Functional Facultative Apomixis in *Arabidopsis thaliana*

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Abstract

Apomixis is associated with occurring in hybrid and polyploid plants. Here a model is tested wherein facultative apomixis might be achieved by impairing intracellular stress-signaling via a hybridization induced miss-regulation of ribosome biogenesis. The HDA6 gene knockout globally up-regulates ribosome biogenesis in *Arabidopsis thaliana*. Embryological analysis revealed reduced meiocyte initiated stress-signaling and associated callose and tetrad formation, hallmarks of sexual reproduction in angiosperms. In its place various forms of unreduced embryo sac formation, hallmarks of apomixis, and the production of viable seed suggest that, based upon a dosage dependent effect, inhibited meiocyte initiated stress-signaling converts the model plant *Arabidopsis thaliana* into a fully functional facultative apomict. More work is needed for confirmation prior to publication.

Introduction:

Apomixis in plants is clonal or asexual seed formation. In crop plants grown from seeds apomixis could be used to stably perpetuate favorable genetic combinations, including hybridity, that increase the yield or value of a crop.

Most of the current research on apomixis is based upon the "broken gene" hypothesis aimed at finding single gene knockouts that may be responsible for the repeated de-novo evolution of apomixis among angiosperms. However apomixis is a two-step process requiring i) apomeiosis, the formation of a genetically unreduced clonal egg and ii) parthenogenesis, the development of an embryo from the unreduced egg without fertilization. As a result researchers are reporting limited success wherein the unreduced gametes formed through gene knockouts fail to develop parthenogenetically (Ravi et. al. 2008, d'Erfurth et al. 2009, Olmedo-Monfil et al. 2010, Singh et al. 2011). Many of the gene knockouts studied thus far lead to the first of two components of apomixis, i.e. apomeiosis, by altering epigenetic processes. For example, Olmedo-Monfil et. al. discovered that ARGONAUTE9 is involved in a small RNA pathway that restricts female gamete formation to the megaspore mother cell (2010). Knockouts involving components of the pathway caused unreduced embryo sac formation but did not induce parthenogenesis. Ravi et. al. discovered that a DYAD/SWITCH1 knockout caused functional apomeiosis but created triploid plants because fertilization was still required to set seed (2008). Other gene knockouts of interest in apomixis research also alter epigenetic processes. For example the loss of function in MULTICOPY SUPPRESSOR OF IRA 1, an evolutionarily conserved Polycomb group (PcG) chromatin-remodeling complex, initiates non-viable parthenogenic embryos from meiotically derived egg cells (Guitton and Berger 2005). Many additional examples regarding the involvement of epigenetic processes that differentiate apomictic and sexual reproduction are given in a review by Rodrigues and Koltunow (2005).

These findings have led many researchers to study the epigenetic differences between related sexual and apomictic taxa despite the confounding effects of Muller's ratchet (Muller 1932). Without the ability for plants to purge deleterious mutations through meiosis non-functional genes accumulate and contribute to the pool of silenced genes. This makes it difficult to identify any epigenetic differences that may be responsible for apomixis.

Even more disturbing is a critical point that researchers have largely failed to discuss or even account for in their research. This is the fact that apomixis often occurs in tandem with sex within an individual plant. This detail limits the practicality of gene knockout hypothesis. However it does provide a foundation leading to a possible paradigm shift in apomixis research. In facultative apomictic plants (Asker and Jerling 1992; Carman et. al. 2011) and cyclically apomictic animals (Suomalainen et al. 1987) the expression or initiation of either the sexual or the apomictic developmental program is influenced by environmental factors, i.e. stress. In such cases the onset of biotic or abiotic stress initiates the sexual pathway. Furthermore a deeper look into the mechanisms of stress signaling and its involvement in both meiosis and in epigenetic reprograming makes clear why single or multiple gene knockouts to date have failed to produce a fully functional apomictic plant.

