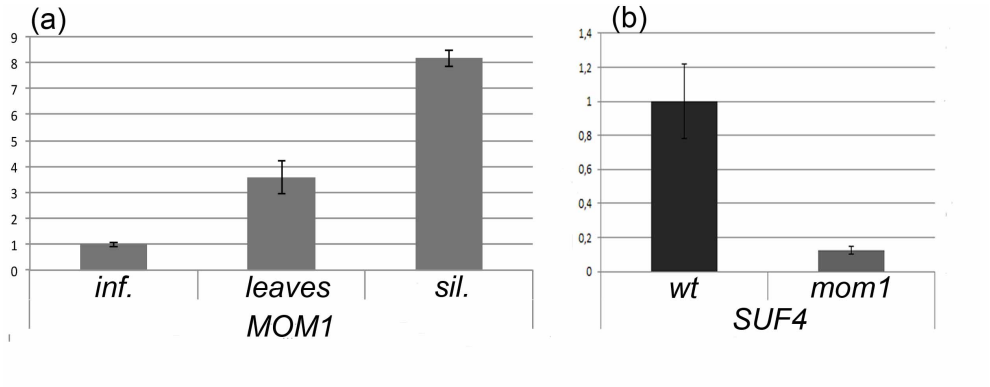

Figure S2. MOM1 and SUF4 expression pattern analyses.

(a) Quantitative RT PCR analyses to monitor *MOM1* expression pattern in leaves, inflorescences, and developing siliques (4-8 DAF).

(b) *SUF4* transcription is significantly reduced in *mom1-3* inflorescences.

Figure S3.

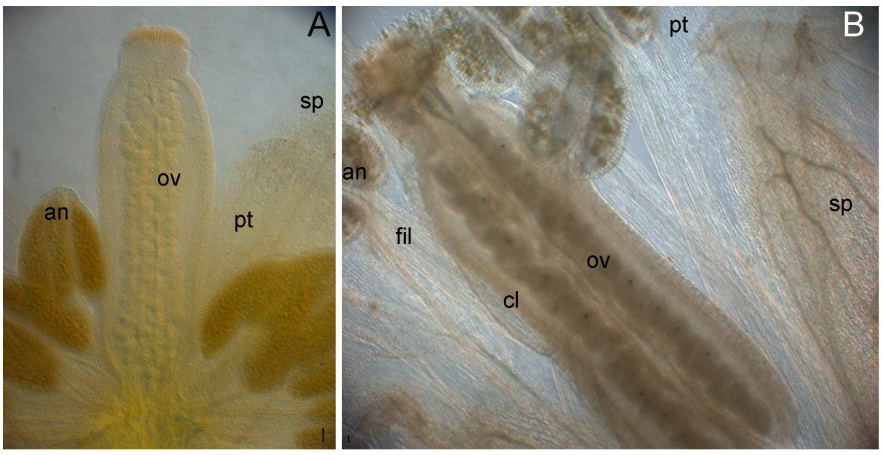

Figure S3. *pEC1.1::GUS* expression in *Col-0* developing flowers

pEC1.1::GUS is not active in flowers at developmental stage 11(a), whilst this promoter drives GUS activity in developing flowers at stage 12; GUS activity is restricted only to the egg cells. An, anthers; cl, carpel leaves; fil, filaments; ov, ovules; pt, petals; sp, sepal.

Scale bar: 40 μm

Table S1

Accession	Description
AT1G0782	Histone 4
AT1G07820	Histone 4
AT2G46980	ASY3, a coiled-coil domain protein
AT1G07820	Histone superfamily protein
AT1G69690	AtTCP15
AT1G01960	embryo sac development arrest 10 (EDA10)
AT1G30970	SUF4
At5g62470	Myb 96
AT3G27670	RESURRECTION1, RST1
AT5G22880	Histone H2B

Table S1. List of proteins able to bind EC1.1 promoter in yeast. The proteins listed have been sequenced more than once.

Table S2

Accession	description	Pearson coefficient (Log)
At1G30970	zinc finger (C2H2 type) family protein, contains Pfam domain	1
At1G78930	mitochondrial transcription termination factor-related /	0,6780
At5G63200	tetratricopeptide repeat (TPR)- protein	0,6676
At1G50910	unknown protein	0,6624
At5G47400	unknown protein	0,6601
At1G08060	**MOM1**	**0,6555**
At3G44530	transducin family protein	0,6551
At4G18600	unknown protein	0,6484
At5G06100	Myb transcription factor (MYB33)	0,6471
AT2G35540	DNAJ heat shock	0,6455
AT2G47820	expressed protein	0,6441

AT5G20200	nucleoporin-related protein	0,6407
AT5G18770	F-box protein	0,6381
AT1G77410	Putative beta-galactosidase	0,6378
AT2G43980	inositol 1,3,4-trisphosphate 5/6-kinase	0,6353
AT4G35930	F-box family protein	0,6345
AT5G05350	unknown protein	0,6295
AT5G05130	SNF2 domain-containing protein /	0,6268
AT4G25540	DNA mismatch repair protein (MSH3)	0,6254
AT1G52620	pentatricopeptide (PPR) repeat-containing protein	0,6248
AT5G13470	unknown protein	0,6222
AT2G40950	bZIP transcription factor	0,6206
AT2G28330	unknown protein	0,6200

Table S2. List of genes co-expressed with SUF4.

Table S3

Atp1576 TCATTGTTTGCCTCCCTGCTG

Atp1682 CGAATTCTGCCTTATGATTTCTTCGG

Atp1683 CGTCTAGATAATTAGTGGGTCTGTTTAGG

Atp1684 CGAATTCCAGACCCACTAATTACG

Atp1685 CGTCTAGACTCAACAGATTGATAAGG

Atp2569 GTATGGCAAATGCCACCTC

Atp2279 CTGAGATTCGTCTGTCTATCGC

Atp2562 CATTTTATAATAACGCTGCGGACATCTAC

AtP2948 AAGCAGTTGTCTTCTACCAG

Atp2949 TTGCCGCTTATTTTGCCTAG

Atp2950 GCGTGGACCGCTTGCTGCAACT

Atp3015 GGGGACAAGTTTGTACAAAAAAGCAGGCT

GATCGACTCTAAACATTATGGG

Atp3016 GGGGACCACTTTGTACAAGAAAGCTGGGT

CATATTCACTGAGAGCACGCAA

AtP_3097 AGTCATTGCCATCACAGTAACC

AtP_3098 CTTGTGTACAGGCTCAAGCTTG

AtP_3160 GGCTTCTAACACAAGTTTCCTC

AtP_3100 ATGACCTCGACGGCTTGACA

AtP_3101 CCGAGTTTGGTCTCACCGTT

AtP_3161 CTCTTCTCCTCGTTCTCAACG

AtP_3103 ATGGCTTCGAACACTACTTTCC

AtP_3104 CCATCAGTCCTCCACTTTGG

AtP_3106 CGGTTCACTCGTACCGGTTTGA

AtP_3171 GACCTTTCATGTCATCACTGTCG

AtpSS1 GATTTGGCATCACACTTTCTACAATG

AtpSS2 GTTCCACCACTGAGCACAATG

AtpSS3 GGGGCTCAGCAACCATCTCAT

AtpSS4 ATCCGCCCAGCAAGCCTACT

AtpSS5 GGGGTCTAGACCACTAATTACGCAGCTTTTAAT AGAGTAATTAC

AtpSS6 GGGGTCTAGACCCAAGTAATTACGAGGGAAGCTCA

AtPSS7 GGGGTCTAGAACGTTTCTACAGTCAAATGCTTTAACGTT

AtpSS8 GGGGTCTAGATTGTGGTTTTGGATGGATTCGAAG

AtpSS9 GGGGTCTAGACTCTT CGCTTGTATCTTTCCGTTAAG

AtpSS10 GGGGTCTAGAATACTGTGATTATGAAA GGATTTTTGAGGT

AtpSS11 GGGGTCTAGAGTTGCTCTTGCTGCATCAAATACATATT

AtPSS12 GGGGTCTAGAGTGTGTTTGTGGAAGGATTTGTTGTG

AtPSS13 GGGGTCTAGAGGGTTTCCATAAAGCCCAATTTAGTT

AtpSS14 GGGGTCTAG ATGTCGTTTAATGTTGTAGTAATTA

AtP_3041 CACCATGGGTAAGAAGAAGAAGAG

AtP_3042 CTAAAACGCCATCCGCCC

ACTIN8F CTCAGGTATTGCAGACCGTATGAG

ACTIN8R CTGGACCTGCTTCATCATACTCTG

UBI10F GGAAAAAGGTCTGACCGACA

UBI10R CTGTTCACGGAACCCAATTC

Table S3. Primers used in this work.

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AtAPOSTART1, an *Arabidopsis thaliana* PH-START domain protein involved in seed germination

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Abstract

In the mature and dry state the seed is metabolically inactive (quiescent) and it is able to withstand extremes environmental conditions, for instance drought and cold.

Germination commences when the dry seed, shed from its parent plant, takes up water (imbibition), and ends when the root emerges through the outer structures of the seed (usually the seed or fruit coat). The seedling establishment uses the reserves stored in the seed, whereas the following vegetative and reproductive growth of the plant is supported by photosynthesis.

Hereby we describe the functional characterization of the *Arabidopsis thaliana* gene *AtAPO1* (*Arabidopsis thaliana APOSTART1*), the putative homologue of *PpAPO1 (Poa pratensis APOSTART1),* a PH-START protein. Using translational fusion of *AtAPO1* promoter and the *uiaD* gene and *in situ* hybridization we show that *AtAPO1* is expressed in mature embryo sacs and developing embryos. The functional analysis of two *at-apostart* mutant alleles suggests that AtAPO1 is involved in the control of seed germination.

Introduction

Angiosperm seeds are the final results of the double fertilization. The pollen grain contains two sperm cells, one fertilizes the egg cell originating the diploid embryo whilst the latter sperm cell fuses with the diploid central cell, forming the triploid endosperm. Embryo and endosperm development proceeds within the maternal tissues of the ovule, the integuments, which turn into the seed coat (West & Harada 1993).

Seed development proceeds through two distinct phases (morphogenesis and maturation) during which the growth and development of the three compartments is strictly coordinated (Berger *et al*., 2006; Gutierrez *et al*., 2007). Soon after fertilization, the zygote undergoes an asymmetric cell division; the apical daughter cell will generate the proper embryo whilst the basal cell is the progenitor of the suspensor. After the first division, the embryo undergoes to several mitosis followed by differentiation processes ending with the establishment of the plant body. The second phase, or post-embryonic development, is characterized by cell maturation, which involves cell growth and the storage of macromolecules such as oils, starch and proteins, needed during germination and precocious seedling growth (West & Harada, 1993).

After reaching physiological maturity, seeds of many plant species, including *Arabidopsis thaliana*, may enter in a state of dormancy during which the dormant seeds will not germinate or germinate very slowly compared to the corresponding non-dormant seeds. Seed dormancy is an adaptive trait, since seeds remain quiescent until germination conditions become favourable (Finch-Savage & Leubner-Metzger, 2006). In mature seeds, the break of dormancy may either occur gradually in

the dry state (after-ripening) or be initiated by imbibition under defined conditions (e.g. cold stratification or chilling at low temperature; Koornneef *et al*., 2002; Donohue *et al*., 2005).

Seed germination is a critical step in plant cell cycle, controlled by several biotic and abiotic factors. However, dormancy maintenance and break is also regulated by several internal growth regulators, such as gibberellins and abscissic acid (Bentsink & Koornneef, 2002) which have antagonistic effects: abscissic acid establishes and maintains seed dormancy, while gibberellins stimulate germination (Steber *et al*., 1998).

Germination begins with the uptake of water by imbibition of the dry seed, followed by embryo expansion. This usually culminates in rupture of the covering layers and the radicle emergence generally ends the germination process. Seed water uptake is triphasic, with a rapid initial uptake, imbibition (phase I) followed by a plateau phase (phase II). A further increase in water uptake (phase III) occurs only when germination is completed, as the embryo axis elongates and breaks through its covering structures (Bewley, 1997; Manz *et al*., 2005). In non-endospermic seeds and in *Arabidopsis*, which has a monolayer cell endosperm, the testa (seed coat, diploid maternal tissue) characteristics are mainly responsible for the coat dormancy (Debeaujon *et al*. 2000).

APOSTART1 (*PpAPO1*) was isolated in *Poa pratensis* by the cDNA-AFLP technique to isolate genes differentially expressed in apomictic and sexual genotypes (Albertini *et al*., 2003). PpAP1 shares high similarity with two *Arabidopsis thaliana* proteins, At5g45560 and At4g19040. The latter one was named EDR2 (Enhanced Disease Resistance 2; Tang *et al*., 2005).

In this manuscript we characterized the expression and function of *Arabidopsis thaliana APOSTART1* (*AtAPO1)*. Interestingly we show that

AtAPO1 is involved in seed dormancy since its down regulation affects germination rate in both fresh harvest and dry stored seeds.

Results

Identification of *AtPAPO1*, the *Arabidopsis PpAPO1* putative orthologue

Sequence comparison revealed that the *Poa pratensis Pp*AP*O1* shares high homology with the *Arabidopsis* protein At5g45560 (66% amminoacidic identity and 80% amminoacidic similarity), thus named *AtAPOSTART1 (AtAPO1), and with* EDR2 (Enhanced Disease Resistance 2 At4g19040; 66% identity and 79 % similarity). *edr2* homozygous plants do not show any obvious developmental defects but they show enhanced capacities to resist to *E. cichoracearum* infections (Tang *et al*., 2005; Vorwerk *et al*., 2007).

As in EDR2, three characteristic domains are recognizable in AtAPO1 (Figure 1a and Supplemental Figure 1): the PH domain (pleckestrin domain) at the N-terminus (from aa 4 to aa 112), the central START domain (from aa 226 to aa 364) and the DUF1336 domain (from aa 503 to 707) at the C-terminus (Albertini *et al*., 2005). The PH domain, firstly identified in pleckstrin (Haslam *et al*., 1993; Mayer *et al*., 1993), together with the START domain, is involved in intracellular signaling (Lemmon & Ferguson, 2000; Soccio & Breslow, 2003). In particular, several PH domains are known to function as lipid-binding domains, and facilitate membrane localization (Maffucci & Falasca, 2001), while the START domain is believed to have important roles in lipid transport and metabolism (Soccio & Breslow, 2003).

The DUF1336 domain lies in the is the C-terminus part of AtAPO and is

around 250 residues long; despite this domain is recognizable in several plant proteins, its function is still unknown.

AtAPO1 is expressed in the female gametophyte and during embryo development

According to quantitative RT-PCR (qRT-PCR) analysis, *AtAPO1* messenger is detected in all plant tissues, such as developing inflorescence, siliques and leaves, however the highest expression levels are recorded in developing flowers and siliques (Supplemental Figure 2).

To investigate in more details the *AtAPO1* expression pattern we transformed *Arabidopsis* plants with the putative *AtAPO1* promoter (*pAtAPO1*, 536 bases) cloned in front of the *uiaD* reporter gene (*pAtAPO1::GUS*). Four independent transgenic lines were analyzed; among them two did not show GUS activity and two lines showed identical expression pattern. In particular GUS activity is detected in mature embryo sacs (Christensen *et al*. 1997; Figure 2a), moreover *AtAPO1* promoter is also active in the zygote (Figure 2b) and in developing embryos till early torpedo stage (Figure 2c-e). After germination, GUS activity appears localized in the primary root of 2-, 3- and 6- days seedlings (Figure 2f, g). In addition *pAtAPO1* drives the reporter gene transcription also in mature pollen grains just before anther anthesis (Figure 2h).

AtAPO1 expression pattern was also confirmed by *in situ* hybridization analyses. To avoid cross hybridization with *EDR2* messenger, *in situ* hybridization experiments were performed in *edr2* mutant background. *AtAPO1* is expressed in mature embryo sacs (Christensen *et al*. 1997; Figure 3a) and in developing embryos (Figure 3b, c) whilst no signal has

been detected when using *AtAPO1* sense probe (Supplemental Figure 3). These *in situ* data further support *AtAPO1* expression pattern produced observed in *pATPO1::GUS* lines.

Identification of *AtAPO1* insertional mutants

To investigate the biological role of AtAPO1, we analyzed two independent T-DNA insertional mutant lines. *atapo1-1,* caused by a T-DNA insertion in the second intron (Figure 1a), 465 bases downstream the ATG, and *atapo1-2* due to a T-DNA integrated at base 2619 from the ATG codon, in the tenth intron (Figure 1a).

The effects of T-DNA insertion have been evaluated by monitoring expression of *AtAPO1* in homozygous insertion mutants (*atapo1-1/atapo1-1* and *atapo1-2/ atapo1-2*). Our RT-PCR (Figure 1b) indicate that *atapo1-2* is a null allele, since RT-PCR analysis performed with primers placed upstream and downstream the T-DNA failed to detect *AtAPO1* messenger. However the T-DNA, designed for activation tagging screenings (pAC106; Rosso *et al*., 2003), drives the transcription of the downstream sequence as shown by RT-PCR analyses (Figure 1b).

About *atapo1-1,* RT-PCR experiments indicate this is not a null allele, since primers located upstream and downstream the insertion fail to detect *AtAPO1* messenger, therefore the second intron is spliced out together with the inserted T-DNA sequence. Coherently, homozygous *atapo1-1* plants develop normally and are indistinguishable from wild-type sister plants (data not shown). Moreover, the offspring of *AtAPO1/atapo1-1* individuals show a normal mendelian segregation (1:2:1). 78 plants were analyzed, and 18 *AtAPO1/AtAPO1*, 46 *AtAPO1/atapo1-1* and 14 *atapo1-1/atapo1-1* plants were identified (Table 1). X^2 test supports the causality of this deviation (0,1< *P value* <0,2).

Differently, a distorted segregation ratio of the three forecasted genotypes was recorded in *AtAPO1/atpo1-2* offspring. In particular a population of 243 plants was analyzed, and we identified 77 *AtAPO1/AtAPO1* plants, 140 *AtAPO1/atapo1-2* and only 26 *atapo1-2/atapo1-2*. The X^2 rejects the hypothesis that this distortion is due to causality (*P value* <0,01; Table 1).

