NAME OF THE PROJECT:

Senescence of unfertilized ovules in *Arabidopsis thaliana*

FINAL REPORT

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INTRODUCTION

Programmed cell death during reproductive development

Sexual reproduction is a key event in most of the multicellular organisms and represents a natural source of genetic diversity. The spermatophytes (seed bearing plants) represented a novelty in the evolution of vascular plants, particularly the angiosperms (flowering plants). Seeds of angiosperm plants constitute a major proportion of the human diet worldwide, thus, from the agronomic point of view, the study of seed biology is very important to improve seed yield and quality. Sexually-derived angiosperm seeds develop after a "double fertilization" process has taken place in a very unique mode, which results in two different fertilization products: the diploid embryo and the triploid endosperm. They are both surrounded by the maternally derived seed coat, and the trinity of these genetically distinct organisms requires a strict communication to coordinate seed growth and development (Ingram, 2010).

Not only proliferative growth is required for a successful reproductive development, but also the controlled instances of cell death. Programmed cell death (PCD) is a genetically encoded, self-destruct mechanism of the cell. It can be triggered both by internal and external factors, leading to signaling events which result in a cascade of hydrolytic activity, a shutdown in the cellular metabolism and a succession of events to promote cell death (Olvera-Carrillo et al., 2012). In higher plants it is a tightly regulated process that occurs in several instances during its life cycle: PCD occurs during tracheary element formation (Turner et al., 2007), leaf and floral organ senescence (Guiboileau et al., 2010), the hypersensitive response to contend the growth of biotrophic pathogens and viruses (Mur et al., 2008), and throughout reproductive development (reviewed in Olvera-Carrillo et al., 2012). While many forms of PCD have been dissected in animals, most prominently apoptosis, the molecular pathways controlling PCD processes and the different forms of cell death in plants are scarcely known to date (van Doorn et al., 2011).

Seed set and thus the yield of seed-bearing crop plants depends mostly on plant reproductive success. Plant reproduction relies on a favourable biotic and abiotic environment during its life cycle and PCD is a common component of the plant's reaction to stress (Taylor et al., 2009). Indeed, the reproductive phase of plants is one of the most sensitive to stress as even short periods of unfavourable conditions can strongly reduce yield (Mittler and Blumwald, 2010). The reproductive phase is defined by the transition from vegetative growth to the differentiation of floral meristems in the apical region of the plant. The timing for its occurrence can vary according to the species and their interaction with the surrounding environment. Land plants have alternating sexual and asexual generations: the haploid gametophytes and the diploid sporophytes. Inside of the flowers of angiosperms, the ovules contain the haploid female gametophyte, and they are the direct precursors of the seeds. The ovule is exceptionally vulnerable to stress and its receptivity to fertilization is an important factor limiting seed set. In many plants, ovules undergo developmentally controlled PCD when they are not fertilized within a certain species-specific time span (Sun et al., 2005). Therefore, it is of great importance to study the perception, signalling and regulation of cell death process during plant reproductive development. We have chosen several techniques to study PCD in the model plant *Arabidopsis thaliana*. I created a toolbox of reporter genes to be used for live cell imaging by creating transcriptional fusions with the promoters of genes which have been shown to be induced at particular stages of cell death. The morphology of degenerating ovules was studied at the ultrastructural level using transmission electron microscopy (TEM), and these changes will be correlated with the transcriptomic profile of discrete degeneration stages using deep sequencing of RNA (RNAseq). The candidate genes obtained from these analyses will be further studied by genetic and functional approaches.

An additional level of regulation occurs at the protein level, and the protein complexes mastering the execution steps of cell death can be studied with the platform that I developed in collaboration with the Proteomics laboratory with the use of Tandem Affinity Purification (TAP)

to unravel the interactions of our candidate genes at the protein level.
During the BELSPO fellowship, I developed diverse molecular and biochemical tools to study the mechanisms of PCD process during reproductive development, with an emphasis on two closely related tissues/organs: the unfertilized ovule and the early stages of seed development. The results obtained in each of the projects will be presented, followed by the conclusions and the definition of future perspectives.