Physiology of stress signaling

At the hub of both biotic and abiotic stress signaling is the formation of reactive oxygen species (ROS) such as H_2O_2 (Shlomai 2010). As a signaling molecule, H_2O_2 and other ROS post-translationally modify target proteins by oxidizing thiol groups thus forming disulfide bonds that reversibly alter protein structure and function. Specificity is achieved by localized production, concatenate hormone or Ca^{+2} signaling, with targeted secondary oxidation occurring via glutaredoxins or thioredoxins (Winterbourn and Hampton 2008). Target proteins containing reduction-oxidation (redox) sensitive thiol groups include *i*) signal transduction pathway proteins, such as phosphatases (Tanner et al. 2011), *ii*) cell cycle regulating Mitogen Activated Protein Kinases (Kovtun et al. 2000), *iii*) embryogenesis regulating proteins (Ufer et al. 2010), as well as proteins that directly regulate the epigenetic landscape including: *iv*) many transcription factors, *v*) RNA binding proteins that direct gene silencing through DNA methylation, and *vi*) proteins involved in histone acetylation, deacetylation or methylation (Sundar et al. 2010; Shlomai 2010). Thus oxidative stress, or a programmed oxidative burst, can globally alter cell function and re-structure the epigenome.

Furthermore, Cavalier-Smith (2010) and Gross and Bhattacharya (2010) argue that stress signaling, particularly ROS signaling, played a central role in the evolution of meiosis during eukaryogenesis and its timing-of-onset during the life cycle.

A specific example of an important role for stress signaling in meiosis is the Mei2 gene. It encodes an RNA binding protein considered to be a master regulator of meiosis and is required for its initiation (Harigaya and Yamamoto 2007; Pawlowski et. al. 2007). In yeast, during mitosis Mei2 transcripts accumulate but the protein remains inactive in the cytoplasm. When under stress (nutrient starvation) the protein binds to a small RNA (meiRNA) and is moved to the nucleus forming a visible dot (Shimada et. al. 2003; Yamashita et. al. 1998). This coincides with the onset of meiosis. As Mei2 is conserved between yeast and plants (Kaur et al. 2006), this process may be another part the complex cascade of events activated by a stress signal thereby regulating meiosis and possibly coupling it to the requirement of syngamy.

The stress-induced meiotic switch

Further evidence clearly shows that the timing and location of the oxidative burst(s) that may initiate meiosis can be traced to the formation of callose, a polysaccharide that is deposited in cell walls as a response to both biotic and abiotic stress. It is found in a thick layer in the cell wall around the developing meiocyte and tetrad of angiosperms. In *Arabidopsis* callose formation in ovules increases under stress conditions in a dosage dependent manner until it leads to apoptosis and ovule abortion (Sun et. al. 2004), a mechanism to balance fecundity with resources. Recently, callose deposition in

Arabidopsis has also been shown to correlate with levels of H_2O_2 production (Luna et. al. 2011). This stress-induced polysaccharide also forms, albeit transiently and to a much lesser extent, around the cell plate of active mitotically dividing cells (Stone 2005). Although an oxidative burst precedes mitosis by stimulating cell expansion (Foreman et. al. 2003), the strength of an oxidative burst and other signals may, in a dosage-dependent manner, initiate a downstream redox signal used to distinguish somatic mitosis from germ-cell meiosis. In this way facultative apomictic plants may use stress as a signal to switch from apomictic to sexual reproduction. This is significant because to date all apomictic angiosperms stained for callose deposition in the ovule, across monocots and dicots, have revealed that most either lack callose or it infrequently occurs in various but indistinct places throughout the ovule (Carman et. al. 1991) but not immediately around the meiocyte in a thick layer as found in related sexual taxa (Tucker and Koltunow 2001). A lack of callose-inducing meiotic-like redox signaling in all apomictic angiosperm germ-cells stained for callose deposition to date substantiates the hypothesis of a redox-based signal transduction pathway regulating the switch between apomictic and sexual reproduction. Facultative apomictic *Boechera* plants, a close relative of *Arabidopsis*, collected in the wild at the end of the growing season show increased callose formation immediately around sexually forming tetrads when compared to their apomictically forming dyads within the same pistle (personal observations).

Therefore, a massively restructured stress-induced sexual epigenome likely prohibits simple gene knockouts from thwarting the ensuing reproductive program. This begs the question: will the absence of a stress signal during megasporogenesis and embryogenesis in apomicts set the stage to maintain an apomeiotic epigenome and parthenogenetic competence?