From reciprocal crosses with wild-type plants we calculated the transmission efficiency (TE) of the *atapo1-2* mutant allele through the male and female gametophytes (Table 1). The TE represents the fraction (%) of mutant alleles that successfully transmit the mutation. This analysis showed that 100% of pollen and of megagametophytes carrying *atapo1-2* successfully transmitted the mutation (TE = 100%). Indeed AtAPO1 does not play any role during microgametogenesis and/or megagametogenesis. Furthermore we have analyzed 60 siliques produced by 12 *AtAPO1/atapo1-2* individuals and we never reported alteration in the normal seed set. In total we have analysed 2988 ovules, 26 of them (1.2%) aborted, analogous abortion percentage has been observed for wild-type sister plant siliques in agreement with previous observations (Acosta-Garcia & Vielle-Calzada, 2004).

AtAPO1 regulates Arabidopsis seed germination

Our genetic data reveal that *atapo1-2* allele is not properly transmitted to the offspring, but reciprocal crosses excluded that AtAPO1 is involved in gametophyte development; therefore ATAPO1 might play a role during embryo and/or seed development (Table 1). To address this point we extensively employed differential interphase contrast microscopy to investigate embryo development in *atapo1-2* plants, but no alteration have been observed (data not shown).

It has been reported that endosperm developmental defects are responsible for poor seed viability (Neufer & Sheridan, 1980). Although *Arabidopsis* endosperm does not store reserves, the reduced embryo growth as a consequence of reduced endosperm size suggests that nutrients are delivered from the endosperm to the embryo (Garcia *et al*. 2003). To verify whether endosperm in *atapo1-2* mutant is affected, *AtAPO1/atapo1-2* heterozygous plants were crossed with plants containing the *pFIS2::GUS* (*FERTILIZATION INDEPENDENT SEED 2*) reporter gene construct. The *FIS2* promoter is active in the embryo sac, and after pollination *FIS2* expression is observed in the nuclei of the coenocytic endosperm (Luo *et al*., 2000). Seven developing siliques produced by a plant homozygous for the *pFIS2::GUS* insertion and *AtAPO1/atapo1-2* were analyzed. *pFIS2::GUS* expression pattern was identical in all analyzed seeds, strongly indicating that endosperm development occurs normally (Figure 2i). Therefore we can conclude that post embryonic defects might affect *atpo1-2* allele transmission.

To address this aspect, preliminary germination tests were performed on dried stored seeds. Seeds produced by *AtAPO1/atapo1-2* individuals were germinated on medium supplemented with 0.5% sucrose and vernalized (24 hours at 4°C). After 7 days at 22°C the sibling wild-type control seeds showed a germination ratio of 97%, while 84,2% of *AtAPO1-2/atapo1-2* seeds did not germinate in analogous conditions (303 out of 360), and 75% of *atapo1-2* seeds were able to geminate (110 out of 148).

These data pinpointed a possible role for AtAPO1 during seed germination. Omission of the cold treatment before the germination assays resulted in substantial differences in the germination rates of wild type and *atapo1-2* fresh harvested seeds (Debeaujon & Koornneef, 2000). As shown in Figure 4a-c, *atapo1-2* fresh harvest seeds do not

germinate (we analyzed 300 seeds obtained by three independent homozygous mutant plants). Also *atapo1-2* seeds, two and four weeks old, retain a strong dormancy. *atapo1-2* seeds twelve weeks old partially lose such feature although their germination ratio is lower than the wild type ones (Supplemental Figure 4).

The germination response of the *atapo1-2* mutants to light and chilling was also investigated. The cold treatments followed by light exposition (100 umol m-2 s-1, 16 hours light -8 hours darkness) promote *atapo1-2* seed germination (Figure 4d-f). Nevertheless, *atapo1-2* seeds show a strong delay in germination response in comparison to wild-type sibling seeds, and the best germination performances were achieved with 96 hour of chilling treatments (Figure 4f).

atapo1-1 seeds showed a milder phenotype in respect to *atapo1-2* ones, in agreement with RT-PCR analysis (Figure 1c). Indeed fresh harvested seeds were able to germinate although slower, moreover stratification treatments can restore normal germination ration.

To verify whether EDR2 participates also in controlling seed germination, we performed germination assays using seeds produced an *edr2* insertional line (Salk-052496; Tang *et al*., 2005); which is a knock-out line (Supplemental Figure 1b). Our germination assays pinpoint that also e*dr2* seeds are dormant (Figure 5a, b) and the germination delay can be partially rescued by vernalisation (Figure 5c, d).

Discussion

AtAPO1 and EDR2 are two paralogues with divergent biological functions

EDR2 and AtAPO1 are two paralogues and both are PpAPO1 ortologues. Here we show that *atapo1* mutants produce dormant seeds, however we have been able to show that also *edr2* seeds germinate in delay. This is a new feature for *edr2* mutant, which has been isolated during a screening for enhanced disease resistance (*edr*) to the biotrophic powdery mildew pathogen (Tang *et al*., 2005; Vorwerk *et al*., 2007). Differently, AtAPO1 exerts its major role during seed germination.

AtAPO1 and *EDR2* expression patterns are in agreement with the proposed roles. *EDR2* is constitutively expressed, as confirmed by in silico analyses using Geneinvestigator (Zimmereman *et al*., 2004) and by promoter fusion analysis, moreover stresses positively enhance *EDR2* expression. On the contrary *AtAPO1* shows a more restricted expression pattern as proved by our *in situ* analysis, and it does not respond to biotic stresses (Vorwerk *et al*., 2007).

AtAPO1 and EDR2 are PH domain proteins. The PH domain is recognized in proteins with quite divergent functions, including the Btk tyrosine kinases, phospholipase C, phosphoinositide 3-kinase, the SOS guanine-nucleotide-exchange factor and the GTPase dynamin. Among these subgroups the pleckstrin one is quite peculiar since it contains proteins with two PH domains.

Individual PH domains possess specificities for phosphoinositides phosphorylated at different sites within the inositol ring. This is important because it makes the recruitment of different PH domain containing proteins sensitive to the activities of enzymes that either

phosphorylate or dephosphorylate these sites, thus modulating the localization of downstream signaling proteins with PH domain able to bind their phospho-lipid products. Vorwerk and collaborators (2007) were able to show that, at least *in vitro*, EDR2 is able to bind phosphatidylinositol-4-phosphate.

Beside the PH domain, ERD2 and APO1 possess also a START domain involved in cellular signaling and cytoskeletal organization that requires association with cell membranes (Lemmon *et al*., 1996). START domain is often found in proteins involved in signal transduction or transcriptional control (Schrick *et al*., 2004).

START domains are ~200 amino acid lipid/sterol binding modules that are conserved from animals to plants (Ponting & Aravind, 1999; Schrick *et al*., 2004). The prototype START domain is found in mammalian StAR, a cholesterol transporter. In humans, mutation or miss-expression of START proteins is linked to inherited disorders, autoimmune disease and cancer (reviewed in Alpy and Tomasetto, 2005). Thus, it is conceivable that the PH-domain may interact co-operatively with the START-domain and that the concerted action of both domains influences the ligand binding ability and function.

The subcellular localization of a protein correlates with its function and is thus used to draw conclusions about its cellular role, interaction partners and function in biological processes. . Few algorithms (iPSORT, MitoPred, Predotar, SLOX) predict that AtAPO1 might be a mitochondrial-localized or a plastidial-localized protein. However these predictions have a low reliability. The output lists appear quite ambiguous since it is forecasted that AtAPO1 could also join the endoplasmic reticulum. One of these algorithms (SLox) also suggests a possible cytosolic localisation although with a poor reliability. Protein sorting is a process not fully understood yet and suck lack of knowledge

strongly limits the predictive power of bioinformatic tools. However, *in vivo* experiments have shown that EDR2 is localized to multiple compartments, EDR2:HA:eGFP was observed in the endoplasmic reticulum, plasma membrane and in small endosomes in young seedlings (Vorwerk *et al*., 2007). Indeed such complicate subcellular localisation pattern explains the incapacity to predict a specific subcellular compartment for AtAPO1.

***atapo1* seeds are dormant**

Germination tests carried on *atapo1-2* mutant show that mutations in *AtAPO1* cause decreased germination potential. Most plant seeds are dispersed in a dry, mature state, and they will undergo through the germination process in case they are non-dormant and the environmental conditions are favorable.

Germination involves the mobilization of storage reserves and the initiation of growth and metabolic activity within the embryo, thus early germination stages show high respiration rate.

Interestingly, our data suggest a role for AtAPO1 during this process. EDR2, AtAPO1 paralogoues, acts as a negative regulator of cell death; specifically the cell death elicited bynpathogen attack and mediated by the salicylic acid defense pathway. Phosphatidylinositol-4-phosphate may have a role in limiting cell death via its effect on EDR2. Vorwerk and co-workers did not clarify whether EDR2 role in cell death is indirect, by helping to target EDR2 to the appropriate membrane, or direct. However the authors linked EDR2 action to the salicylic acid pathway, although it is not clear whether it acts up- or down- stream.

The salicylic acid is an important signaling molecule that modulates plant responses to pathogen infection. Upon infection, salicylic acid

biosynthesis is stimulated, together with pathogenesis-related genes. These events are the prelude to the hypersensitive response, which induces necrotic damages at the attack site to block the pathogens entry (Durrant & Dong, 2004).

Recently it has been clarified that salicylic acid also plays a role in germination under stressful conditions, although the molecular mechanisms involved are still non fully described (Borsani *et al*., 2001; Rajjou *et al*., 2006; Alonso-Ramırez *et al*., 2009).

Salicylic acid inhibits seed germination in a dosage-dependent manner in maize (*Zea mays*) (Guan & Scandalios, 1995), *Arabidopsis thaliana* (Nishimura *et al*., 2005), and barley (*Hordeum vulgare*) (Xie *et al*., 2007). Germination of salicylic acid deficient seed is severely delayed compared with that of wild-type seeds in the presence of high salt, and this trait is recovered by exogenous application of salicylic acid (Rajjou *et al*., 2006). The involvement of EDR2 in the resistance response mediated by the salicylic acid pathway and the contribution of salicylic acid during germination, led us to speculate that also AtAPO1 could be involved in salicylic acid metabolism. In addition to powdery mildew resistance, *edr2* also shows an enhanced ethylene-induced senescence phenotype (Tang *et al*., 2005).

Recently, to better understand the molecular basis of plant defense responses secondary screenings to identify *edr2* suppressors have been set up (Nie *et al*., 2011; Nie *et al*., 2102). Indeed a gain-of-function mutation in the calmodulin-binding motif of SR1 (SIGNAL RESPONSIVE 1) is able to suppress *edr2* phenotypes (the mediated resistance to powdery mildew and the enhanced ethylene-induced senescence). Calcium is an important second messenger involved in biotic and abiotic stress signalling, calmodulin is one calcium sensor. SR1, a calmodulin-binding transcription factor, contributes to plant

defense responses by binding to the CGCG box in the promoter of its target genes to regulate their expression (Yang & Poovaiah, 2002). SR1 binds to EDS1, a positive regulator of SA signaling (Du *et al*. 2009), to NDR1 and ETHYLENE INSENSITIVE3 (EIN3). These data put in strict relation EDR2 and ethylene. Intriguingly, ethylene biosynthesis and signaling regulate seed germination, ethylene promotes endosperm cap weakening of *Lepidium* and endosperm rupture either in Lepidium or in Arabidopsis and negatively acts on ABA the abscisic acid (ABA) that inhibits these two processes.

We strongly favour the idea that similarly to EDR2, also AtAPO1 acts upstream ethylene regulation, and this regulation can indeed explain *atapo1* seed dormancy.

Material and methods

Plant Materials

The *atapo1-1* (GK_237G04), *atapo1-2* (GB 846D05) and *edr2* (Salk-052496) mutants were found by screening the insertion flanking database SIGnAL (Alonso *et al*., 2003; http://signal.salk.edu/cgi-bin/tdnaexpress). The T-DNA element positions were confirmed by sequencing analysis.

The endosperm cell marker line *pFIS::GUS* (At1g02580; Chaudhury *et al*., 1997) wan kindly provided by R. Gross-Hardt.

Plants were grown under long-day conditions (14 hours light/10 hours dark) at 22°C. Seeds were surface-sterilized, chilled at 4° C for 2 days, and then germinated and grown on plant growth medium (Murashige & Skoog, 1962).

Plasmid Construction and *Arabidopsis* Transformation

Clonings were done using the Gateway™ system (Invitrogen). To generate the *pAtAPO1*:GUS construct, 536 base long genomic region upstream of the *AtAPO1* ATG was amplified by PCR employing Atp582 with Atp583 (supplementary material). The final destiny vector was pBGWFS7 vector (Karimi *et al*., 2002).
All the constructs were sequenced and used to transform Col-0 employing the "floral-dip" protocol (Clough & Bent, 1998).

Determination of Genotypes by PCR and Complementation of the *apo1-2* Mutant

Genomic DNA was extracted as previously described (Masiero *et al*., 2004). *AtAPO1* wild-type alleles were amplified with Atp154/Atp1722, or with Atp1441/Atp1544 (see supplementary material). The two mutant alleles were amplified using the T-DNA left border specific primer Atp1247 together with a gene specific primer, respectively Atp1722 for *atapo1-1* or Atp1441 for *atapo1-2*. *EDR2* wild-type allele was amplified with Atp155 and Atp219, whilst the mutant allele with Atp219 in combination with Atp171.

Whole-Mount Preparation

To investigate ovule and embryo development, developing flowers and siliques were cleared with chloral hydrate:glycerol:water solution 8:1:2 (Colombo *et al*., 2008). Siliques were dissected under a stereomicroscope

and observed using a Zeiss Axiophot D1 microscope (http://www.zeiss.com/) equipped with differential interface contrast (DIC) optics. Images were recorded with an Axiocam MRc5 camera (Zeiss) using the Axiovision program (version 4.1).

Reverse Transcriptase–Mediated PCR

Total RNA was extracted from various tissues using the LiCl method (Verwoerd *et al.,* 1989). DNA contamination was removed using the Ambion TURBO DNA-*free* DNase kit according to the manufacturer's instructions. Reverse transcription was done using the ImProm-II™ Reverse Transcription System (Promega). *AtAPO1* transcript was amplified with Atp173 and Atp 1573. Mutated *atapo1-2* was amplified using Atp1981 and Atp1573 (supplementary material).

GUS Staining Assays and *in situ* hybridisations

GUS staining assays were done as described by Vielle-Calzada *et al*. (2000).
In situ hybridisations with dioxigenin-labelled antisense RNA-probe were performed as previously described (Brambilla *et al*., 2008). *AtAPO1* specific probe was amplified from developing silique cDNAs with Atp 2187 and Atp 2188 and afterwards cloned in pGEM T-easy vector (Promega).

Germination Assays

All germination experiments were performed in 6-cm Petri dishes on filter paper and described in Debeaujon & Koornneef (2000). The

average germination percentage was determined after 1,2,3 and 4 days in a climate room (25°C, 16 hours light/8 hours dark). In some experiments, the seeds sown on water-soaked filter paper were submitted to 5 d of cold treatment at 6°C (chilling) to break dormancy.

Acknowledgements

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Captions

Figure 1

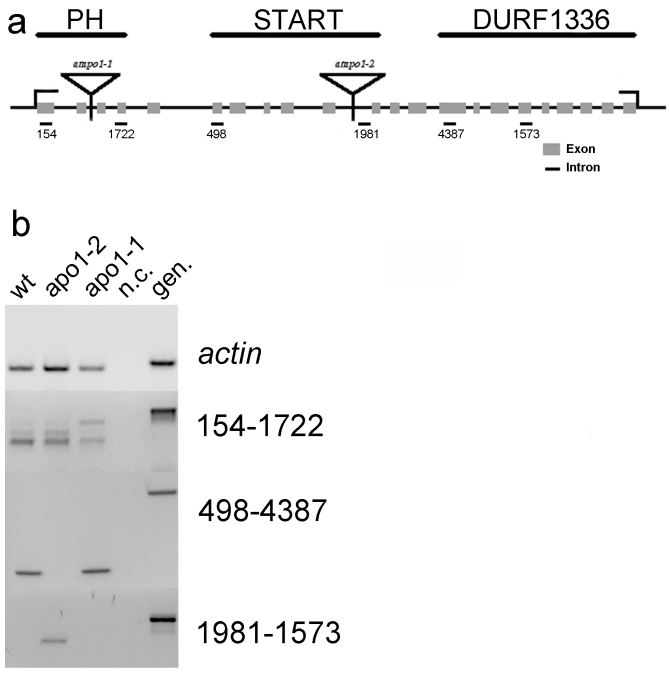

Figure 1. *AtAPOSTART1* gene structure and RT-PCR experiments

(a) Schematic representation of *AtAPOSTART1* gene. T-DNA insertions responsible for the *atapo1-1* and *atapo1-2* mutant alleles are outlined. The PH, START and DUF1336 domains are underlined. The annotation data predict that *AtAPO1* consists of 22 exons and 21 introns, whilst AtAPO1 is 720 aa long.

The primers used for genotyping *atapo1-1* and *atapo1-2* are indicated.

(b) Gene-specific RT-PCR performed on total RNA isolated from *atapo1-1* and *atapo1-2* developing siliques. The primers used are indicated, *atapo1-2* is a knock out allele, in homozygous atapo1-1 plants we can still detect some residual *AtAPO1* transcript. n.c. negative control; gen. genomic DNA has been used as template.