METHODOLOGY AND RESULTS

I. Morphological characterization of ovule degeneration in unfertilized pistils.

The morphological characterization was used as a first step to describe the processes that can be observed at the cellular level in degenerating ovules. Previous work of a Master student from the Seed Development group (Matthias van Durme) using Confocal Laser Scanning Microscopy (CSLM) allowed us to define four discrete degeneration stages, and it was complemented with a fine characterization at the ultrastructural level. The morphology of fully receptive, wild type ovules, was compared to the morphology of ovules in a degeneration series from 2 to 6 days after emasculation . The latter consists in the removal of anthers from the flower to prevent the fertilization of the ovules. I carried out this analysis using Transmission Electron Microscopy (TEM) in collaboration with Riet de Riecke from the TEM facility in VIB-Ledeganck. Two methods for the tissue fixation were compared: chemical fixation and high pressure freeze substitution (HPFS) to ensure that the morphological changes were associated with the degeneration process and not as a result of technical artefacts. The information provided by CLSM and TEM has enabled our research team to identify the morphological changes during the degeneration of unfertilized ovules undergoing cell death and to highlight those that can be studied in further detail. We have selected for further research the degeneration process of synergid cell as one of the first hallmarks of programmed cell death undergoing in unfertilized ovules. Synergid viability is expected to limit the receptivity of the entire ovule, as its function is crucial for pollen tube attraction and receptivity. The integuments are also one of the first tissues from maternal origin undergoing PCD during age dependent degeneration of ovules. The order in which the inner and outer integumental layers degenerate follow a similar pattern to the one that occurs naturally during seed development, where the seed coat is reduced to a single layer after the progressive degeneration of the integuments.

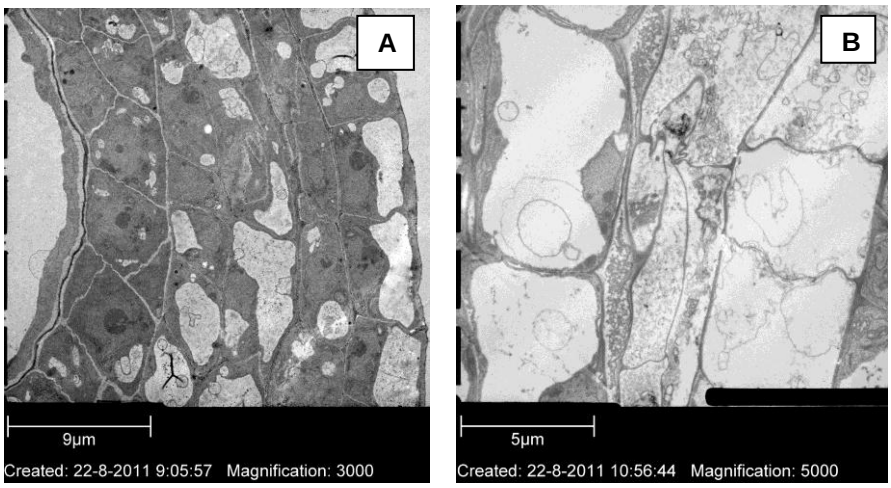

Figure 1. Representative pictures comparing the integument layers surrounding the female gametophyte, during an early stage of the decay series (4 DAE), when the ovules are no longer receptive to fertilization (panel A) and the following degradation of the inner integument layers showing clear signs of cell death, such as clearance of the cytoplasm and plasma membrane interdigitation (panel B).

II. Promoter cloning of tissue specific genes

The cloning of several tissue specific promoters has been a common effort in the seed development laboratory leaded by Dr. Moritz Nowack, to set up the tools for further studies at the molecular, cellular and genome wide level. During the first months of the fellowship I contributed to the cloning of ovule specific promoters to create transcriptional fusions with a nuclear localized GFP (which is used as a reporter gene), visible under fluorescent light microscopy in a transactivation system (Figure 2, see below for explanation).