Many genetic approaches can be used to miss-regulate a stress-response signal within a cell. The key to finding a candidate target lies in the fact that ROS are primarily produced in the mitochondria by the over-reduction of the electron transport chain (ETC) (Møller et. al. 2001). Calcium released in localized bursts from the endoplasmic reticulum (ER) is absorbed by closely associated mitochondria and regulates their metabolic rate (Rizzuto et. al. 1998). This occurs because calcium increases the mitochondrial proton motive force thereby increasing ATP production, and possibly ROS, by two mechanisms. Increasing mitochondiral calcium activates TCA cycle enzymes (McCormack and Denton 1993) as well as increases the mitochondiral pH gradient through a complex exchange of ions (Poburko et. al. 2011). In this way energy production is coupled with calcium bursts that simultaneously activates energy demanding cellular processes.

This type of calcium signaling is dependant upon the signaling molecule inositol 1,4,5 trisphosphate (IP3), which is formed by PHOSPHOLIPASE C (Williams and Katan 2004). IP3 works in concert with Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and locally produced NADH to bind to the cystolic side of the inositol tris-phosphate receptor protein (IP3R) channel to release calcium from the ER (Rizzuto et. al. 1998; Patterson et. al. 2004) . The system is designed as a feedback loop that tightly regualtes cellular levels of free-ATP (Mak et. al. 1999).

However the initiation of calcium induced stress-signaling requires the oxidation of the IP3R channel from luminal side of the ER (Kang et. al. 2008) which then mitochondira amplify into a ROS stress signal via the Respiratory Burst Oxidase Homologue (RBOH) pathway outlined below. Li et. al. clearly showed that the luminal side oxidation of IP3R is regulated by ERO1α and that the RNAi knockdown of ERO1α inhibits IP3R calcium release induced apoptosis (2009). This approach, driven by a megaspore

mother cell (mmc) specific promoter, is a likely candidate for testing the stress-induced switch hypothesis.

Another approach is to prevent mitochondira from amplifying the stress signal that comes from the ER. Outlining the RBOH pathway makes clear that many methods can be used to thwart the amplification of the stress signal. When an abundance of calcium over-stimulates the mitochondrial metabolic rate the availability of ADP becomes limiting and the localized area becomes saturated with ATP. A limited amount of ADP available for the ATPsynthase to convert the H+ gradient into ATP causes the ETC to "back up" and become over-reduced with electrons. This activates specific proteins that help alleviate it. This includes AT1G07180, an alternative NADH dehydrogenase (Rasmusson et. al. 2004), and two proteins that amplify the ROS signal and fine-tune the spatial control of ROS production. These are RBOHD, a NADPH Respiratory Burst Oxidase (AT5G47910) and its partner AtRBOH_F (AT1G64060). RBOHD produces superoxides by transferring electrons directly from NADPH to oxygen (Torres et.al. 2002). This restores NADP+ so that is can alleviate the over-reduction of the ETC. Superoxide dismutase FSD1 (AT4G25100) then catalyzes the dismutation of superoxide into oxygen and H_2O_2 which is subsequently buffered by the ascorbate-glutathione redox buffer system that transmits the stress signal.

Mitochondria can be prevented from amplifying the stress signal by alleviating the over-reduction of the electron transport chain. This can be done by uncoupling the H+ gradient from ATP production via uncoupling proteins in the membrane that provide a channel to simply allow the H+ gradient to dissipate releasing the energy as heat. It can also be accomplished by ensuring an ample supply of ADP, a method which may occur in nature via a hybridization induced imbalance in ribosome gene copy number. Ribosomal transcripts comprise the majority of the RNA in an average cell and are normally tightly regulated according to current metabolic demand. Increased biosynthesis beyond natural levels is a very energetically costly process (Moss et. al. 2007) and could quantitatively impact the ATP/ADP ratio. A pre-meiotic lowering of the ATP/ADP ratio would prevent the ETC from becoming saturated with electrons and stimulating ROS production during meiocyte maturation. This could circumvent redox signaling and the cascade of events needed to induce meiosis and couple it with syngamy. In its place an alternative embryogenic program could then progress thereby coupling apomeiosis with parthenogenesis.