Figure 2

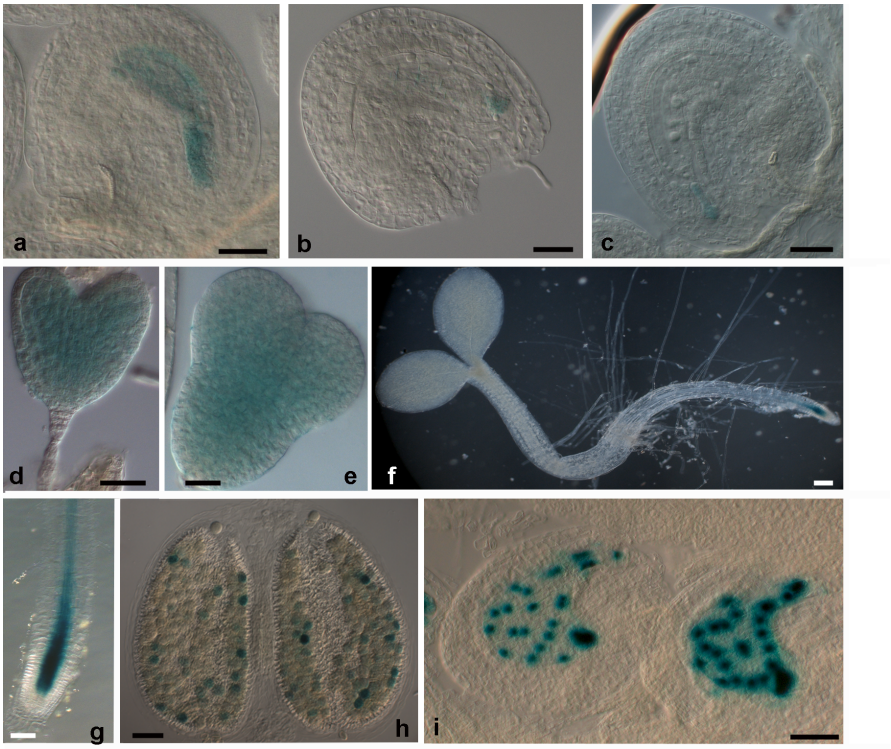

Figure 2. *AtAPO1* spatial and temporal expression pattern.
pAtAPO1::GUS lines have been analyzed.
(a) Arabidopsis mature ovule, the GUS signal is mainly restricted to the mature embryo sac FG7 (Christensen *et al*. 1997). (b-e) GUS activity in developing embryos. GUS signal is detected form the zygote (b) till torpedo stage (e) In 2 and 6 days seedlings, the GUS activity is restricted to the developing roots (f-g). (h) *AtAPO1* is transcribed also in mature pollen grains. (i) *pFIS2::GUS*-driven GUS expression in *ATAPO1/atapo1-2* plants is visible in all developing seeds suggesting that endosperm formation is normal.
Scale bars in (a) to (f) and (i) = 50 µm, in (g) and (h)= 150 µm.

Figure 3

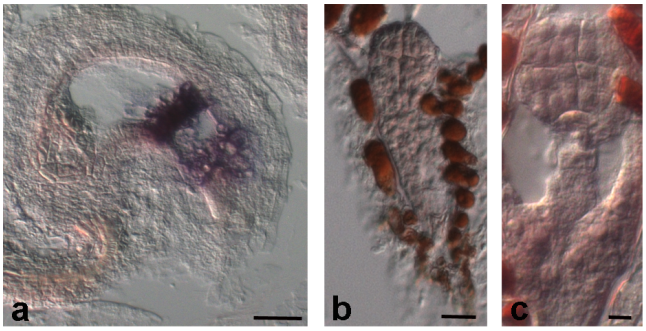

Figure 3. ***in situ*** **hybridization using** ***AtAPO1*** **anti-sense probe.**
(a) Longitudinal section of a mature ovule, *AtAPO1* messenger is detected in the mature embryo sac. *AtAPO1* is transcribed in developing embryos at globular stage (b-c).
Scale bars =50 μm.

Figure 4

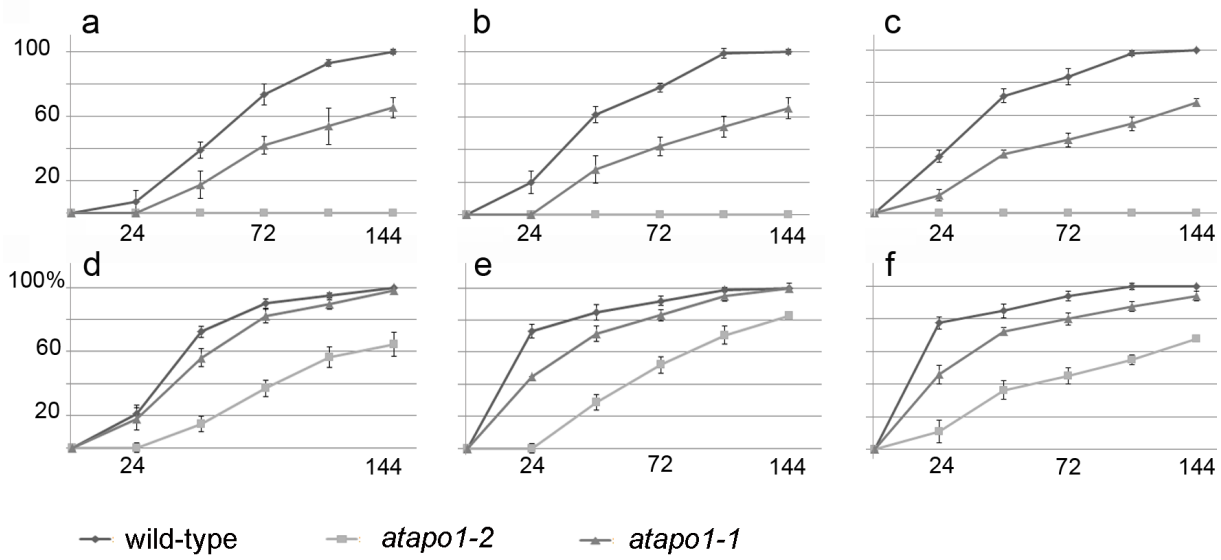

Figure 4. *atapo1-1* and *atapo1-2* germination tests

Germination test of *atapo1-2*, *atapo1*-1 and wild-type sibling seeds.

The graphics report the proportion of germinated seeds (y-axis) over time (x-axis) expressed in hours.

(a,d-f), fresh harvest seeds; (b and c) seeds two and four weeks after harvesting respectively.

Effect of light and chilling on dormancy breaking and germination of wild types and single mutants, were evaluated. (d) seeds have been vernalized for 24 hours, (e and f) vernalization have been prolonged for 48 and 96 hours respectively.

Wild-type seeds closed circle dark grey, *atapo1-1* seeds closed triangles (grey), *atapo1-2* closed squares (light grey).

Figure 5

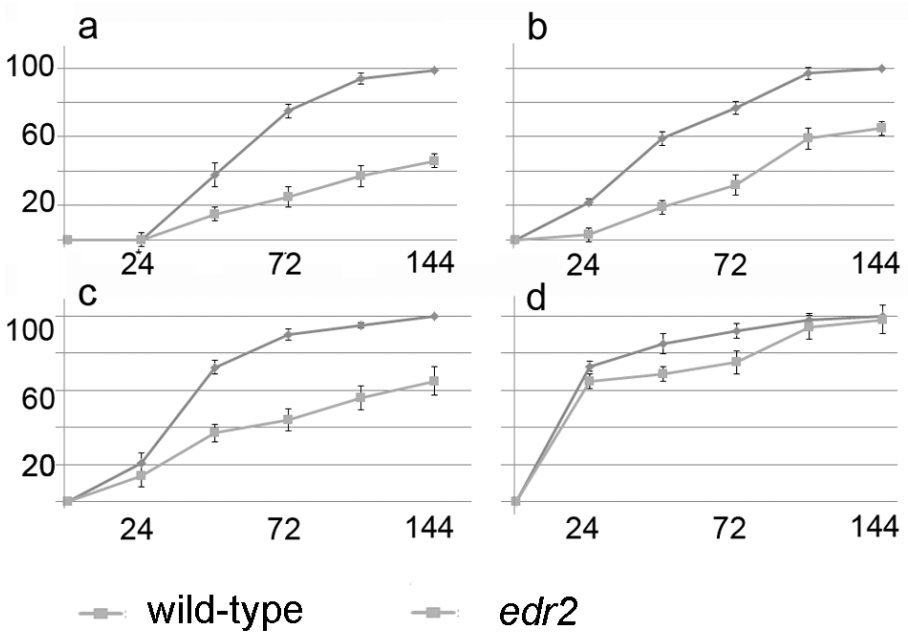

Figure 5. *edr2* germination tests

Germination test of *edr2* (Salk-052496) and wild type seeds.

The graphics report the proportion of germinated seeds (y-axis) over time (x-axis) expressed in hours. (a,c-d), fresh harvest seeds; B two and four weeks after harvesting

seeds. Effect of light and chilling on dormancy breaking and germination of wild types and single mutants, were evaluated. (d) seeds have been vernalized for 24 hours, (e and f) vernalization has been prolonged for 48 and 96 hours respectively.
Wild-type seeds closed circle (dark orange), *edr2* closed squares.

Supplementary information

Figure S1

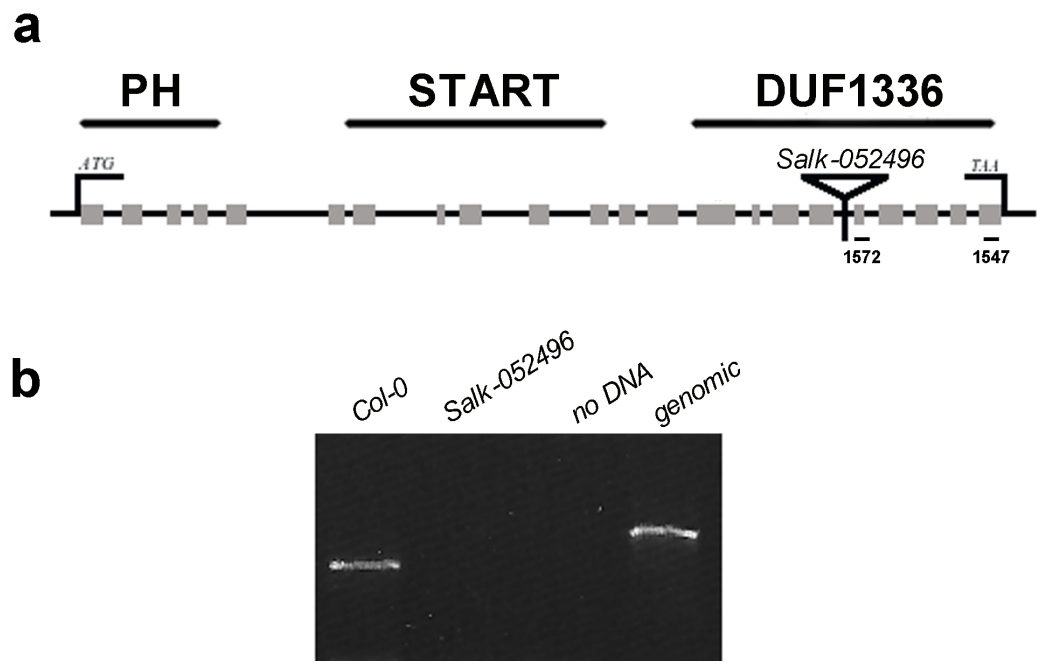

Figure 1. *EDR2* gene structure and RT-PCR experiments

(a) Schematic representation of *EDR2* gene. T-DNA insertions responsible for the *edr2* (Salk-052496) mutant allele is indicated The PH, START and DUF1336 domains are outlined.

(b) Gene-specific RT-PCR performed on total RNA isolated from *edr2*.

Figure S2

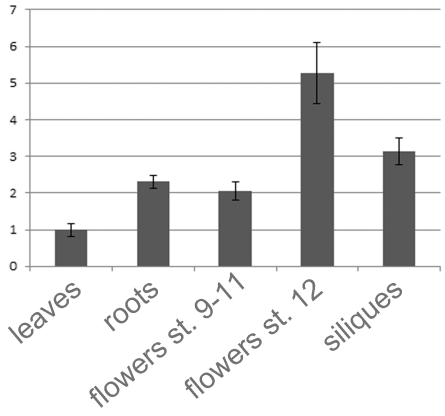

Figure 2. Quantitative RT-PCR for *AtAPO1* expression pattern

AtAPO1 is widely expressed, it is detected in leaves, roots, flowers and siliques. Flower stages are according to Smyth et al (1990).

Figure S3

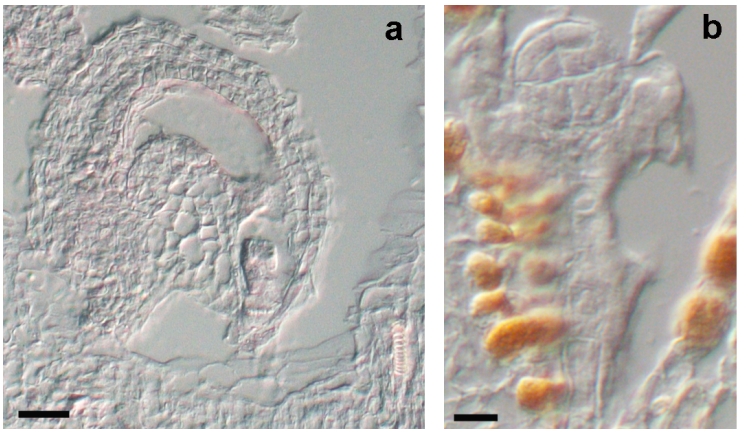

Figure 3. In situ hybridization using AtAPO1 sense probe.

AtAPO1 sense probe in *erd2* developing ovules (a) and globular developing embryos (b) . Bars =50 μm.

Figure S4

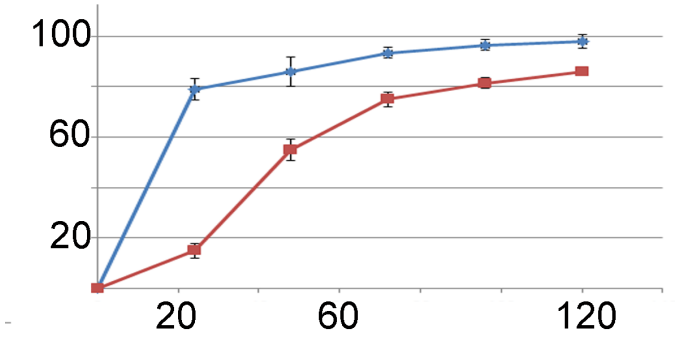

Figure 4. Germination tests

Germination tests of *atapo1-2* (red), and wild type sibling seeds (blue) 8 weeks old.

Table S1

Sequences of primers used in this work

Atp154 5′-GGTAGTGTACGAAGGATGGATGG-3′

Atp155 5′-GACTCCAGATTCGGAACTATAGCC-3′

Atp171 5′-CGATTTCGGAACCACCATCAAACAGGA-3′

Atp173 5′-GGTCCACAACCTGGATGTGTTCG-3′

Atp219 5′-GGCATCATCTTCCTTGAGC-3′

Atp498 5′-CGTAGCTTAATGAGGAGGACAAC-3′

Atp582 5′-
GGGGACAAGTTTGTACAAAAAAGCAGGCTCATGTGTGTTCATGTGAAA
AGC-3′

Atp583 5′-
GGGGACCACTTTGTACAAGAAAGCTGGGTTTCTATTGCCACTTACTCTC
AC-3′

Atp1247 5′-CCCATTTGGACGTGAATGTAGACAC-3′

Atp1441 5′-TTTAGTCAAGCACCATCATTTACG-3′

Atp1544 5′-CACATCTGCATAAGCCTTG-3′

Atp1573 5′-TTTCGTTATCATCGTGTCGC-3′

Atp1722 5′-GACCTGATCTATGACACACTC-3'

Atp1981 5'-CAAGGCTTATGCAGATGTG-3'

Atp2187 5'-GAGTTCTTGGTCTTGTCATC-3'

Atp2188 5'-GATTTGGTAATAGCATATGTGTTG-3'

Atp4387 5′-ACAGGGATCCTGATCGGTTG-3′

PART III

- accepted -

BbrizAGL6 Is Differentially Expressed During Embryo Sac Formation of Apomictic and Sexual *Brachiaria brizantha* Plants

Larissa Arrais Guimarães • Diva Maria de A. Dusi • Simona Masiero • Francesca Resentini • Ana Cristina M. M. Gomes • Érica Duarte Silveira • Lilian Hasegawa Florentino • Júlio Carlyle M. Rodrigues • Lucia Colombo • Vera Tavares de C. Carneiro

Plant Mol Biol Rep
DOI 10.1007/s11105-013-0618-8

BbrizAGL6 Is Differentially Expressed During Embryo Sac Formation of Apomictic and Sexual *Brachiaria brizantha* Plants

Larissa Arrais Guimarães · Diva Maria de A. Dusi · Simona Masiero · Francesca Resentini · Ana Cristina M. M. Gomes · Érica Duarte Silveira · Lilian Hasegawa Florentino · Júlio Carlyle M. Rodrigues · Lucia Colombo · Vera Tavares de C. Carneiro

Abstract Species of the genus *Brachiaria* comprise plants with different modes of reproduction, sexual and apomictic. In apomixis, the embryo sac differentiates from an unreduced cell, and the embryo develops in the absence of egg cell fertilisation. In this work, the characterisation and expression analyses of a MADS-box gene from *Brachiaria brizantha*, named *BbrizAGL6,* was described in sexual and apomictic plants. Phylogenetic analyses indicated that *Bbriz*AGL6 belongs to the AGL6-like subfamily of proteins and clusters together with the AGL6-like protein of other monocots. *Bbriz*AGL6 and AGL6 show conservation of the protein complex. Furthermore, *BbrizAGL6* expressed preferentially in reproductive tissues and corresponding transcripts were detected in anthers and ovules. In ovules of *B. brizantha*, where the main differences among sexual and apomictic reproduction occur, *BbrizAGL6* was differentially modulated. Transcripts of *BbrizAGL6* were localised in the megaspore mother cell of ovaries from apomictic and sexual plants and, additionally, in the region where aposporic initial cells differentiate, in the nucellus of apomictic plants. For the first time, a role of an *AGL6*-like gene in megasporogenesis of apomictic and sexual plants is suggested.

Keywords Apomixis · *Arabidopsis* · *Brachiaria* · MADS-box · Monocot · Plant reproduction

Electronic supplementary material The online version of this article (doi:10.1007/s11105-013-0618-8) contains supplementary material, which is available to authorized users.