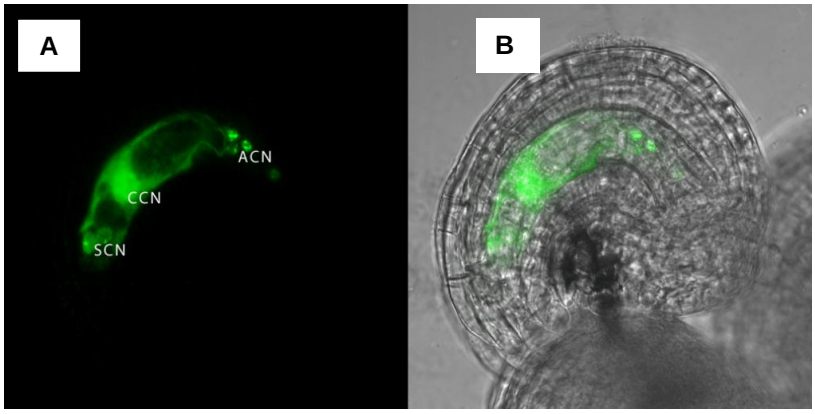

Figure 2. Representative picture showing the expression of the reporter gene (a nuclear localized GFP, H2A-GFP), driven by the female gametophyte specific promoter MYB98, shown as an example of the activator constructs. In panel A the fluorescent signal is seen in the nucleus of antipodal cells (ACN), central cell nucleus (CCN) and synergid cells nuclei (SCN). In panel B an overlay of the GFP signal with a bright field image of the female gametophyte in a fully receptive ovule.

Additionally, seed compartment specific promoters were cloned as part of the common effort to obtain a toolbox of tissue specific promoters as transcriptional fusions to express in a transactivation system (Table 1). The transactivation system depends on the use of separate transgenic lines carrying activator or effector constructs. In the lines carrying the activator construct, the transcription of GAL4/VP16 is controlled by our tissue-specific promoter. These transgenic plants can activate the transcription of genes that are cloned behind an upstream activating sequence (UAS), which allows transcriptional activation by GAL4/VP16. The activator lines can thus redirect the activity of the original promoter *in trans* to multiple other genes, which can be either reporter genes or candidate cell death effector genes. The two separate constructs can be combined by co-transformation or by crossing plant lines for further analyses (Figure 3).

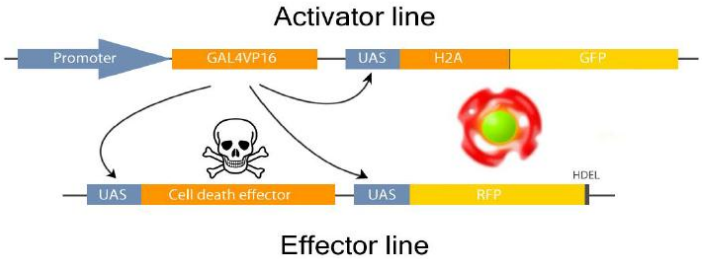

Figure 3. Schematic representation of activator and effector lines. In the activator lines the transcription of GAL4/VP16 is under the control of a tissue specific promoter. The activator can initiate the transcription of genes that are cloned behind an upstream activating sequence (UAS), thus redirecting the activity of the ovule promoter *in trans* to other genes. These constructs can be combined by crossing or co-transformation. To verify the transactivation each construct has a unique reporter (H2A-GFP, a nuclear localized green fluorescent protein, or RFP, red fluorescent protein).

The analysis of the transgenic plants in the T1 generation was performed to check their expression patterns using fluorescence microscopy, and the seeds recovered from the plants which showed the expected pattern, were harvested individually to find single insertion lines, as indicated by their 3:1 segregation ratio of the resistance marker. In the next generation (T2) I carried out a second screen of the selected lines to find homozygous plants for the desired promoter fusion. Some candidate cell death effector genes have been also cloned by other members of the Seed Development group and transformed into Arabidopsis plants. These effector lines will be used for crossing with our tissue specific promoter and to analyse their phenotype in the reproductive stage.

Table 1. List of seed compartment and ovule specific promoters cloned with a Gateway compatible system, which will be used as activator lines in the transactivation system based on GAL4/VP16. The constructs were transformed to Arabidopsis plants and the selection marker resistant plants were screened in the T1 generation to assess its expression levels.

AGI code	Name	Project
At4g04460	APR_1	Seed, cellularized endosperm
At1g48930	GH9C1	Seed, cellularized endosperm
At2g43660	F18019.23	Seed, early endosperm
At1g02450	NIMIN1	Seed, early endosperm
At5g46950	INVI	Seed, embryo and early endosperm
At5g54640	KS117/FH5	Seed, marker for early endosperm
At3g29770	MES11	Seed, cellularized endosperm, weak in embryo
At1g68510		Seed, peripheral endosperm onwards, weak in embryo
At2g38900		Seed, predominantly seed coat
At1g62080	UP1	Seed, strong late seed coat
At4g00220	LOB	Seed
At3g12110	ACT11	Ovule
At4g18770	MYB98	Ovule