Ribosomal genes are generally grouped together into tandem arrays of hundreds to thousands of genes termed nucleolar organizer regions (NOR) that are dynamically regulated epigenetically as an entire unit (Tucker et. al. 2010). Interestingly, the ectopic insertion of ribosomal genes has been shown to stably escape silencing (Lewis et. al. 2007). The mapping of genomic locations of ribosome genes in Arabidopsis has revealed that they exist in multiple copy numbers due to duplications of whole chromosome segments and many post-duplication insertions and deletions (Barakat et. al. 2001). Therefore it is possible to create a hybridization-induced imbalance in genomic copy number of ribosome genes that are miss-regulated. Also of interest is the observation that an increase in the number of NOR's is correlated with apomixis in fish (Sola et. al. 1997) and in apomictic dandelions (Dijk and Bakx-Schotman 2004).

For testing purposes a simpler genetic approach can be used to miss-regulate the entire NOR and cause a large increase in ribosome biogenesis. For example, the RNAi knockdown of HDA6 and other components of the RNA directed DNA methylation (RdDM) pathway that are specific to ribosome

regulation de-represses rRNA gene silencing and enables a global increase in ribosome transcripts (Tucker et. al. 2010; Earley et. al. 2010). This also causes an increase in the mobility of transposable elements (TE) (liu et. al. 2011) which are often associated with apomixis. Key proteins involved in this pathway are localized in Cajal bodies adjacent to and are strongly associated with the nucleolus (Li 2008). These proteins include RNA DEPENDENT RNA POLYMERASE2 (RDR2), DICER-LIKE3 (DCL3), ARGONAUTE4 (AGO4), and NRPD1b, the largest subunit of Polymerase IV complex known to contribute to downstream DNA methylation rather than upstream siRNA biosynthesis (Pontier et. al. 2005). In addition to the above mentioned proteins two additional RdDM components involved are NRPD2 and DOMAINS REARRANGED METHYLTRANSFERASE2 (DRM2). They are located immediately adjacent to silenced 45s rDNA sequences in smaller more discrete nuclear bodies, termed AB-bodies (Li 2008).

The impact that the global up-regulation of ribosome biogenesis can have on stress signaling has already been implicated in the fact that the gene knockout, or RNAi knockdown, of HDA6 has rendered *Arabidopsis* hypersensitive to ABA and salt stress (Chen et. al. 2010). Presumably this occurs because, due to significantly reduced ROS signaling, the plants aren't able to up-regulate the antioxidant buffer system needed to physiologically adapt the plant to the stress conditions. Therefore a hybridization-induced large increase in the biosynthesis of ribosomes or other energy consuming processes may be responsible for thwarting the ROS signal needed to stimulate a sexual developmental program in apomictic plants. It also accounts for the observation that the sexual pathway can be restored under stress conditions in facultative apomictic plants.

I hypothesize that female meiocyte development and callose formation in the *Arabidopsis* knockout of HDA6 will mimic patterns seen in facultative apomictic *Boechera* plants, a close relative of *Arabidopsis.*

Materials and Methods

Initial Analysis

Arabidopsis seed accessions (CS66153, CS66154, CS1854) of the HDA6 knockout were grown in 4" pots in a 50/50 mix of sand and soilless media (sphagnum peat) mixed with compost in a growth chamber at 25^{o}C under a 16 hour photoperiod. Plants were difficult to grow and appeared to suffer from water and/or drought and nutrient stress. Young inflorescences were fixed in formalin acetic acid alcohol (FAA) and cleared in 2:1 benzyl benzoate : dibutyl phthalate, dissected and mounted (Crane and Carman 1987). Slides were analyzed using the Olympus BX51 microscope with differential interference contrast and UV fluorescence optics and pictures taken with an Olympus DP71 Digital camera.