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Introduction

The transition between haploid and diploid generations in the life cycle of angiosperms occurs in the ovule. The ovule is also the site of most of the processes relevant to sexual reproduction and to the asexual mode of reproduction through seeds, known as apomixis (Koltunow and Grossniklaus 2003). Sexual reproduction and apomixis diverge in two main steps: meiosis at megasporogenesis and fertilisation (Koltunow and Grossniklaus 2003). In sexual reproduction during the development of the megagametophyte, or embryo sac, after chromosome reduction by meiosis of the Megaspore Mother Cell (MMC), three megaspores degenerate and one functional megaspore is obtained. After three mitotic divisions and differentiation, a seven-celled embryo sac of the Polygonum-type is generated, consisting of: one egg cell, one central cell, two synergids and three

antipodals. Upon double fertilisation, the egg cell and the central cell are fertilised by two spermatic cells, giving rise to the seed embryo and endosperm.

Apomixis is characterised by the omission of meiosis and egg cell fertilisation; it produces a progeny identical to the mother plant by autonomous development of the embryo from an unreduced egg cell. Gametophytic apomixis is classified into apospory or diplospory, depending on the origin of the cell that initiates unreduced embryo sac formation (Nogler 1984; Rodriguez-Leal and Vielle-Calzada 2012). Differentiation of the precursor cell from a MMC that failed to effect meiosis is called diplospory (Nogler 1984). Apospory, observed in *Brachiaria*, a tropical forage grass, is characterised by differentiation of multiple precursors from sporophytic cells, called aposporic initials (AI), in a position other than that occupied by the MMC (Asker and Jerling 1992; Dusi and Willemse 1999; Araujo et al. 2000). Simultaneously, in facultative apomixis, the MMC can form a reduced embryo sac by sexual mode of reproduction. Embryogenesis occurs autonomously without fertilisation of the egg cell in aposporic embryo sacs. Endosperm formation may be autonomous or may require fertilisation (pseudogamy) (Nogler 1984), as in *Brachiaria* (Alves et al. 2001). In *Brachiaria* the unreduced Panicum-type embryo sac differs morphologically from the reduced embryo sac, which is of the Polygonum-type. The Panicum-type embryo sac is composed of just four cells: two synergids, one egg cell and one central cell.

In sexual plants, the formation of the germ line is actively restricted in somatic cells by an epigenetic pathway (Olmedo-Monfil et al. 2010, Singh et al. 2011). In aposporic apomictic plants, somatic cells can originate embryo sacs, thus, its origin is not restricted to the megasporocytes. However, the ability of somatic cells to form embryo sacs in aposporic plants depends on sexual pathway initiation, as shown in apomictic *Hieracium*, where AI appearance requires MMC and tetrad differentiation. These data suggest that an interaction between sporophyte and gametophyte is needed for apomixis (Koltunow et al. 2011). In *Brachiaria brizantha*, AIs are visualised surrounding intact or degenerating tetrads (Araújo et al. 2000). Isolating and characterising genes that are putatively related to the determination of sporophytic and gametophytic fates of the ovule in apomictic and sexual plants can contribute to understanding the molecular basis of these modes of reproduction. Gene expression information is being built in the female reproductive organs, where the main differences between apomictic and sexual plants occur (Pessino et al. 2001; Rodrigues et al. 2003; Polegri et al. 2010; Sharbel et al. 2010; Zhang et al. 2012). In *B. brizantha*, a differential pattern of expression in developing ovaries of apomictic and sexual plants was detected by macroarray analysis (Silveira et al. 2012). Among the genes expressed in ovaries a MADS-box gene similar to the AGL6 of *Zea mays*, *BbrizAGL6*, showed a differential pattern of gene expression in apomictic and sexual plants. This work aims to investigate the association among *BbrizAGL6* expression and the main events of embryo sac differentiation.

MADS-box genes are transcription factors that are involved in all major aspects of plant development (Bowman et al. 1991; Coen and Meyerowitz 1991) including the differentiation of the cells of the ovule (Matias-Hernandez et al. 2010); for review, see Ng and Yanofsky (2001). MADS-box genes were extensively studied in gymnosperms and angiosperms including *Eucalyptus globulus* (Southerton et al. 1998), *Nicotiana tabacum* (Jang et al. 2002), *Ginkgo biloba* (Jager et al. 2003), *Petunia hybrida* (Vandenbussche et al. 2004), *Alpinia hainanensis* (Song et al. 2010), *Coffea arabica* (Oliveira et al. 2010), *Gnetum gnemon* (Wang et al. 2010), *Prunus serotina* (Liu et al. 2010), *Brassica campestris* (Liu et al. 2012a, b), *Pyrus pyrifolia* (Liu et al. 2012a, b) and *Citrus* (Hou et al. 2013). The MADS-box proteins are characterised by a highly conserved DNA binding domain, the MADS-box, which binds a consensus sequence named CArG box (Riechmann et al. 1996). Modulation of DNA-binding specificity and transcriptional activity is determined by the formation of complexes with other MADS proteins (Theissen and Saedler 2001). For instance, ovule identity is determined by a multimeric complex formed by the C-, D- and E-class MADS-Domain factors, SEEDSTICK (STK), SHATTERPROOF1 (SHP1), SHP2 and SEPALLATA3 (SEP3), respectively (Favaro et al. 2003). Interactions between MADS-box proteins have been demonstrated to be conserved in monocot and eudicot plants (Favaro et al. 2002; de Folter et al. 2005). MADS-box transcription factors were extensively studied in various plant species and were able to form specific homodimers and heterodimers (de Folter et al. 2005). AGL6 from *Arabidopsis* and its homolog in rice, OsMADS6, have shown interaction with SEP-like, SQUAMOSA (SQUA) and AGL6 subfamilies (Moon et al. 1999, de Folter et al. 2005). The SEP subfamily is closely related to the AGAMOUS-LIKE6 (AGL6) and SQUA subfamilies (Favaro et al. 2002; de Folter et al. 2005; Zahn et al. 2005). In *Petunia*, AGL6 was able to interact with proteins that form multimeric complexes determining carpel and ovule identity such as SEP, AG and STK proteins (Rijpkema et al. 2009). Recently, it has been shown in *Arabidopsis* that *VERDANDI*, a gene from B3 superfamily, is a direct target for this complex, affecting the identity of antipodal and synergid cells. The evidence indicates that the MADS-box transcription factors are also involved in embryo sac ontogeny (Matias-Hernandez et al. 2010). The involvement of MADS-box genes in the differential development of apomictic plants has not been reported, apart from DEFICIENS in *Hieracium* (Guerin et al. 2000). In this work, we demonstrate that *BbrizAGL6* is differentially expressed in apomictic and sexual *B. brizantha* ovules and is able to interact with those MADS-box proteins that regulate carpel and ovule identity. The involvement of this gene in the regulation of the apomictic pathway is discussed.

Materials and Methods

Plant Material

Two accessions of *B. brizantha* (Syn. *Urochloa brizantha*) from Embrapa's germplasm collection were used in this work: BRA 002747 (B105), a sexual diploid (2*n*=2*x*=18), and BRA 000591 (B030), a facultative apomictic tetraploid (2*n*=4*x*=36) named *B. brizantha* cv. Marandu, with up to 98 % of apospory (Araújo et al. 2000). Both were cultivated in the field at Embrapa Genetic Resources and Biotechnology (Brasília, DF, Brazil). Ovaries from *B. brizantha* were previously classified in four different stages of development. Stages I and II correspond to megasporogenesis, while stages III and IV correspond to megagametogenesis (Araújo et al. 2000; Rodrigues et al. 2003). Reproductive structures and leaves were collected in the field, whilst roots were collected in the greenhouse.

Amplification of a *Brachiaria AGL6* Homologue

A contig from an EST library of ovaries of *B. brizantha* (Silveira et al. 2012) with 83 % to 95 % similarity to the predicted proteins of ZAG3 and ZAG5 members of AGL6 clade (Mena et al. 1995) was used to design primers AG29F (5'-ATCGATCACCAGCAGGCAGAGAG-3') and AG1052 R (5'-CCACGCACACCACAATCACATAG-3') to amplify a *AGL6* homologue. RNA extraction using TRIZOL® (Invitrogen™) and cDNA amplification from a pool of ovaries at the four stages of development were performed as described previously (Rodrigues et al. 2003).

Phylogenetic Analysis

Phylogenetic analysis of 27 MADS-box protein sequences (Table S1) was conducted using MEGA 5 (Tamura et al. 2011) with default settings. The K and C terminal regions of MADS-box genes were used for the alignment using program ClustalW (Thompson 1994). The gap opening penalty was ten, and the gap extension penalty was 0.1 for pairwise alignments, and they were ten and 0.2, respectively, for multiple alignments. The Gonnet matrix was selected, and residue-specific and hydrophilic penalties were ON. The gap separation distance was four, and end gap separation was OFF. Phylogenetic tree was constructed using the neighbour-joining method (Saitou and Nei 1987). The bootstrap values (%) of 1,000 replicates are shown at the branching points.

Yeast Two-Hybrid Assays

The two-hybrid assays were performed using the Ah109 *Saccharomyces cerevisiae* strain. pBD and pAD vector constructs were selected on yeast synthetic dropout (YSD) media lacking leucine (Leu) and tryptophan (Trp), respectively. Two-hybrid interactions were assayed on two selective YSD media, the first lacking Leu, Trp and adenine, and the second lacking Leu, Trp and histidine (His) supplemented with 3-aminotriazole at concentration [5 mM]. Genes used for the yeast two-hybrid assays were cloned in the Gateway vector GAL4 system using vectors adapted from pADT7 (Clontech) for activation domain fusions and pGBKT7 (Clontech) for binding domain fusions passing through pDONOR207 (Life Technologies). The cDNA of the genes was amplified by polymerase chain reaction (PCR) with specific primers containing the attB1 and attB2 sequences for homologous recombination. Cloning of AG, SHP1, SHP2 and STK proteins in AD or BD vectors were previously described (Favaro et al. 2003). The SEP3Δ192 cDNA fragment was amplified and cloned according to Brambilla and co-workers (Brambilla et al. 2007). The coding sequences of *AGL6* and *BbrizAGL6* containing the attB1 and attB2 sequences for homologous recombination were amplified using the following primers AtP3094 (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTCGATGGGAAGAGGGAGAGTG-3') and AtP3095 (5'-GGGGACCACTTTGTACAAGAAAGCTGGGTGTCAAAGAACCCAACCTTGGACG-3') for *AGL6* from *Arabidopsis thaliana* and AtP3139 (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGGGAGGGGACGGGTC-3') and AtP3140 (5'-GGGGACCACTTTGTACAAGAAAGCTGGGTGTCAAAGAACCCATCCCAGCATG-3') for *BbrizAGL6* from *B. brizantha*.

RT-qPCR

RNA extraction from *B. brizantha* developing flowers was performed using TRIZOL® (Invitrogen™) as previously described (Rodrigues et al. 2003). RNA from leaves and roots was extracted using LiCL as previously described (Sambrook 2001). For the RNA extraction experiment, four pools of 1,000 ovaries and four pools of 50 anthers of each stage of pistil development were prepared. Ovaries and anther pools were prepared for apomictic and sexual *B. brizantha*. In addition, young leaves, root tissues and a pool of the whole spikelets containing a mixture of the corresponding four pistil developmental stages were isolated from both *B. brizantha* accessions.

Oligonucleotide pairs for *BbrizAGL6*, RT38F1 (5'-TCTGCAAATCGGGTATCCTC-3') and RT38R1 (5'-CCATCCCAGCATGAAGTTG-3') were designed using Primer 3.0 program (Rozen and Skaletsky 2000) with Tm of 60 °C and primer lengths between 19 and 20 bp. The reference gene used was *BbrizUBCE*, an ubiquitin-conjugating enzyme, previously described as the best reference gene for these samples in *B. brizantha* (Silveira et al. 2009). PCR reactions were performed using Syber Green Rox Plus kit (LGCBIO™) to detect dsDNA synthesis according to manufacturer's protocol. Reactions were run

in a Mastercycler Realplex (Eppendorf™) device using the following cycling parameters—95 °C for 15 min; 40 cycles of 95 °C for 15 s, 58 °C for 15 s, 72 °C for 20 s. The dissociation curve was obtained by heating the amplicon from 60 °C to 95 °C. No-template controls were included for each primer pair. Each amplicon was then analysed by electrophoresis on 1 % agarose gel to verify the specificity of each amplification reaction. Two biological replicates for each of the samples were used for RT-qPCR analysis, and three technical replicates were analysed for each biological replicate. Relative gene expression was calculated using Q-Gene software (Simon 2003).

In Situ Hybridisation

The RNA probe was synthesised using the DIG RNA labelling kit (Roche) according to the manufacturer's protocol. The same PCR fragment of 120 bp from *BbrizAGL6* amplified for RT-qPCR was cloned into pGEM-T Easy Vector System I (Invitrogen™ life technologies) and used as a template for in vitro transcription with SP6 and T7 polymerases, used as sense and antisense probes, respectively. In situ hybridisation was performed in sections of ovaries, at megasporogenesis and megagametogenesis and of anthers at microsporogenesis of the sexual and the apomictic accessions. In situ hybridisation was performed according to Alves et al. (2007). Sections were observed with a Zeiss Axiophot light microscope.

Results

BbrizAGL6 Shares High Sequence Similarity with *AGL6* Isolated from Other Monocot Species

A sequence of 1,159 bp, similar to the predicted proteins of ZAG3 and ZAG5 members of AGL6 clade, was amplified from cDNA of ovaries of apomictic and sexual *B. brizantha* plants. This sequence, with an identical amino acid predicted sequence in sexual and apomictic plants, was named *Bbriz*AGL6.

*Bbriz*AGL6 shows two conserved motifs (I and II) in the C-terminal region, a typical feature of AGL6 clade members (Ohmori et al. 2009) (Fig. 1). The first motif is well conserved in monocots and represents a ten amino acid residue sequence, DCEPTLQIGY; however, in *Bbriz*AGL6, glutamic acid is substituted by aspartic acid. The second motif is at the end of the C-terminal region and is composed of a ten amino acid residue sequence, ENNFMLGWVL.

Phylogenetic analyses have also proved that *Bbriz*AGL6 belongs to the AGL6-like subfamily of proteins and clusters together with the AGL6-like protein of other monocots (Fig. 2).

*Bbriz*AGL6 Interacts with Proteins that Form Ovule and Carpel Identity Complex in *A. thaliana*

To investigate whether *Bbriz*AGL6 is able to interact with MADS-box proteins related to ovule and carpel identity, a GAL4-based yeast two-hybrid experiment was performed between *Bbriz*AGL6 and *A. thaliana* MADS-box proteins, those that determine ovule and carpel identity. AG, SHP1 and SHP2 are members of the C-class; the D class STK and SEP3Δ192 from E-class were tested. A deleted version of SEP3 was used since the full protein auto-activates the reporter gene (Brambilla et al. 2007). Analyses proved that *Bbriz*AGL6 homodimerises and heterodimerises with AG, SHP1, SHP2 and STK. However, *Bbriz*AGL6 interacted with SHP1, SHP2 and STK only when *BbrizAGL6* coding sequence was fused to the activation domain (AD), and SHP1, SHP2 and STK to the binding domain (BD) (Table 1). None of the proteins tested were able to interact with empty vector AD or BD when the transformed yeast was grown in media lacking Trp, Leu and His, supplemented with 5 mM 3-aminotriazole.

BbrizAGL6 Is Sporophytically Expressed in Flowers

The pattern of *BbrizAGL6* expression in apomictic and sexual *B. brizantha* was firstly investigated by RT-qPCR in reproductive and vegetative structures (Fig. 3). Expression of *BbrizAGL6* was restricted to the reproductive tissues; the strongest signal being observed in ovaries. Ovaries from sexual plants showed higher expression compared with those of apomictic plants. The highest *BbrizAGL6* expression was observed at stage II, corresponding to ovules at megasporogenesis (Dusi and Willemse 1999; Araújo et al. 2000). In developing anthers, *BbrizAGL6* expression was lower compared with developing ovules.

In situ hybridisation analysis was performed to investigate in detail the spatial and temporal pattern of *BbrizAGL6* expression. During microsporogenesis, hybridisation signal was stronger in pollen mother cells (PMC) and lower in the tapetum cells (Fig. 4a, d). Transcripts of *BbrizAGL6* were localised in the MMC of ovaries from apomictic and sexual plants (Fig. 4b, c, e, f). In apomictic plants, expression was also present in the nucellus surrounding the MMC (Fig. 4b, c). During megagametogenesis, *BbrizAGL6* expression decreased in the ovaries of apomictic and sexual plants (Fig. 4g, h). Control in situ hybridisation with a *BbrizAGL6* sense probe confirmed that the probe employed is specific since we could not record any signals (Fig. 4i, j).

Discussion

Knowledge about molecular pathways involved in different types of reproduction in flowering plants is still elusive. MADS-box genes are much conserved among living organisms

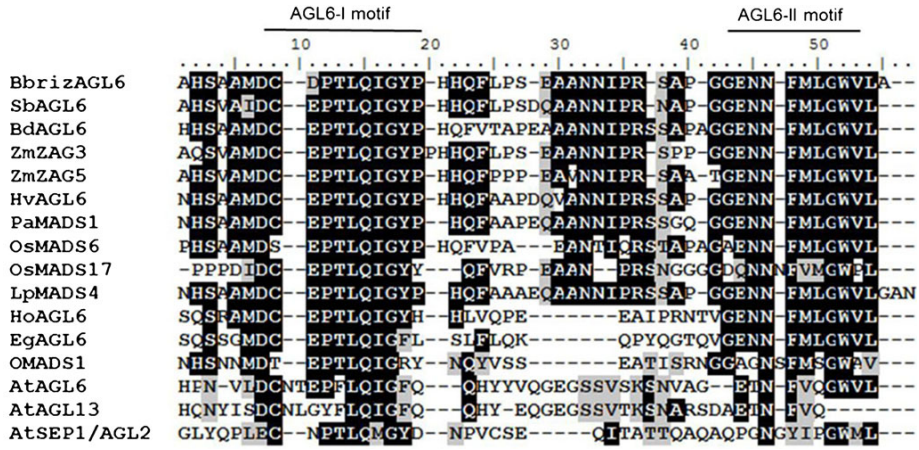

Fig. 1 Alignment of C-terminal region of AGL6-like MADS-box proteins. The C-terminal regions of 15 AGL6-like MADS-box proteins from various monocot plant species and from *Arabidopsis* were used (see Supplemental Table 1) *Arabidopsis* SEP-like protein (at SEP1/AGL2) sequence was inserted in the alignment for the identification and comparison of the motifs. The positions of two conserved motifs are indicated *above* the sequences. Identical and similar amino acids are *shaded black* and *grey*, respectively. Analyses were done using ClustalW program

(Purugganan et al. 1995). They are widely studied in plants, contributing to the explanation of various aspects related to floral organ formation. This work sheds light on the involvement of MADS-box genes in apomictic and sexual reproduction, since they are poorly described in apomictic plants.