III. Subcellular characterization of the degeneration processes in unfertilized ovules.

In order to study the changes that occur at the subcellular level in the cell biology of PCD in degenerating ovules we wanted to investigate the dynamics of subcellular compartments and organelles that are important as executers or targets of PCD processes: nucleus, ER and vesicle transport machinery, Golgi, endosomes, vacuole, cytoskeleton, tonoplast, etc. Around 20 marker lines for subcellular compartments are available in the Department of Plant Systems Biology, they belong to the WAVE collection, a multicolour set of marker lines from the group of Niko Geldner in Germany (Geldner et al., 2009). They are translational fusion proteins of subcellular compartment markers driven under the control of commonly used constitutive promoters. Hence, first thing to do was to test whether these promoters are active in the female gametophyte and during ovule degeneration. Two ubiquitouos promoters were analysed in the CLSM and both were seen to be active in the integuments (which are of maternal origin), promoter 35S from CaMV was often seen as patches in the fluorescent signal, promoter UBQ10 was weaker in the signal intensity, but more evenly expressed. Unfortunately, they were not visible in the female gametophyte. Therefore, the available subcellular markers were cloned as translational fusion proteins with two fluorescent reporter proteins (YFP and RFP), to be available to combine them with the female gametophyte specific promoters in a Gateway compatible system. These marker lines will enable the analysis of the cell biological dynamics in aborting ovules. Table 2 shows the clones of the subcellular compartment markers that I created during the first term of the fellowship.

Table 2. List of marker lines cloned as translational fusions with YFP and RFP reporter proteins, which will be used under the control of ovule specific promoters to analyse the cellular dynamics of selected sub-cellular compartments during the degeneration process of unfertilized ovules.

Line	Name	Localization	Reporter
Wave 1	Tag only	Cytosol/nucleus	YFP/RFP
Wave 2	RabF2b (ARA7)	Late endosome/prevacoular compartment	RFP
Wave 3	RabC1 (Rab18)	Post-golgi/endosomal	YFP
Wave 5	RabG3f	Late endosome/vacuole	YFP
Wave 6	NIP1;1	ER/plasma membrane	YFP
Wave 9	VAMP711	Vacuole	YFP
Wave 11	RabG3c (Rab 7D)	Late endosome/vacuole	YFP/RFP
Wave 18	Got1p homolog	Golgi	YFP
Wave 24	Rab A5d	Endosomal/recycling endosome	YFP/RFP
Wave 25	Rab D1 (ATFP8)	Post-golgi/endosomal	YFP
Wave 27	Rab E1d (Rab8C)	Post-golgi/endosomal	YFP/RFP
Wave 29	Rab D2a	Golgi/endosomal	YFP
Wave 33	Rab D2b (Rab1A)	Golgi/endosomal	YFP/RFP
Wave 34	Rab A1e	Endosomal/recycling endosome	YFP/RFP
Wave 127	MEMB12	Golgi	YFP/RFP
Wave 129	Rab A1g	Endosomal/recycling endosome	RFP
Wave 138	PIP1;4	Plasma membrane	RFP

IV. Locus identification of cell death markers during reproductive development.

As the embryo develops inside the seeds embedded in endosperm tissue, an active process of cell death and autolysis occurs in the embryo surrounding region of the endosperm (ESR), providing nutrients and space for the expanding embryo. Enhancer trap promoter lines have been widely used to study the expression patterns at the transcriptional level and also to reveal its localization at the sub-cellular level. Two marker lines were shown to be expressed in the ESR, they belong to two different enhancer trap mutant collections, N9185 and G222, which are visible with the reporter genes GFP and GUS, respectively (Ingouff et al., 2005). As these markers are closely correlated with the occurrence of cell death in the ESR, we asked whether they also mark cell death processes in other developmental contexts, especially in aborting unfertilized ovules. Although in a first examination we could identify N9185 expression in differentiating tracheary elements -a process also accompanied by PCD- no N9185 expression was detected in aborting ovules. G222 expression, on the contrary, was located in ovules, but no clear correlation of the expression with the ovule abortion process could be established. Nevertheless, the fact that G222 is expressed in ovules and that N9185 is associated with differentiating tracheary elements convinced us to try to determine the enhancer trap loci in these marker lines. This procedure might yield gene identities that are functionally involved in PCD as well as ovule development control. The locus identification of these marker lines was carried out by the genome walk method followed by sequencing of PCR products. For both lines, candidate loci were successfully identified. For the G222 marker line, it was found that a single T-DNA was inserted in a coding region of a strictosidine synthase-like gene, in a 5' LB-RB orientation. In the N9185 marker line, tandem repeats of T-DNAs were inserted as inverted repeats between two genes, they encode a protein of unknown function and a calcineurin B-like interacting protein kinase. The promoter of these candidate genes were cloned, combined with nuclear localized GFP as reporter gene in the transactivation system, and transformed to Arabidopsis. The transformants from the T1 generation were screened for the expression pattern in the ESR, but the GFP signal did not follow a clear pattern, each independent insertion transformant showed the signal either in the embryo, endosperm, testa, and/or suspensor, but this pattern was not as the expected one from the G222/N9185 enhancer trap lines.