Controlled Stress Test

To improve growth conditions *Arabidopsis* seed accessions (CS66153, CS66154, CS1854) of the HDA6 knockout were sown in 4" pots of soilless media (a 50/50 mix of sphagnum peat and vermiculite) leached with 2 volumes of distilled water after thorough wetting and then watered with one volume of Peter's 21-5-20 fertilizer nutrient solution diluted to 100ppm nitrogen (Bugbee, B. 2004), and allowed to drip dry before weighing. Seeds were vernalized at 4 ^{o}C for 7 days prior to placement in the growth chamber under the same conditions as above. Two additional pots were weighed (heavy pot: 459.8g, and light pot: 388.0g) and baked at 70 ^{o}C until dry, and weighed again (heavy: 121.6g, light 96.0g). At full saturation the pots held about 73.5% (heavy pot) or 75.2% (light pot) water by weight. Therefore a

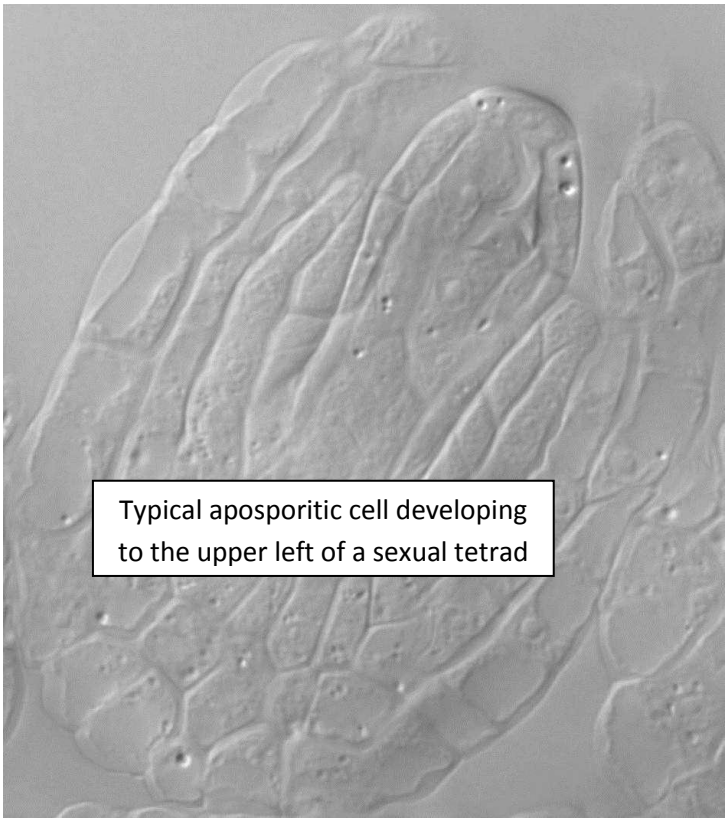
74% water holding capacity will be used to calculate the weight at which individual pots in each treatment will be maintained. The control plants will be maintained at about an 80% water capacity by watering with nutrient solution whereas the drought-stressed plants will be allowed to slowly dry down to about a 50% water capacity by the 14th day after sprouting. Young inflorescences will be fixed and analyzed as above. The percentage of ovules that revert back to normal sexual development will be calculated among the three lines. Plants will be thinned to 4 per pot with two pots per treatment.

Results

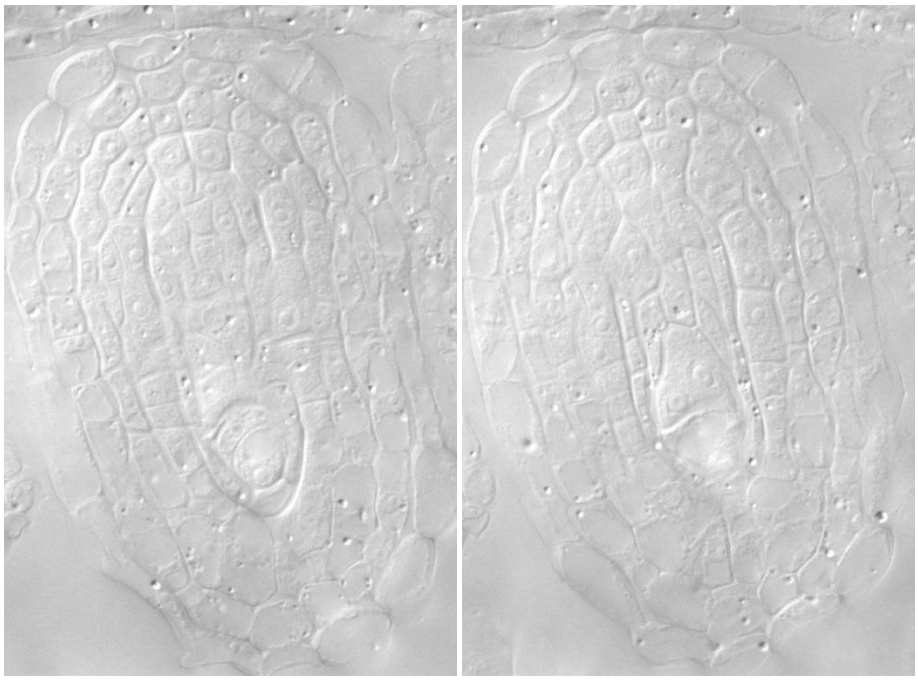
Initial analysis of the *Arabidopsis* seed accessions:

- CS1854 – Inflorescences developed abnormally. The pistil grew an additional 4 pistils that were fused along the length of the central pistil. Ovules growing inside the central pistil were more mature than ovules growing inside the surrounding pistils (pictures are forthcoming). A few of the megaspore mother cell's (MMC) within the ovules of this plant progressed through meiosis and formed distinct sexual tetrads that were lined with callose and appeared normal. However, the majority of the MMC's grew very large and did not divide. Rather, they appeared to skip meiosis and eventually develop into normal looking embryo sacs. The embryo sac of more mature ovules appeared to develop normally and form endosperm. Additional work needs to be performed and more ovules need to be analyzed to determine if this development occurred after normal fertilization or if it was the result of fully autonomous parthenogenesis or parthenogenesis initiated by pseudogamous fertilization. As the plant matured and appeared more stressed the percentage of ovules forming normal sexual tetrads appeared to increase.

- CS66153 – The pistils of this accession developed normally but the ovules exhibited high rates (> 90%) of apospory occurring next to normally developing sexual tetrads. Apospory is the development un-reduced non-germline cells into an embryo sac. In some apomictic species these aposporitic cells compete with and outgrow sexual tetrads so that the majority of seeds are formed apomictically. In such cases it is common for multiple aposporitic cells to initiate development but they only occur transiently until one continues to grow into a viable embryo sac. In this particular accession the aposporitic cells only initiated during meiosis and further apospory events were very rarely seen in later stages. Interestingly in one case two embryo sacs were seen developing within what appeared to be a sexual tetrad. It is possible that the inner



embryo sac is forming from an unreduced dyad while the embryo sac at the tip is developing from an apospory event (see pictures of the ovule in two different focal planes to the right).



- CS66154 – This line was difficult to grow and even though it was stunted and purple it set some seed. It will be analyzed in the next growth trial.

Detailed analysis of the *Arabidopsis* seed accessions under a controlled stress test: (This portion of the analysis is not yet available.)

- CS66153
  - Optimal growth conditions:
  - Controlled stress conditions:
- CS66154
  - Optimal growth conditions:
  - Controlled stress conditions:
- CS1854
  - Optimal growth conditions:
  - Controlled stress conditions:

Discussion

The HDA6 knockout reduces the ability of the plant to respond to stress. As a result the plants are more sensitive to normal stresses making it difficult to decrease the impact of a preprogramed stress signal in the meiocyte. A more effective approach will be to target the knockdown of HDA6 exclusively in the meiocyte via the DMC1 promoter (Klimyuk and Jones 1997). RNAi knockdown of genes using the DMC1 promoter has proven a successful strategy to study meiosis (Siaud et. al. 2004; Higgins et. al. 2005, Liu and Makaroff 2006).

It is interesting that the meiocyte of sexual plants is preprogramed to initiate a strong enough stress signal to induce callose formation during meiosis. This suggests that it is difficult for the signal to be suppressed in most species through genomic perturbations such as hybridization or polyploidization. Conversely other plant families that exhibit a hybridization induced predisposition towards apomixis may be more susceptible to genomic perturbations that thwart the stress signal returning them to facultativeness or even rendering them obligate apomicts.

The results of this study suggest that thwarting the preprogramed stress signal in the meiocyte poses a paradigm shift for apomixis research. Previous gene knockouts that led to partial apomictic phenotypes can now be reevaluated in the new light that they were limited by being positioned within a sexual epigenome programed by a pre-meiotic stress signal.

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