MADS-box transcription factors classified as MIKC are exclusive to plants and are composed of a MADS (M-), intervening (I-), keratin-like (K-) and C-terminal (C-) domain (Theissen et al. 1996). The MADS-domain is responsible for DNA binding. K domain mediates interactions, specified by I-domain, between other MIKC-type proteins (Davies and Schwarz-Sommer 1994). The C-terminal domain is the most divergent among different MADS-box genes but displays conserved motifs among members of the same clade (Davies and Schwarz-Sommer 1994), determining their divergent biological functions and possibly forming a specific network of interactions (Egea-Cortines et al. 1999; Honma and Goto 2001). *Bbriz*AGL6 shows two motifs that are similar to those previously described in different species, especially among monocots (Zahn et al. 2005) and which resemble SEP proteins. This region is known to be essential for protein–protein interaction and transcriptional activation (Honma and Goto 2001). The AGL6-I and AGL6-II motifs were described in rice as transcriptional activation domains, and the AGL6-II has stronger activity than the AGL6-I one (Ohmori et al. 2009). Replacing the glutamic acid, found in the other monocot sequences, with aspartic acid, in the first motif of *Bbriz*AGL6, might not be relevant as both are polar and negative amino acids. The *Bbriz*AGL6 second motif is an acidic-rich domain and is identical to the AGL6-II motif identified in maize. The negative charge in this motif is critical for the structure of activation domain (Ohmori et al. 2009).

Phylogenetic analysis indicated that the predicted amino acid sequence of *Bbriz*AGL6 is grouped together with AGL6 from other monocot species and is close to the SEP group, which is highly conserved among various plant species (Reinheimer and Kellogg 2009). *Bbriz*AGL6 can be included in the same clade formed by ZAG3 and ZAG5 from maize, OsMADS6 from rice and SbAGL6 from sorghum. *AGL6*-like genes from grasses were shown to be expressed in paleas, lodicules, ovules and floral meristems and show a SEPALLATA-like function (Reinheimer and Kellogg 2009, Li et al. 2011). It was demonstrated that *AGL6* has a role in the formation of floral organs in monocots and eudicots (Viaene et al. 2010).

Studies in various plants indicated AGL6's ability to form multimeric complexes with MADS-box transcription factors related to ovule and carpel identity, similarly to SEP (Moon et al. 1999, de Folter et al. 2005). SHP1 and SHP2, related to the shattering of dehiscent fruits (Liljegren et al. 2000), act redundantly with STK in *Arabidopsis* ovules (Pinyopich et al. 2003) being grouped in an AG monophyletic group (Parenicova et al. 2003). *Bbriz*AGL6 interaction with

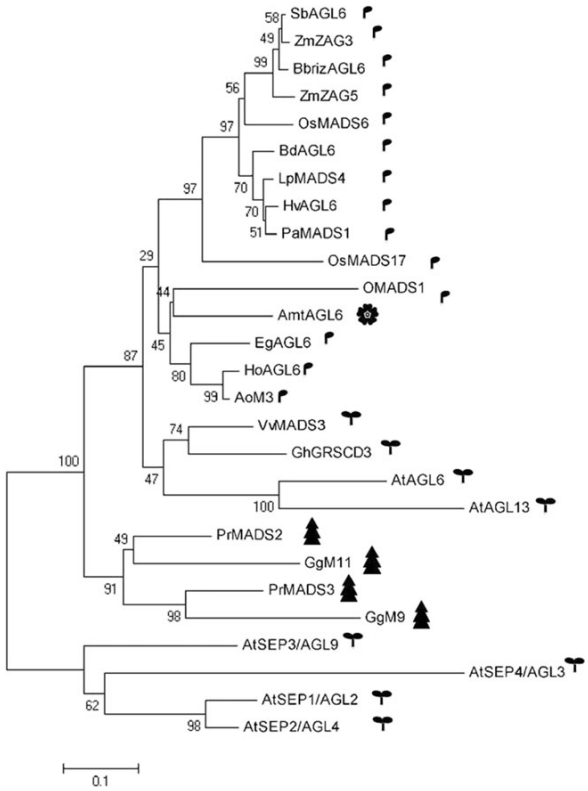

Fig. 2 Phylogenetic tree of MADS-box proteins constructed by the neighbour-joining method using 27 MADS-box protein sequences (see Supplementary Table S1). The bootstrap values (%) of 1,000 replicates are shown at the branching points. represent eudicot, monocot, magnoliid and gymnosperm, respectively

Table 1 Yeast two-hybrid study using the GAL4 system for testing interactions of *Bbriz*AGL6 with other MADS-box proteins: AG, SHP1, SHP2, STK, AGL6 and SEP3Δ

*Bbriz*AGL6	C-class			D-class	E-class		
	AG	SHP1	SHP2	STK	AGL6	SEP3Δ	*Bbriz*AGL6
−W-L-A[a]	+a	+a	+a	+a	−b	+a	–
−W-L-H[b]	+	–	–	–	++	++	+

[a] Selective dropout medium lacking Trp, Leu and adenine (−W-L-A)

[b] Selective dropout medium lacking Trp, Leu and His (−W-L-H) supplemented with 3-aminotriazole at concentration [5 mM]

++ Interactions clear in both directions, + interactions positive only in one direction, – no growth, *a* interactions could only be tested in one direction due to autoactivation of the GAL4 reporter gene by intrinsic transcription activation domain, *b* interactions could not be tested due to autoactivation of the GAL4 reporter gene by intrinsic transcription activation domain

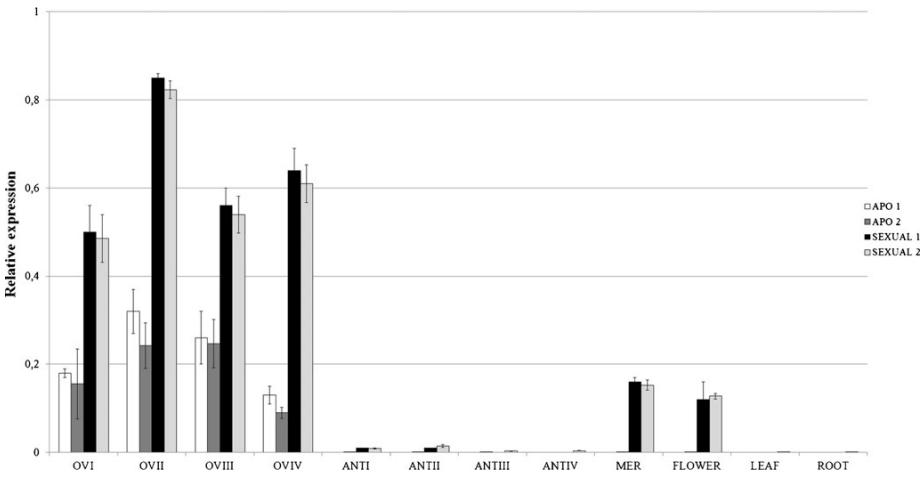

Fig. 3 Expression profile of *BbrizAGL6* by RT-qPCR in ovaries in megasporogenesis (ovI, ovII) and megagametogenesis (ovIII, ovIV); anthers in microsporogenesis (ANTI, ANTII) and microgametogenesis (ANTIII, ANTIV); inflorescence meristem (MER); flower; leaf and root from apomictic and sexual plants. *White* and *black columns* represent first biological replicate from apomictic (APO 1) and sexual (SEXUAL 1) plants, respectively. *Dark* and *light grey columns* represent second biological replicate from apomictic (APO 2) and sexual (SEXUAL 2) plants, respectively. *Bars* represent the standard error of technical variation. *BbrizAGL6* is more expressed in sexual plants than in apomictic plants, especially in ovaries. Higher *BbrizAGL6* expression was detected in sexual ovII; the stage where MMC starts its development

SHP1, SHP2 and STK can be due to its SEP-like function since SEP3 gene was designated as glue in the formation of the multimeric complex in *Arabidopsis* (Immink et al. 2009). This multimeric complex can be related to *Brachiaria* reproduction, since *BbrizAGL6* is modulated in apomictic and sexual plants.

Yeast two-hybrid of OsMADS6, a rice AGL6 homolog, did not show any interaction with B and C class of MADS-box. Moreover, it was recently demonstrated that OsMADS6 is a key regulator of transcription levels of B-, C- and E-class genes (Li et al. 2011). However, ZAG3 from maize showed interaction with C class of MADS-box genes (Moon et al. 1999; Thompson et al. 2009), results similar to that observed with *Bbriz*AGL6. Interestingly, the rice AGL6-II motif is slightly different from those of *Bbriz*AGL6 and ZAG3, which are identical. Analysis using yeast two-hybrid demonstrated that BbrizAGL6 has the same role as AGL6 from *Arabidopsis* and as observed in the literature with maize. *AGL6* belongs to MIKC MADS-box type genes. Generally, genes from this group are expressed only in diploid tissues that control various aspects of sporophyte development. They are involved in almost all levels of the regulatory network that controls reproductive development. Some of them are affected by stress treatment regulating flowering time (Gramzow and Theissen 2010).

The expression of *BbrizAGL6* in the ovary during megasporogenesis of sexual *B. brizantha* is consistent with data obtained in other species.

In *Arabidopsis*, rice and maize, *AGL6* expression is more abundant in ovule sporophytic tissues, and its expression is restrained to flower organ (Mena et al. 1995; Ohmori et al. 2009; Reinheimer and Kellogg 2009; Koo et al. 2010). In rice, *AGL6* mRNA was strongly detected in MMC and microspores (Zhang et al. 2010). The peak of expression of *BbrizAGL6,* observed at stage II ovaries of apomictic and sexual plants, could be related to events that are more recurrent in this stage, such as meiosis. The localisation of expression at this stage was preferentially detected inside MMC and PMC, cells which will enter meiosis. Remarkably, in apomictic plants, a broader expression of *BbrizAGL6* included the nucellar region near the MMC, where AI cells differentiate to form unreduced embryo sacs. These data suggest a deregulation of the spatial expression pattern of *AGL6* during sporogenesis of apomictic plants. It is possible that the presence of *AGL6* in the nucellar region of apomictic plants enables AI formation; alternatively, *AGL6* could mark the differentiation of sporogenous cells, either the MMC in sexual plants, or the AI precursor cells in apomictic plants, suggesting that the expression of *BbrizAGL6* could be involved at sporogenesis, regardless of mode of reproduction.

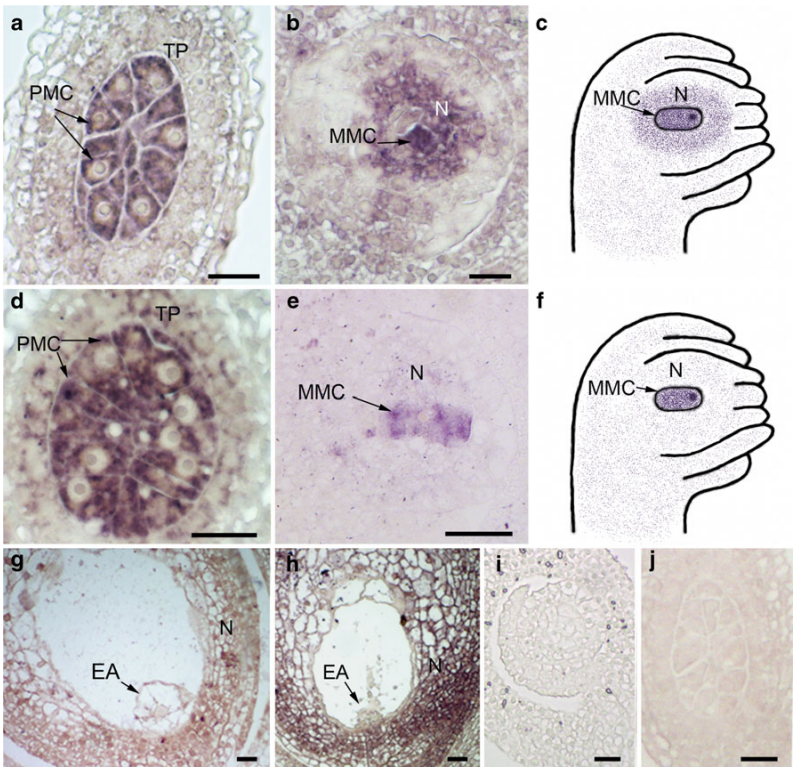

Fig. 4 Detection of *BbrizAGL6* transcripts by in situ hybridisation in semi-thin sections of anthers (**a**, **d**) and ovaries (**b**, **e**, **g**, **h**) of apomictic (**a**, **b**, **g**) and of sexual (**d**, **e**, **h**) *B. brizantha*. Schematic representation of in situ hybridisation at megasporogenesis of apomictic (**c**) and sexual (**f**) plants. Hybridisation of sense probe in ovary (**i**) and anther (**j**) of apomictic *B. brizantha*. *EA*, egg apparatus; *MMC*, megaspore mother cell; *N*, nucellus; *PMC*, pollen mother cell; *TP*, tapetum. *Bar*=20 μm

The presence of *AGL6* in male sporogenous cells of sexual and apomictic *B. brizantha*, also observed in rice (Zhang et al. 2010), suggests that the latter is most likely the case.

Based on in situ analysis showing that *AGL6* is not present in gametophytic cells in rice (Zhang et al. 2010) and *B. brizantha* sexual and apomictic plants (this paper), this could be a conserved element in *AGL6* regulation, regardless of mode of reproduction. Therefore, *AGL6* appears to be required not only for sexual reproduction but also for the apomictic process. This corroborates the hypothesis that apomixis is a consequence of deregulation of the sexual pathway (Koltunow and Grossniklaus 2003; Rodriguez-Leal and Vielle-Calzada 2012).

We have successfully cloned and characterised a MADS-box gene, *BbrizAGL6*, from *B. brizantha* plants. The phylogenetic association with MADS-box genes of other monocots was shown. In ovaries, *BbrizAGL6* was expressed only in the MMC of sexual plants while in apomictic plants it was expressed more broadly in the MMC and surrounding cell types. *BbrizAGL6* can be a useful marker for the characterisation and study of apomixis in *Brachiaria*.

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CONCLUSION AND FUTURE PERSPECTIVE

Differently from animals, plant life cycle is characterised by alternation of generations, one diploid and one haploid. The female haploid generation develops and grows protected inside the maternal tissue, thus for long time has been referred as the hidden generation (Brukhin *et al*., 2005) (Figure 11).

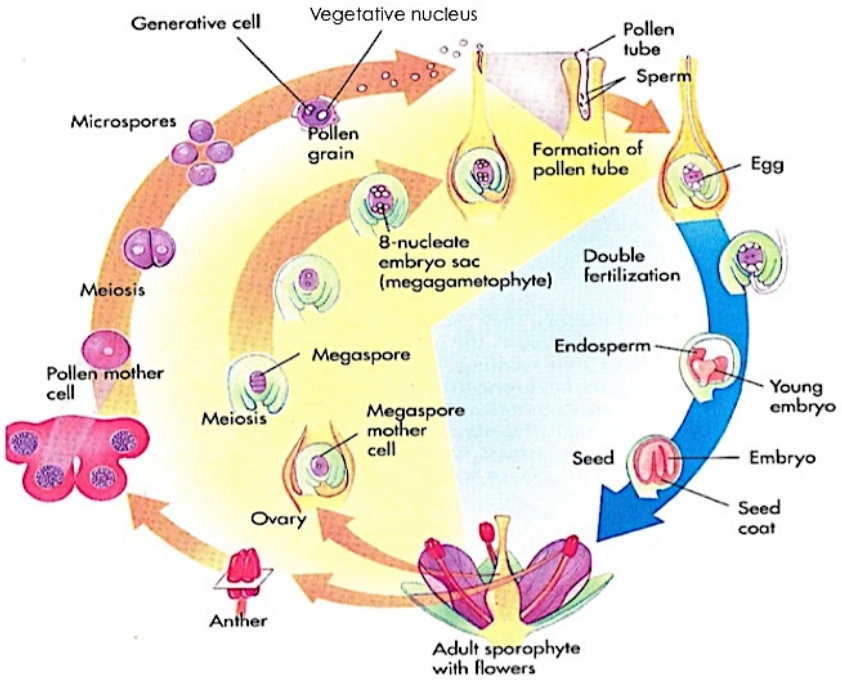

Figure 11. Angiosperm life cycle.

Although genetic and molecular approaches have identified many factors involved in male and female gametophyte development (Borg *et al*., 2009; Brukhin *et al*., 2005; Dresselhaus and Marton, 2009; Kägi and Gross-Hardt, 2007; Liu and Qu, 2008; Sundaresan and Alandete-Saez, 2010; Yadegari and Drews, 2004; Yang *et al*., 2010) the molecular networks controlling embryo sac cell differentiation are still elusive.

The screening of T-DNA insertion lines for functionally important gametophytic genes has led to the identification of several T-DNA-tagged gametophytic mutants in *Arabidopsis* (Feldman *et al*., 1997; Bonhomme *et al*., 1998; Howden *et al*., 1998; Christensen *et al*., 2002), also

transposon-based gene trap systems have been successfully used (Sundaresan *et al*., 1995; Pagnussat *et al*., 2005). Such efforts ended with lists of genes whose products are necessary for embryo sac formation and/or its proper differentiation (Christensen *et al*., 2002; Drews and Yadegari, 2002; Grini *et al*., 2002; Huck *et al*., 2003; Kwee and Sundaresan, 2003; Rotman *et al*., 2003). However the dissection of the female gametophyte developmental pathways into defined genes and functions is still an unaccomplished goal.