During the time the candidate promoters were cloned and transformed in Arabidopsis, we searched for available mutants in the candidate genes, to find loss of function mutants and check if they had any phenotype during seed development due to alterations in the ESR. The seeds were ordered and received from the NASC and GABI-Kat collections and were

genotyped using gene specific primers to confirm the insertion site. Their phenotype was screened in the developing siliques from the heart stage embryos onwards. The mutants for N9185 candidate genes did not show any phenotype during seed development, and the plants showed normal seed set. On the contrary, all the alleles for the G222 mutants showed a lower amount of ripe seeds per silique and no homozygous mutants were recovered. Two phenotypes were observed, some seeds shrivelled early during development and others produced abnormal seeds which did not ripe at the same pace as the Wt siblings. The siliques of three mutant allelles of heterozygous plants were mounted in chloral hydrate to clear up the tissues and a few days later were observed with light microscopy using differential interfering contrast (DIC).The embryos from the shrivelled seeds were arrested after the early globular stage (Figure 4a, left) and the other aberrant seeds showed defects in the embryo shape, as well as a higher proportions on chalazal cysts, but the endosperm nuclei appeared normal (Figure 4b). The plants were backcrossed with wild type plans to ensure that the T-DNA was segregating with the phenotype observed. The same phenotype was observed in the F1 plants, but since the phenotype is related to an embryo defect rather than a defect in the endosperm, the mutants were not further studied for the purposes of cell death of the endosperm in the ESR.

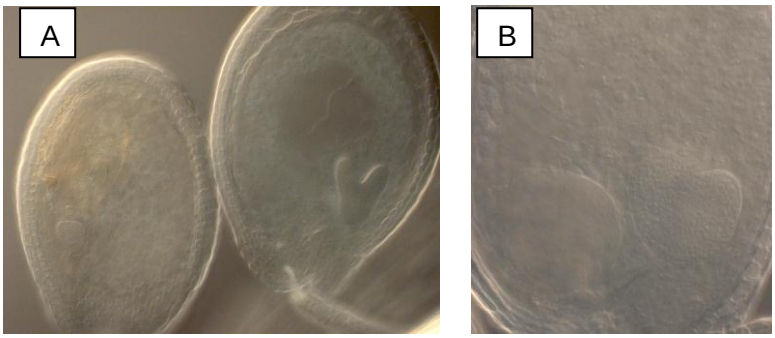

Figure 4. Representative pictures comparing the embryo development of the mutant seeds from the promoter of the candidate G222 marker line in heterozygous plants. Shrivelled seeds were the result of an arrest in the globular stage embryos (panel A, left), as compared to the normal development of the wild type siblings (panel A, right). Other abnormal seeds sin the same silique showed alterations in the embryo shape and the presence of a higher proportion of chalazal cysts in many seeds as compared to the wild type panel B).

V. Protein-protein interactions of candidate inducers of programmed cell death in Arabidopsis cell suspension cultures.

The suspensor is a short-lived organ, undergoing developmentally controlled PCD during seed development; however its initiation and execution signals, and how cell death is prevented in the adjacent cells of the embryo proper is not known (Kawashima and Goldberg, 2010). KISS OF DEATH (KOD) encodes a 25 amino acid peptide that is specifically expressed in suspensor cells before degeneration, as well as after biotic and abiotic stresses. Loss of KOD function lead to a decreased rate of suspensor degeneration and heat-shock induced PCD in root hair cells. Ectopic KOD expression in tobacco leaves and Arabidopsis seedlings lead to induction of PCD, loss of mitochondrial membrane potential and induced caspase-3 like DEVDase activity (Blanvillain et al., 2011).