More recently several laboratories have worked to determine the total set of genes expressed in the embryo sacs, by combing cell isolation, genetic subtractions and high through-put sequencing (Tzafrir *et al*., 2004; Sprunck *et al*., 2005; Yu *et al*., 2005; Jones-Rhoades *et al*., 2007; Johnston *et al*., 2007; Wuest *et al*., 2010; Xiang *et al*., 2011). These approaches produced long lists of genes differentially expressed in the developing embryo sacs. A spatially resolved view of gene expression allows the elucidation of transcriptional networks that are linked to cellular identity and function. Moreover it clearly emerged that differentially expressed post-transcriptional regulatory modules and metabolic pathways characterize the four distinct embryo sac cell types (Wuest *et al*., 2010; Sprunck *et al*., 2005; Lê *et al*., 2005).

The dissection of gene regulatory networks requires identification of transcription factors conferring cell-specific expression, as well as the *cis*-regulatory elements through which these transcription factors act to activate downstream genes (Levine and Davidson, 2005). Sometimes, transcription factors conferring cell-specific expression have been identified using forward-genetics approaches in which mutants were identified and analysed (Steffen *et al*., 2007).

Alternatively it is very informative to identify genes expressed in specific cell types, to characterize the *cis*-regulatory elements within

these genes able to confer cell-specific expression, and consequently identify the transcription factors able to bind these (Levine and Davidson, 2005).

Isolation of wheat egg cells and subsequent EST analyses resulted in the identification of the large, egg cell-specific transcript cluster *TaEC-1* (*Triticum aestivum ECA1-like*) specifically expressed in the egg cell and sharing high sequence similarity to the barley *ECA1* (Sprunck *et al*., 2005; Vrinten *et al*., 1999). The *TaECA1*-like genes encode small proteins of circa 151 amino acids with six conserved cysteine residues and a putative extracellular localization signal: these features indicate their involvement in cell signalling. However only reverse genetic studies have been able to shed light into the biological function of this small gene family (Sprunck *et al*., 2012).

During my PhD we tried to shed light into embryo sac cell differentiation and to identify those transcription factors involved in female gamete formation. In particular we wanted to identify the key regulators of genes specifically expressed in the egg cell, namely *EGG CELL1.1* (*EC1.1*), *EC1.2*, *EC1.3*, *EC1.4* and *EC1*.5, by using a yeast one-hybrid approach.

I have been able to show that SUF4, SUPPRESSOR OF FRIGIDA 4, is a candidate. Our *in vivo* and *vitro* data indicate that SUF4 is a real regulator of *EC1.1* and coherently with its role SUF4 is detected from FG1 to the female gametophyte mature, where it is present in the synergids and in the central cell.

Additionally, all together our data indicate that SUF4 is able to regulate all the five *EC1* genes, as demonstrated by gel retardation assays and by real time PCR analyses.

We speculate that SUF4 regulates the *EC1* genes by acting together with other transcription factors. *EC1* regulation is not achieved through

a SUF4-FRI dimer since the *EC1.1* expression pattern, either by *in situ* either by analysing transgenic plants (*pEC1.1:GUS, pEC1.1:GFP;* Sprunck *et al*., 2012), has been studied using the *Arabidopsis* ecotype Columbia (*Col-0*). *Col-0* lacks FRI activity because of a small deletion at the *FRI* locus (Lempe *et al*., 2005). At the moment we are trying SUF4 partners involved in *EC1* gene regulation.

We used a bioinformatics approach to verify whether *SUF4* is co-expressed with other genes. These analyses revealed that *MOM1* (*Morpheus's molecule1*) is co-expressed with *SUF4*; MOM1 is required for transcriptional gene silencing maintenance (Amedeo *et al*., 2000). Conversely also MOM1 contributes to *SUF4* expression and to *EC1.1* spatial-temporal regulation. It is not clear how MOM1 acts on transcriptional gene silencing, indeed in *mom1* mutant poor alteration in heterochromatin state are recorded; indeed MOM1 does not affects DNA and histone methylation (Vaillant *et al*., 2006). Recently it has been shown that MOM1 promotes gene-silencing interaction with RNA polymerase IV and V (Yokthongwattana *et al*., 2009; Wierzbicki, 2010).

Definitely a full comprehension of MOM1 molecular action will allow to shed light also into its role in egg cell differentiation and will clarify the its relation with SUF4.

Right now I planned some experiments finalised to better clarify how MOM1 regulates *EC1.1*, we found very interesting that MOM1 prevents *EC1.1* transcription in the carpel leaves. At the moment I am trying to identify which molecular epigenetic mechanism regulates *EC1.1*, verifying whether the *EC1.1* histone code is alterated in *mom1* mutant. In particular I'm exploring the histone code in of pEC1.1 focusing on histone acetylation (Figure 12).

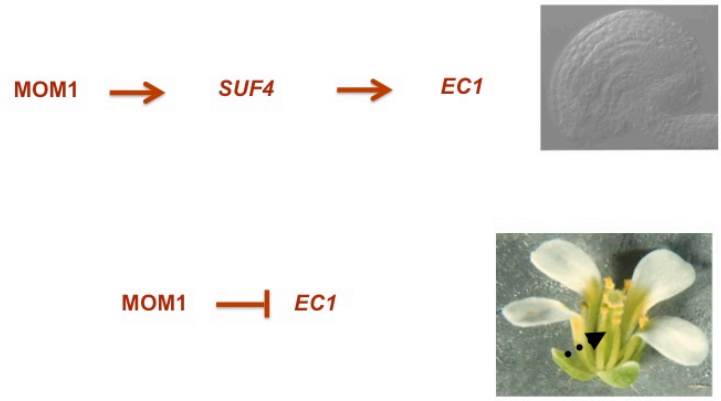

Figure 12. Possible role of MOM1 for EC1 gene regulation in *Arabidopsis* ovule.

Plant can produce progeny without sexual reproduction, and one example is apomixis.

The inheritance of apomixis has been established in some species and a molecular mapping study has been initiated. The molecular relationships between apomictic and sexual reproduction, however, were still unknown.

Considerable information has been accumulated concerning the regulation of ovule and female gametophyte development in sexual plants, and this provides molecular tools for the comparative analysis of sexual and apomictic reproduction (Koltunow and Grossniklaus, 2003). Tucker *et al*. (2003) provided strong evidences that, sexual and apomictic, reproduction in *Hieracium* is related developmental pathways that share common regulatory programs (Eckardt, 2003).

The exploitation of the genetic pathways controlling apomixis are quite complicated by lack of recombination, irregular segregations and polyploidy are even more difficult are analyses of apomictic seed formation itself because the processes of interest take place deeply embedded within the ovule, even if sexual and asexual reproduction take place inside the same organ.

In order to further discover which is the mechanism that is the responsible for sexual or asexual reproduction I collaborated with two groups that work on apomixis and use as *Poa pratensis* and *Brachiaria brizantha* as models.

Kentucky bluegrass (*Poa pratensis*) is an important fodder and turf grass. Considerable effort is being expended to breed improved cultivars, particularly in Europe and North America. It is an aposporous pseudogamous facultative apomict and highly variable as to reproductive, chromosomal, and phenotypic features.

APOSTART1 (*PpAPO1*) was isolated in *Poa pratensis* by the cDNA-AFLP technique to isolate genes differentially expressed in apomictic and sexual genotypes (Albertini *et al*., 2003). We showed that *PpAPO1*shares high similarity with two *Arabidopsis thaliana* genes, At5G45560 and At4g19040. The latter one was named EDR2 (Enhanced Disease Resistance 2; Tang *et al*., 2005).

It has already been published that *edr2* homozygous plants do not show any developmental defects but they show enhanced capacities to resist to *E. cichoracearum* infections (Tang *et al*. 2005; Vorwerk *et al*., 2007).

We characterized the expression and function of *AtAPO1* (*Arabidopsis thaliana APOSTART1*) and we demonstrated with our data that *AtAPO1* is involved in seed dormancy since its down regulation affects germination rate in fresh harvest and dry stored seeds.

Germination involves the mobilization of storage reserves and the initiation of growth and metabolic activity within the embryo, thus early germination stages show high respiration rate.

Interestingly, our data suggest a role for *AtAPO1* during this process; in fact EDR2 acts as a negative regulator of cell death, and phosphatidylinositol-4-phosphate may have a role in limiting cell death

via its effect on EDR2. Vorwerk and co-workers linked also EDR2 to the salicylic acid pathway, although it is not clear whether it acts up- or down- stream.

All together our data lead us to speculate the role of *AtAPO1* in salicylic acid metabolism and to demonstrate that apomixis can be a consequence of alterations in hormonal networks.

Taking into account these data, it could be interesting in a future verify in *Poa pratensis* if alterations in homeostasis could activate or inactivate apomixis.

On the other hand we tried to shed light on the involvement of MADS-box genes in apomictic and sexual reproduction, since they are poorly described in apomictic plants. MADS-box transcription factors classified as MIKC are exclusive to plants and are composed of a MADS (M-), intervening (I-), keratin-like (K-) and C-terminal (C-) domain (Theissen *et al*., 1996).

Among the genes expressed in ovaries a MADS-box gene similar to the *AGL6* of *Zea mays*, showed a differential pattern of gene expression in apomictic and sexual plants.

In *Brachiaria brizantha*, a differential pattern of expression in developing ovaries of apomictic and sexual plants was already detected by macroarray analysis (Duarte Silveira *et al*., 2012).
BbrizAGL6 shows a region essential for protein-protein interaction and transcriptional activation (Honma and Goto, 2001) and is close to the SEP group.

We were able to demonstrate that *BbrizAGL6* is also able to interact with MADS-box proteins that regulate carpel and ovule identity; the interaction with SHP1, SHP2 and STK can be due to its SEP-like function. These interactions demonstrated that *BbrizAGL6* has the same role as *AGL6* from *Arabidopsis*.

Based on our data *AGL6* appears to be required not only for sexual reproduction but also for the apomictic process. This corroborates the hypothesis that apomixis is a consequence of deregulation of the sexual pathway.

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APPENDIX

TCP14 and TCP15, together with DELLA, regulate *Arabidopsis* seed germination

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TCP14 and TCP15, together with DELLA, regulate seed germination

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Introduction

Angiosperm seeds are the final results of the double fertilization, in which one of the two sperm cells fertilizes the haploid egg cell, giving rise to a diploid embryo, and the other sperm cell fertilizes the polar nuclei in the central cell, giving rise to the triploid endosperm (Chaudhury *et al*., 1997). As the embryo and the endosperm develop, the ovule enlarges into a seed and the maternal ovule integuments, protecting the embryo sac, turn into the seed coat (West & Harada 1993). Seed development proceeds through two distinct phases (morphogenesis and maturation) during which the growth and development of the three compartments are strictly coordinated (Berger *et al*. 2006; Gutierrez *et al*. 2007). Soon after fertilization, the zygote undergoes an asymmetric cell division; the apical daughter cell will generate the proper embryo whilst the basal cell is the progenitor of the suspensor. The second phase, or post-embryonic development, is characterized by cell maturation, which involves cell growth and the storage of macromolecules such as oils, starch and proteins needed during germination and precocious seedling growth (West & Harada 1993; Baud *et al*., 2008). During this phase embryo can increase in size up to 100 fold (Gendreau *et al*., 1997).

At the end of embryonic development, most seeds dehydrate to about 5% moisture content, acquisition of desiccation tolerance is part of the seed maturation program (Manfre *et al*., 2006). Hydrophobic interactions with the aqueous solution are important for maintaining the integrity of the lipid bilayer (Lodish *et al*., 2000).

After reaching physiological maturity, seeds of many plant species, including *Arabidopsis thaliana*, may enter in a state of dormancy (Chibani *et al*., 2006; Debieu *et al*., 2013). *viviparous* mutants fail to undergo the

maturation program leading to seed dormancy but instead germinate directly (White *et al*., 2000).

Hormonal mechanisms and genetic programs strictly control the maturation phase and the dormancy organisation. Abscisic acid (ABA) is necessary to induce the expression of genes involved in maturation and desiccation tolerance. *viviparous* mutants are either ABA deficient or insensitive (Koornneef *et al*., 1982). Seed dormancy is an adaptive trait, since seeds remain quiescent until germination conditions become favourable; the primary factors are water availability and season (Finch-Savage & Leubner-Metzger, 2006). In mature seeds, the break of dormancy may either occur gradually in the dry state (after-ripening) or be initiated by imbibition under defined conditions (e.g. cold stratification or chilling at low temperature (Koornneef *et al*., 2002; Donohue *et al*., 2005).

In *Arabidopsis thaliana,* as in many other species, both dormancy and germination potential are determined by the interaction between genetic and environmental factors and these processes are mediated mainly by the ratio of two antagonistic hormones: ABA and gibberellins (GAs). ABA promotes the establishment of seed dormancy whilst GA opposes this effect favouring the breaking of seed dormancy and triggering germination (Richards *et al*., 2001). GAs are tetracyclic diterpenoid growth factors that are essential regulators of stem elongation and other plant developmental processes (Itoh *et al*., 2002; Nakata *el al*., 2009; Rueda-Romero *et al*., 2012).

Germination begins with the uptake of water by the dry seed and ends with the elongation of the embryonic axis (Liu *et al*., 2009; Chakraborty and Kar, 2008; Sarkar *et al*., 2009). The protrusion of the radicle tip through the seed envelopes is the visible consequence of germination. The embryo, the envelopes or a combination of both factors to an extent

that depends on the plant species can impose seed dormancy. Embryos play a key role in both activating their own growth potential and providing signals to the endosperm to weaken physical constraint (Tatematsu *et al*., 2008). Activation of embryonic growth potential is triggered by the enhanced activity of cell elongation rather than cell division and so requires a local qualitative change in cellular activity (Tatematsu *et al*., 2008; Ogawa *et al*., 2003).

TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR1 (TCP) proteins are plant-specific transcription factors that are involved in growth-related processes, such as branching, floral organ morphogenesis, and leaf growth (for review, see Martín-Trillo and Cubas, 2010). The *Arabidopsis* (*Arabidopsis thaliana*) genome encodes for 24 TCP transcription factor genes, which, based on sequence homology, are divided into two classes: class I and class II TCPs. The TCP transcription factors share the TCP domain, a 59-amino-acid-long, non-canonical basic helix–loop–helix domain responsible for nuclear targeting, DNA binding, and mediating protein–protein interactions (Cubas *et al*., 1999; Kosugi and Ohashi, 2002).

TCP14 and TCP15 are members of the class I sub-group of TCP factors that has 13 members in *Arabidopsis* and they are closely related (Kieffer *et al*., 2011; Martín-Trillo and Cubas, 2010) and few of them as been functional characterised. AtTCP14 and AtTCP5 act redundantly to regulate plant stature by promoting cell proliferation in young internodes (Kieffer *et al*., 2011). This defect is associated with a reduction of cell proliferation, characterized by reduced expression levels of a range of effectors of cell division.

Here we shed light into the role and the mechanism of TCP14 and TCP15 as key regulators of *Arabidopsis* seed germination. Tatematzu and co-workers (2008) have shown that *attcp14* mutant seeds are dormant,

however we prove that also *tcp15* single mutants and *tcp14*/*tcp15* double mutant are characterised by a strong delay in germination. This delay can be partially rescued by either adding gibberellins or by prolonged vernalisation, suggesting a possible role of these two transcription factors in gibberellin homeostasis. DELLA proteins are negative regulators of gibberellin signalling and they act immediately downstream of the GA receptor (Wen and Chang, 2002). Either TCP14 or TCP15 are able to heterodimerise with DELLA proteins. All together our data indicate that the joint regulation of germination, by gibberellin and TCPs, occurs through physical interactions with DELLA.

Results

***tcps* seeds are dormant**

TCP14 and TCP15 are two class I TCP transcription factors (Kieffer *et al*., 2011; Martín-Trillo and Cubas, 2010) close related as shown by phylogenetic reconstruction (Figure 1). *tcp14* seeds germinate in delay (Tatematsu *et al*. 2008) as well as *tcp15* ones. For our tests we used *tcp15-3* seeds (Kieffer *et al*., 2011), as control we also evaluate germination delay of seeds obtained by *tcp14-4* plants confirming that *TCP14* disruption causes germination delay (Tatematsu *et al*., 2008). Germination tests have been performed using fresh harvest seeds and two and four weeks old seeds. Germination delay is rescued by vernalisation treatments and by gibberellin application (Figure 2E).
TCP14 and TCP15 share high similarity moreover they collaborate to regulate internode length and leaf development (Kieffer *et al*., 2011). These observations suggested to perform germination assays with the double mutant seeds (*tcp14-4tcp15-3*), which further confirmed the

redundancy between these two proteins. Interestingly vernalisation and gibberellin application have been able only to partially rescue the germination delay (Figure 2C, D, E).

Two types of seed dormancy have been recognized, coat-imposed dormancy and embryo dormancy (Baskin and Baskin, 2004). To discriminate between these two scenarios *tcp14-4* and *tcp15-3* pistils have been pollinated by wild type (*Col-0*) pollen grains; the obtained seeds had mutated seed coat protecting wild type (heterozygous) embryos. These seeds still retained germination delay (data not shown) suggesting that *tcp15* and *tcp14* are characterised by a coat-imposed dormancy. There are several basic mechanisms of coat-imposed dormancy, one is the mechanical constraint. To verify whether the mechanical constraint is the problem affecting *tcp14* and *tcp15* seeds, we have settle germination assays using mutant seeds whose testa has been mechanically damaged. At least 100 seeds, produced by *tcp14-4*, *tcp15-3*, *tcp14-4tcp15-3* and *Col-0* plants have been manipulated. Normal germination ratio have been scored for all the genotypes under-investigation when the testa were removed, regardless the seed genotype. The undamaged mutant control seeds still showed strong germination delay in comparison to the *Col-0* seeds (Figure 2F).

Close relation between *TCP14, TCP15* and GA

We have shown that GA application can rescue the germination delay observed of *tcp15* and *tcp14* seeds, pinpointing that TCP14 and TCP15 might be involved into GA metabolism. To uncover the relationship between TCPs and GA, we used quantitative RT-PCR analyses to explore *TCP14* and *TCP15* expression level in the *ga1-7* mutant and we observed that both genes were down-regulated (Figure 3A). In *ga1-3*,

GA synthesis is blocked at the conversion of geranylgeranyl pyrophosphate (GGPP) to enf-kaurene (Sun *et al*., 1992).
Conversely in *tcp14* and *tcp15* mutants, the expression level of genes codifying for protein involved in GA synthesis do not change. We analysed the expression levels of Gibberellin (*GA) 20-oxidase (GA 20-ox)* and *GA 3 β-hydroxylase (GA 3 β-hy*), which encode two enzymes that catalyse the late steps in the formation of active Gas and are potential control points in the regulation of GA biosynthesis. Quantitative PCR analyses indicate that the expression level of these two enzymes is not affected in *tcp14-4*, *tcp15-3* and *tcp14-4tcp15-3* roots (Figure 3B and C).
All together these data pinpoint a close relation among *TCP14*, *TCP15* and GA but this does not occur at GA biosynthesis, rather in GA signal transduction.

***tcp14tcp15* RAM are smaller**

Kieffer and co-workers (2011) have shown that TCP14 and TCP15 regulate plant stature by promoting cell proliferation in young internodes. We have hypothesized that seed coat germination defects in *tcp14tcp15* might be caused by defects in cell proliferation. To verify this hypothesis, we measured cell size and number at the root apical meristem (RAM). Wild type and mutants seed have been vernalized and imbibed overnight, to favour the manual seed coat removal. The protoderma of the RAM of the double mutants develops less and smaller cells respect to the wild type (Figure 4A and B). The defects observed in *tp14* and *tcp15* single mutants are quite mild and fully restored by GA application. Hormone addition cannot restore the normal cell number in the RAM of the double mutants *tcp14 tcp15* (Figure 4C).

TCP14 and TCP15 form dimers with GAI

Loss-of-function mutations in RGA (REPRESSOR OF GA1-3) and GAI (GA INSENSITIVE) can suppress some of the effects of GA deficiency, suggesting that RGA and GAI negatively regulate a subset of GA responses in *Arabidopsis* (Peng *et al*., 1997; Silverstone *et al*., 1997). RGA and GAI may function as transcriptional regulators that directly or indirectly repress the expression of GA-induced genes.

GAI and RGA belong to the DELLA subfamily within the GRAS family of plant regulatory proteins (Pysh *et al*., 1999). GAI and RGA are distinguished from other GRAS family members by an N-terminal DELLA domain (Peng *et al*., 1997; Silverstone *et al*., 1998). This domain is involved in modulating the activity of the RGA and GAI proteins in response to GA (Peng *et al*., 1997; Dill and Sun, 2001).

To investigate TCPs and DELLA interaction we tested by yeast two-hybrid experiments whether TCP transcription factors were able to interact with GAI. This analysis revealed that TCP14 strongly heterodimerises with TCP15. Moreover either TCP14 either TCP15 can form dimers with GAI. The strength of the interaction was tested by selecting fro growth on medium without His and different concentrations of 3-amino-1,2,4 triazole (3-AT) (Figure 5C).

To validate *in planta* the results obtained by the yeast interaction experiments, a bimolecular fluorescent complementation assay in tobacco (*Nicotiana benthamiana*) leaves was performed. The coding sequence of each gene was fused with a part of the yellow fluorescent protein, and then they were introduced into the cells through *Agrobacterium tumefaciens*-mediated transient transformation. Using this system, the interaction between TCP14 and GAI has been confirmed (Figure 5D-G).

To examine the effect of the interaction on the TCP14 activity, we performed another transcriptional assays by transient expression in leaves of *N. benthamiana*. For this aim, we prepared a synthetic promoter consisting of six concatemerized copies of the TCP class I binding site separated by 6 nucleotides, a minimal *35S* promoter, and the viral translational enhancer Ω controlling the expression of the reporter gene *LUCIFERASE* (*LUC*) (see Materials and Methods for details).

Expression of a translational fusion of TCP14 to the strong transcriptional activator VP16 caused an increase in the LUC activity. Remarkably, the activation activity was largely reversed when GAI-TAP was co-expressed with TCP14-HA, whereas GAI-TAP alone did not affect significantly the LUC activity. Taking together, these results support the model that GAI inactivates TCP14 by direct, physical interaction.

TCP14 and TCP15 action on DELLAs expression

To check if GA really regulate TCP expression as suggested by initial experiments we performed Real time PCR in a transgenic line that expresses a gain-of-function version of the DELLA protein GAI under the control of a temperature-inducible promoter, HS::gai-1. To confirm that the inductive treatment resulted in an increase of GAI activity, we used as a control *GA20ox1* (Javier Gallego-Bartolomé *et al*., 2011) (data not shown).

As expected, transcripts of *GA20ox1* accumulated strongly in seedlings following the heat shock, but the expression of *TCP14* and *TCP15* transcripts did not change significantly in response to the temperature treatment (data not shown).

More importantly, Western blot analysis in roots with anti-GAI, could prove that GAI protein accumulation is not affected in mutant background (*tcp14-4*, *tcp15-3* and *tcp14-4tcp15-3*).

TCPs regulate cell cycle genes

We previously proposed that *tcp14tcp15* seed coat germination defects are caused by defects in cell proliferation.
To strengthen this hypothesis we performed in situ hybridization with a *HISTONE 4*, antisense probe. *H4* is a marker for cell division (Fobert *et al*., 1994) (Figure 6A and D) and its expression appears strongly reduced in *tcp14-4* and *tcp15-3* developing embryos. These evidences clearly indicate that cell cycle progression is under TCP14 and TCP15 control (Figure 6B, C, E, F).
It is has been proven (Tatematzu *et al*., 2008; Li *et al*., 2012) that *CYCB1:1* is directly regulated by TCP14. We introgressed *CYCB1.1::GUS* in *tcp15* mutant to verify whether also TCP15 is able to regulate *CYCB1.1* expression; as expected the GUS activity was not detected in *tcp15-3* mutant indicating that both TCP14 an TCP15 are necessary for stimulating *CYCB1.1* transcription (Figure 6G and H). Interestingly GA application was able to restore GUS activity in *tcp15-3* germinating seeds and roots (Figure 6I). DELLA also act on cell cycle progression; in fact in has already been published that DELLAs restrain cell production by enhancing the levels of the cell cycle inhibitors *Kip-related protein 2 (KRP2*) and *SIAMESE (SIM*) (Achard *et al*., 2009). Therefore we planned series of qRT-PCR on cell cycle genes like *SIM, SMR1 and KRP2* (Achard *et al*., 2009) and we saw that the expression profile of these genes in the double mutant is overexpressed (Figure 6J).

TCPs affect root elongation

All together our data suggest that TCP14, TCP15 and GAI collaborate to promote cell cycle in RAM, these interactions appear very important during seed germination but we asked whether they are involved also in RAM organisation in post embryonic phases.
We first verified root elongation. We followed root growth and elongation in wild type, *tcp14-4, tcp15-3* and *tcp14-4tcp15-3* mutants, for 7 days and we observed that root growth was in delay for the single mutants compare to the wild type and even worst for the double (Figure 4D). As expect, GA treatment could partially rescue the root growth but not completely for the double mutants. Using also a camera that take pictures every 30 minutes we were able to measure step by step the root size until 15 days after germination 8 (Figure 4E).

Discussion

Plant growth involves the integration of many environmental and endogenous signals that together with the intrinsic genetic program determine plant size. At cellular level, growth rate is regulated by the combined of two processes: cell proliferation and expansion.
Gibberellins (GA) are plant-specific hormones that play a central role in the regulation of growth and development with respect to environmental variability (Olszewski *et al*., 2002; Achard *et al*., 2006; Achard *et al*., 2008).
It is well established that GA promote growth through cell expansion by stimulating the destruction of growth-repressing DELLA proteins (DELLAs) (Peng *et al*., 1997; Silverstone *et al*., 1998; Achard *et al*., 2009) (Figure 7).

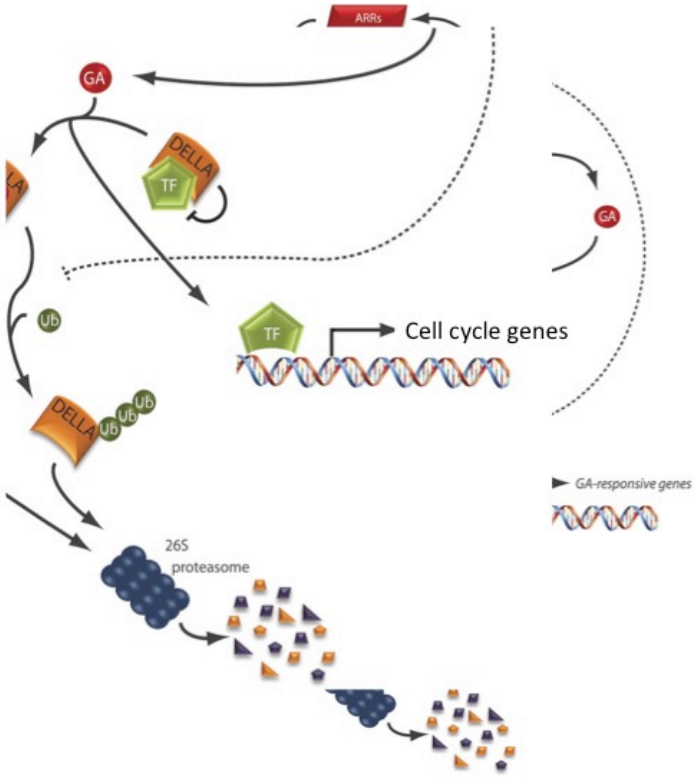

Figure 7. DELLAs regulate cell cycle genes expression through an unknown transcription factor (TF) (Stamm and Kumar, 2010).

However the mechanism by which DELLAs regulate the transcription of cell cycle genes remains unknown. It is already been published that also activation of embryonic growth potential is triggered by the enhanced activity of cell elongation rather than cell division. Activation of embryonic growth potential therefore requires a local qualitative change in cellular activity (Tatematsu *et al*., 2008; Barrôco *et al*., 2005; de Castro *et al*., 2000).

Growth regulators have been characterized extensively by genetic and genomic approaches and the TCP family transcription factors, which belong to the family of bHLH-type transcription factors, are thought to be key regulators of morphological traits and they have been reported to

be specifically linked to the regulation of cell proliferation and cell differentiation during plant development (Tatematsu *et al*., 2008).

Kieffer *et al*., discovered that TCP14 and TCP15 are dynamically expressed in young proliferating tissues and they act redundantly to regulate plant stature by promoting cell proliferation in young internodes. This defect is associated with a reduction of cell proliferation, characterized by reduced expression levels of a range of effectors of cell division, including cyclins and cyclin-dependent kinases (Kieffer *et al*., 2011; Li *et al*., 2012).

In this study we deeply understood the role of TCP14 and TCP15 as key regulators of *Arabidopsis* seed germination. As we shown *tcp14* and *tcp15* single mutants and the *tcp14*/*tcp15* double mutant are characterised by a strong delay in germination but it can be partially rescued by either adding gibberellins or by prolonged vernalisation, suggesting a possible role of these two transcription factors in gibberellin homeostasis. DELLA proteins are negative regulators of gibberellin signalling and they act immediately downstream of the GA receptor. Both TCP14 and TCP15 are able to heterodimerise with DELLA proteins.

All together our data indicate that the joint regulation of germination, by gibberellin and TCPs, occurs through physical interactions with DELLA.

Taking into account all the data and the fact that we demonstrated that TCPs/DELLA complex participate in cell cycle gene regulation, we propose a model (Figure 8), which integrates all these elements, similar to the one already proposed (Stamm and Kumar, 2010).

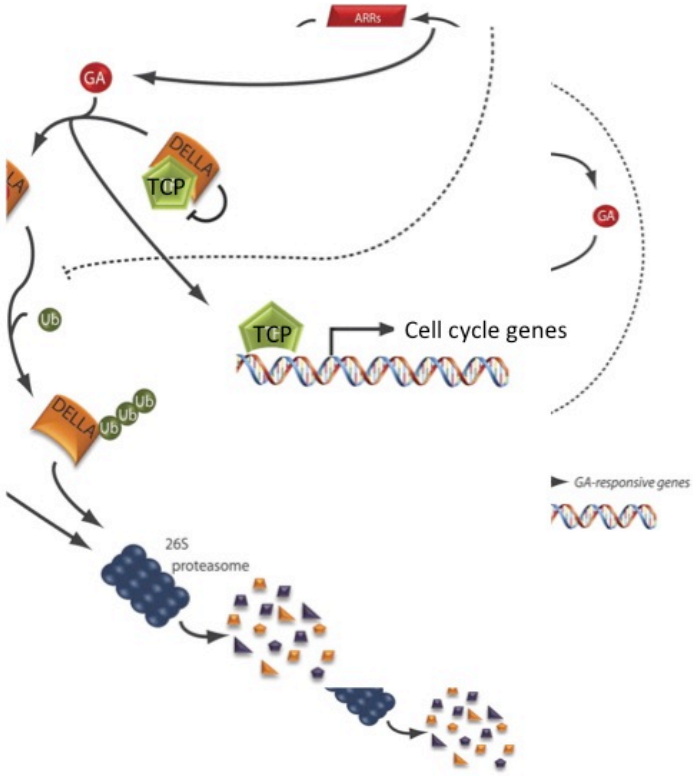

Figure 8. Our proposed model. DELLAs regulate cell cycle genes expression through TCP (according to Stamm and Kumar, 2010).

It is also known that *CYCA2;3* and *RETINOBLASTOMA* (*RBR*) are two important endoreduplication negative regulators and that loss of *CYCA2;3* function showed increased endoreduplication in cotyledon and rosette leaves (Imai *et al.*, 2006). Li and co-workers (2012) also demonstrated the direct binding of AtTCP15 protein at *CYC2;3* and *RBR* promoters; one possible model of *AtTCP15* in cell-cycle modulation.

It could be interesting at this point analysing the expression profile of *CYCB1;1* and *RBR* in single and double mutants. This raises the attractive possibility that TCPs are real regulator of cell cycle genes through DELLAs.

Material and methods

Plant Materials and genomic DNA extraction

Arabidopsis thaliana Columbia accession (*Col-0*) was used as the wild type in this study.

The *tcp14-4* and *tcp15-3* single mutants were found by screening the insertion flanking database SIGnAL (Alonso *et al*. 2003; http://signal.salk.edu/cgi-bin/tdnaexpress). The T-DNA element positions were confirmed by sequencing analysis. The double mutants *tcp14-4tcp15-3* were given by Prof. Brendan Davies (University of Leeds). *HS::gai* were provided by Prof. Miguel Blazquez (Instituto de Biología Molecular y Celular de Plantas, Valencia). *CYCB1;1:GUS* seeds were donated by Arp Schnittger (Universitè de Strasbourg) and *ga1-7* by Lucio Conti (Università degli Studi di Milano).

Plants were grown under long-day conditions (14 hours light/10 hours dark) at 22°C. Seeds were surface-sterilized, chilled at 4° C for 2 days, and then germinated and grown on plant growth medium (Murashige and Skoog, 1962). Seeds were harvested when plants had ceased flowering and siliques were starting to dehisce and stored in the dark at 22°C and 30% relative humidity.

Genomic DNA extractions have been performed has previously described (Masiero *et al*., 2004).

Germination Assays

Wild type and mutant seeds were collected at the same time and obtained from plants grown in the same conditions. Storage conditions were 22°C and 30% relative humidity in the dark for 1 week for freshly harvested seeds and for 3 months for after-ripened seeds.

For each genotype, approximately 50 seeds were placed onto filter papers (Whatman No. 3, UK) moistened with 2 ml of sterile water or aqueous solution of GA_3 (10^{-3} mM) in 6 cm diameter Petri dishes. The average germination percentage was determined after 1, 2, 3 and 4 days in a climate room (25°C, 16 h light/d). In some experiments, the seeds sown on water-soaked filter paper were submitted to 5 d of cold treatment at 4°C (chilling) to break dormancy. All germination assays were carried out in triplicate with at least two independent seed batches.

In situ hybridization and histochemical GUS analysis

In situ hybridization with digoxigenin-labelled antisense RNA were performed as previously described (Masiero *et al*., 2004). A specific *TCP15* cDNA fragment was amplified. The same protocol was performed also for in situ on *H4*.

GUS staining was performed as reported by Vielle-Calzada and collaborators (2000). Developing seeds were cleared according to Yadegari *et al*. (1994) and observed using a Zeiss Axiophot D1 microscope (http://www.zeiss.com) equipped with differential interface constrast (DIC) optics. Images were recorded with an Axiocam MRc5 camera (Zeiss) using the Axiovision program (4.1).

Quantitative Reverse Transcription PCR

Expression analyses of *TCP14* and *TCP15* were performed using the iQ5 Multi Color real-time PCR detection system (Bio-Rad). Normalisation was performed using *UBIQUITIN10* (*UBI10*), *ACTIN2-8* and PROTEIN PHOSPHATASE 2A SUBUNIT A3 (*PP2A3)* as internal standards. Transcript abundances were confirmed by two independent biological

experiments and three technical repetitions. qRT-PCR was also perfomerd for *GA20ox1*.

Bimolecular fluorescent complementation

TCP14 ORF was amplified from *A. thaliana* cDNA and cloned into the pDONR207 plasmid by the BP Gateway reaction. Both GAI and TCP14 were fused in frame with the N- and C-terminal fragments of yellow fluorescent protein (YFP), respectively, by LR Gateway recombination. The fluorescence emission was observed after 24 h of incubation at 22 °C in the dark under a fluorescence Zeiss Axiophot microscope (Carl Zeiss, Germany) with the following filter set: excitation, 450–490 nm; emission, 520 nm. Images were captured with a CCD colour Leica DFC300FX camera and processed with the Leica Application Suite 2.8.1 build 1554 software (Leica, http://www.leica.com). Complementation was confirmed in two independent assays.