In collaboration with the Functional Interactomics group of PSB headed by Geert De Jaeger, the identification of protein-protein interaction of two cell death inducers, KOD and BAX proteins, is an ongoing project. The nature of these proteins as potential cell death triggers required the use of an inducible system to avoid a premature death of the transformed Arabidopsis cell suspension cultures. As no inducible system for the TAP tag cell suspension culture was available in Geert De Jaeger's lab, an established heat shock / dexamethasone activated inducible expression system was adapted for the use in Arabidopsis cell suspension cultures

(Joubès et al., 2004; Figure 5). The first step was the cloning of TAP-tagged versions of these proteins, both in N- and C-terminal fusions, using the Fusion PCR technique, followed by sequencing to confirm the fusion version of each protein, its recombination with the inducible vector and its transformation to cell cultures via *Agrobacterium tumefasciens* co-cultivation.
The inducibility of the system was validated in the first place, by analyzing a fluorescent reporter protein (RFP), to test the levels of induction, as well as the phenotype of the non-transformed suspension cells after the induction treatment using 10 μM Dexamethasone and 2 hours of heat shock at 37ºC.

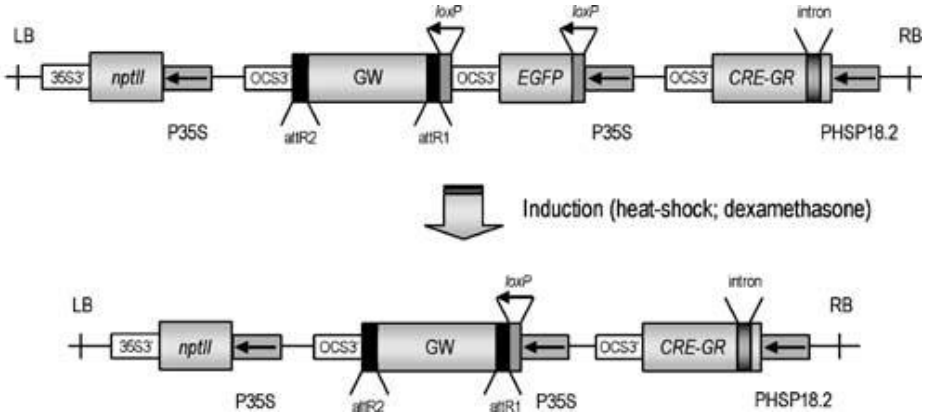

Figure 5. Schematic representation of the T-DNA construct which was transformed into Arabidopsis cell suspension cultures. The system was first validated using RFP as a bait and afterwards the N and C terminal TAP-tagged versions of the KOD and BAX proteins. The genes were recombined using Gateway technology in the GW cassette to be able to obtain fusion proteins with a tag that allows the purification of protein complexes of the bait proteins with its possible interactors. Figure taken from Joubès *et al*., 2004.

The leakiness of the expression of the bait proteins was scored by Western blot analysis of treated cells and mock-treated cells (heat shock treatment and DMSO as solvent for dexamethasone) before and after induction. Very low levels of the RFP protein were observed before induction (low leakiness), therefore the system was validated for its use with the TAP tagged versions of the cell death inducers KOD and BAX, by replacing RFP (shown in Figure 5) with the two versions of the KOD peptide (N- and C-terminal tag) and the two versions of BAX (N- and C-terminal tag).
The cell cultures of KOD protein fused to the TAP tag in the C and N terminal fusions showed a low level of leakiness, scored by western blot analysis (Figure 6A). Several time points during a range of 4 to 72 hours after induction were sampled to assess their inducibility and the expression levels.