Yeast Two-hybrid interaction assays

cDNA of each candidate gene was used to perform confirmation of the interaction. RNA was extracted from *Arabidopsis* seeds development using the RNeasy Plant Mini Kit (Qiagen). cDNA was synthesized with SuperScriptII (Invitrogen™) according to manufacturer's instructions. The cDNA of the candidate genes was amplified by PCR with Phusion High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA) and purified from agarose gel using the kit Wizard Promega. The cDNA sequence of each gene was individually cloned into the pGADT7 vector (Clontech). Each bait/prey pair was introduced in the AH109 yeast strain (Clontech). As a control for autoactivation false-positives, each bait was also co-transformed into the yeast strain with the empty

AD vector, and each prey was co-transformed with the empty BD vector. Bait/prey pair colonies were grown on permissive and selective media (−Trp-Leu-Adenine and/or -Trp-Leu--His supplemented with increasing concentrations of 1 mM to 2.5 mM 3-Amino-1,2,4-triazole).

Reporter construct for transcriptional assays

To prepare the reporter construct, the promoter, synthesized by GenScript, was cloned into the *Pst*I and *Nco*I sites of the *pGreenII 0800-LUC* vector. As effector proteins, we used GAI-C-TAP and HA-VP16-TCP14, which was obtained by cloning the *TCP14* CDS into the *Alligator1* vector by Gateway. The assay was performed in Miguel Blazquez's lab (University of Valencia).

Root lenght tests

To determine the length, seedlings were grown for one week in continuous light at 22ºC in a vertical orientation on plates containing half strength MS medium (Duchefa) with 0.8% w/v phytoagar and without sucrose, and supplemented with mock or 10^{-3} mM GA_3. Every hour the plates were photographed using CCD cameras coupled to Metamorph software as described by Schepens *et al*., 2008. Root growth was measured using Image J software.

mPS-PI staining and cell size measuemnts

mPS-PI staining has been done as following Truernit *et al*., 2008. Samples have been observed using a confocal Leica TCS SP5. Samples have been exciteted at 488 nm and emission was collected at 520 to 720 nm.

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Captions

Figure 1.

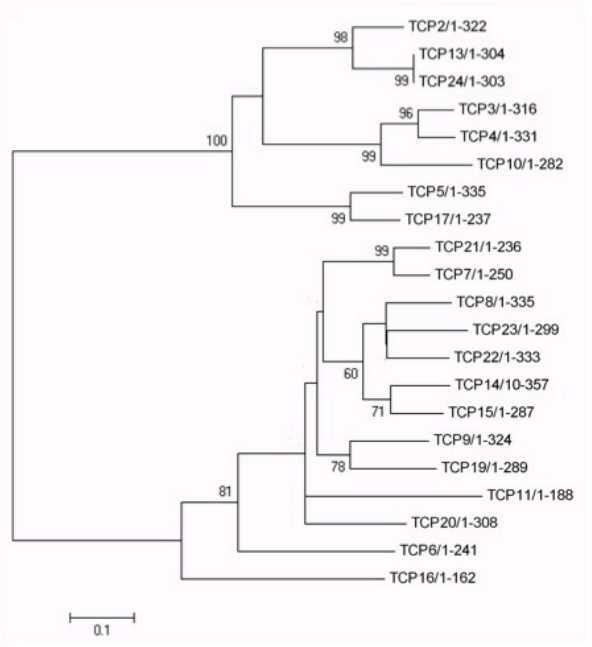

Figure 1. Phylogenetic reconstruction of the Arabidopsis TCP gene family obtained using Neighbor Joining. TCP14 and TCP15 share high homology sequence.

Figure 2.

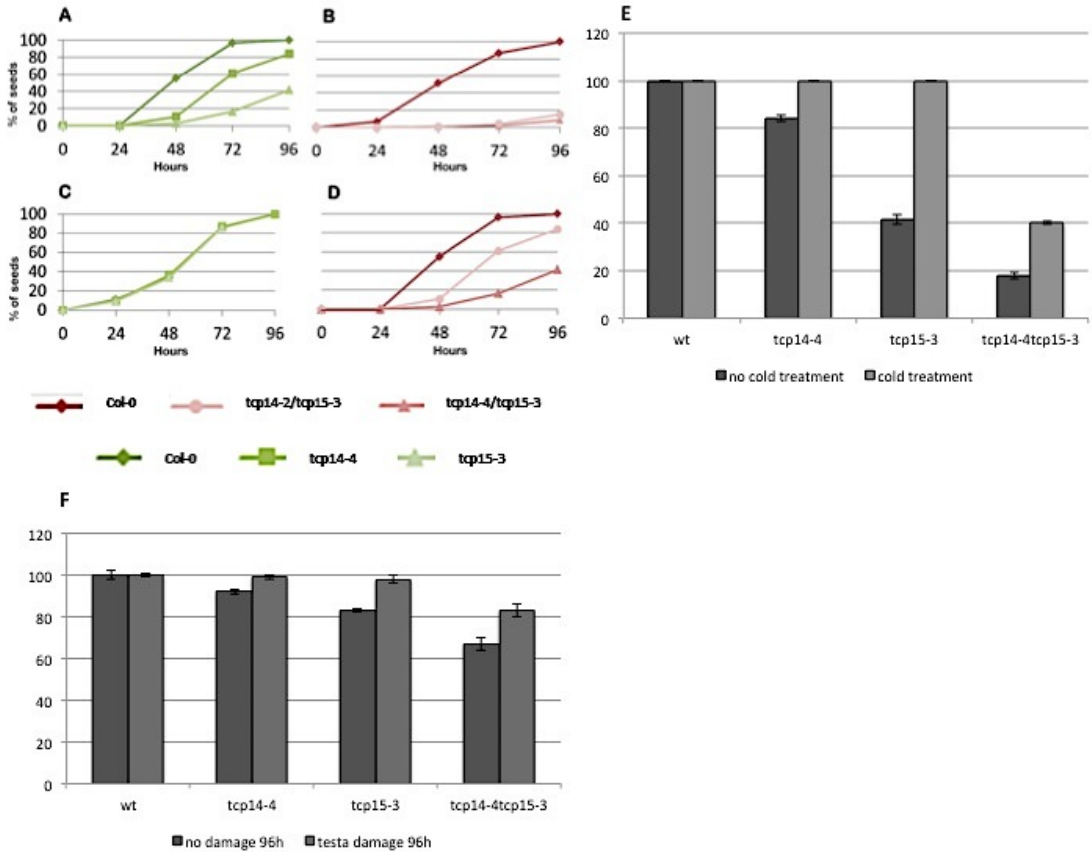

Figure 2. ***tcp14*** **and** ***tcp15*** **are characterized by strong delay in germination** (A). However they completely rescue the phenotype after treating them with gibberellins (GA) (C). Germination delay is even rescued by vernalisation treatments (Figure 2E, light grey). The delay in germination is stronger in the double mutant *tcp14tcp15* (B) where Gas (D) and vernalisation (E) are not able to rescue the phenotype.

Normal germination ratio have been scored for all the genotypes under-investigation when the testa were removed, regardless the seed genotype. The undamaged mutant control seeds still showed strong germination delay in comparison to the *Col-0* seeds (Figure 2F).

Figure 3.

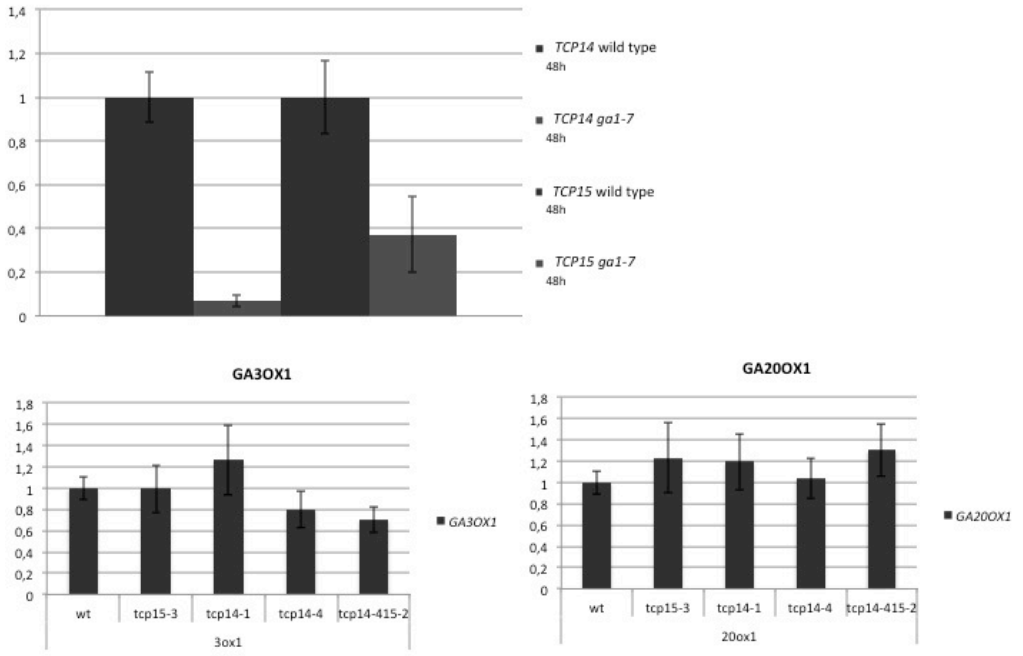

Figure 3. Close relation between *TCP14, TCP15* and GA

RT-PCR analyses to explore *TCP14* and *TCP15* expression level in the *ga1-7* mutant; both genes were down-regulated (Figure 3A).

Conversely in *tcp14* and *tcp15* mutants, the expression level of genes codifying for protein involved in GA synthesis do not change. Quantitative PCR analyses indicate that the expression level of these two enzymes is not affected in *tcp14-4*, *tcp15-3* and *tcp14-4tcp15-2* and *tcp14-4tcp15-3* roots (Figure 3B and C).

Figure 4.

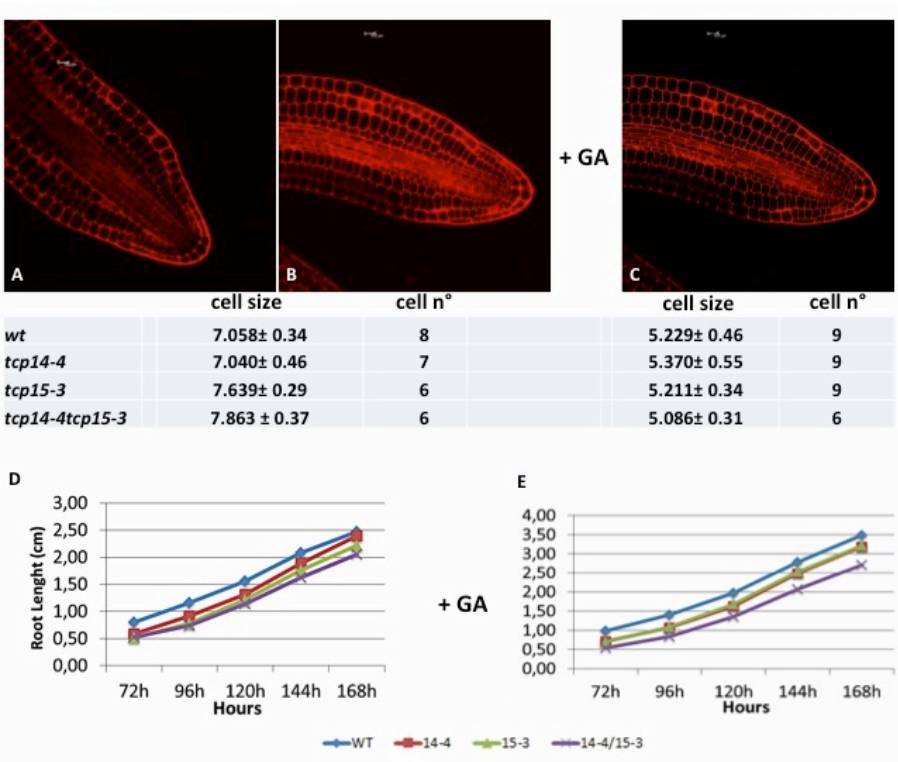

Figure 4. ***tcps*** **mutant present defects in root growh.**

MP-spi staining in wild type (A) and double mutant (B) embryos. Cells of the RAM are counted in single and double mutant compare to the wild type. After treating them with GA, wild type present higher number of cells, with cell size reduced while the double didn't show any alteration compare to a normal situation (C).

The phenotype of *tcp14* and *tcp15* single and double mutant is evident in root lenght (D). GA increase the root growth in all the samples without restoring normal conditions (E).

Figure 5.

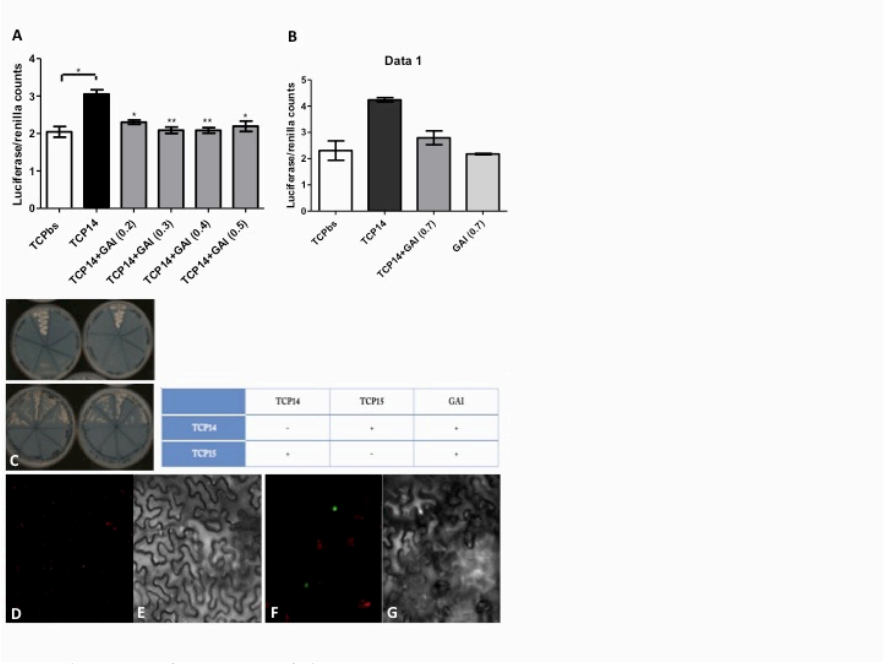

Figure 5. TCP14 and TCP15 interact with GAI.

(A, B). Expression of a translational fusion of TCP14 to the strong transcriptional activator VP16 caused an increase in the LUC activity. Remarkably, the activation activity was largely reversed when GAI-TAP was co-expressed with TCP14-HA, whereas GAI-TAP alone did not affect significantly the LUC activity.

Moreover TCP14 strongly heterodimerises with TCP15 and either TCP14 either TCP15 can form dimers with GAI in yeast (C). BiFC performed in *Nicotiana benthamiana* confirm the interaction between TCP14 and GAI (5D-G).

Figure 6.

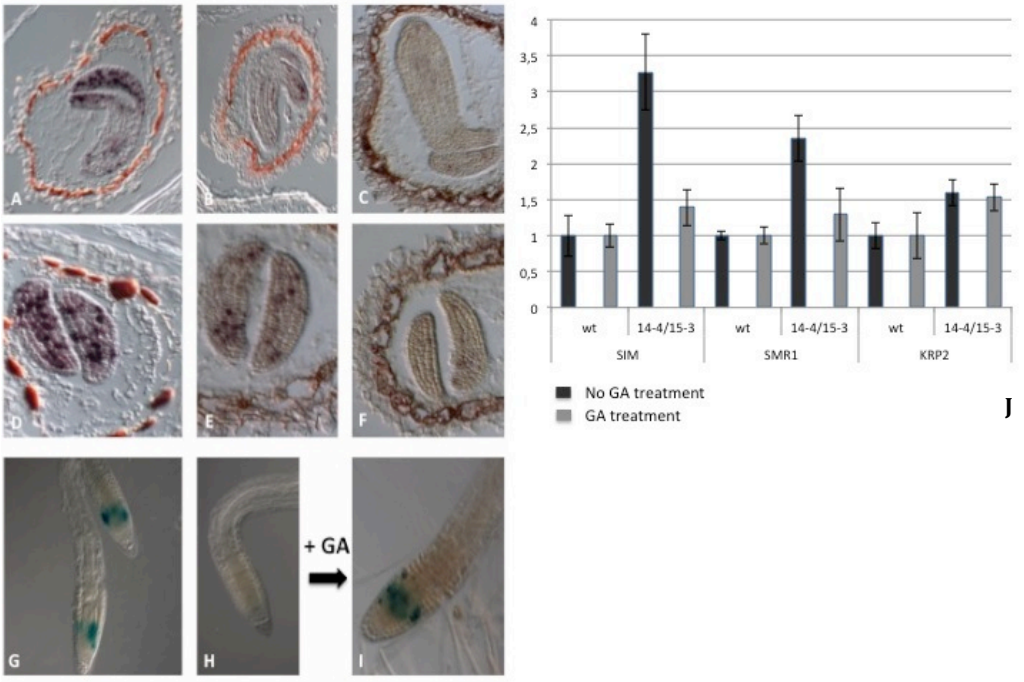

Figure 6. TCP14 and TCP15 control cell cycle genes.

(A,D) *In situ hybridization* of the *HYSTONE 4* in wild type embryo. Lower signal is detected in *tcp14* (B, E) and *tcp15* single mutant (C, F). *CYCB1:GUS* marker line expressed in seedlings 4 DAG (G). Tatematsu *et al.* already published a lower GUS expression in *tcp14* embryo compare to the wild type. Interesting the absence of GUS expression in *tcp15* mutant (H). After treating *tcp15* single mutant with GA, a rescue of the expression is observed (I).

Series of qRT-PCR on cell cycle genes like *SIM, SMR1 and KRP2* (Achard *et al*., 2009); the expression profile of these genes in the double mutant is overexpressed (J).

To all the people that help and support me in this long journey.

Your friend is your needs answered.
He is your field which you sow with love and
Reap with thanksgiving.
And he is your board and your fireside.
For you come to him with your hunger, and you
Seek him for peace.
[Kalhil Gibran]