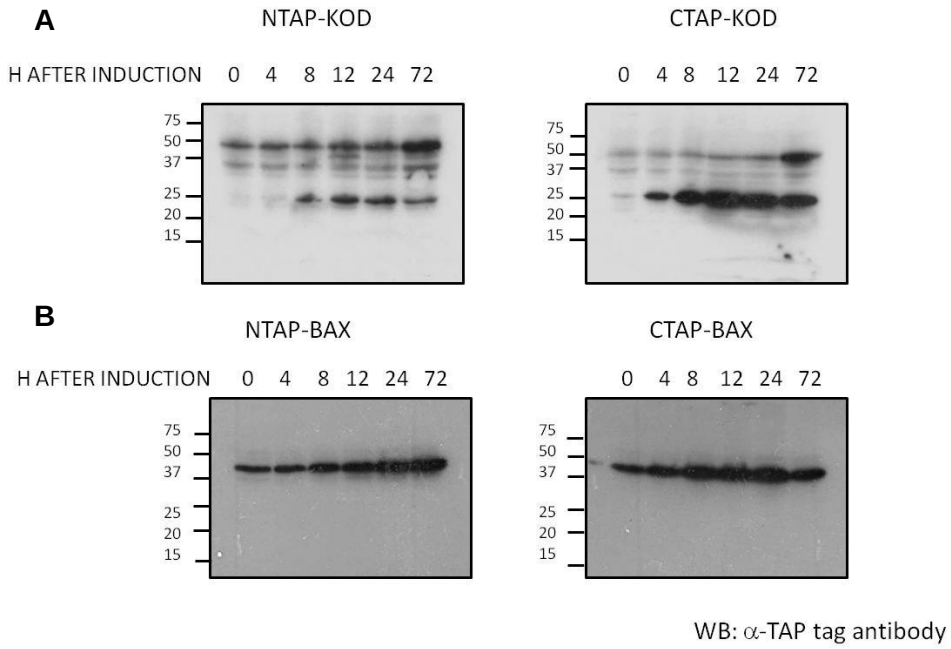

Figure 6. Expression analyses of N and C TAP tagged versions of candidate cell death inducers, KOD and BAX. Western blot analyses using polyclonal antibodies against a portion of the TAP tag and total protein extracts from A) KOD and B) BAX transformed cell suspension cultures. Samples from each cell culture were taken before induction (time 0) and from 4 to 72 hours after induction with heat shock and dexamethasone.

After induction of the expression of the cell death peptide KOD and the BAX protein, the cell suspension cultures showed some extent of cell death as indicated by staining with propidium iodide and Evans blue. However, the control samples also showed some positively stained cell clumps in the culture, so we could not conclude that a true effect in the death of the cell cultures was due to the expression of the bait proteins. We therefore moved on to express these four constructs in stably transformed Arabidopsis plants. The selection of T1 seedlings was carried out in selective plates and the transgenic seedlings were grown to maturity, their seeds were collected individually. From each plant, a segregation analysis (3:1 ratio of resistant versus sensitive seedlings) was performed by plating in selective media, to choose only those independent transgenic lines were a single T-DNA was inserted. I carried out an induction of the expression of the baits in the chosen T2 lines using 2 week old seedlings, with the same induction parameters as with the cell cultures. Only one of the constructs was able to effectively promote the cell death in the seedlings after induction, and the other three were not functional. The N-terminal fusion of BAX (NTAP-BAX) was the functional fusion protein, while the C-terminal fusion (BAX-CTAP) was not capable to induce the same effect (Figure 7A). On the contrary neither of the KOD fusions caused any phenotypic effect on the seedlings, even after testing 10 independent transgenic lines with a 3:1 segregation ratio (Figure 7B).

The BAX containing constructs were upscaled to obtain seeds from the T3 generation in order to obtain the necessary amount of biomass for the purification of the proteins in complex with its putative interaction partners. The purification of protein complexes from the plants after induction with Dexamethasone and heat shock will be carried out using the functional construct and the construct of the C-terminal fusion will be used as control. The identification of peptides will be performed to uncover the proteins which interact with the bait. The candidate proteins will be identified after LC-Mass Spectrometry analysis. Functional analysis (loss/ gain and function) and further confirmation of the interactions by other methods *in vivo* an *in vitro* (yeast two hybrid, pull down) could follow.

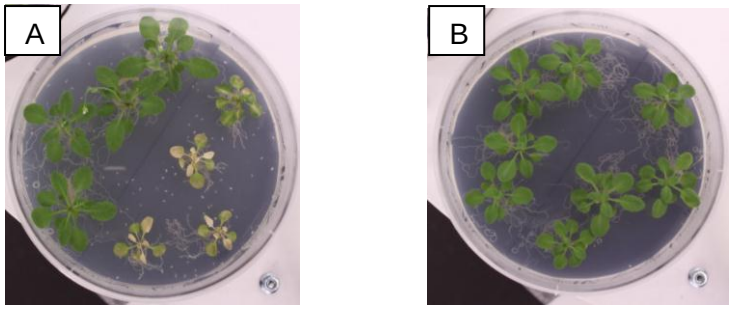

Figure 7. Representative pictures of the constructs transformed in Arabidopsis plants, with N and C terminal fusions of candidate cell death trigger proteins, BAX and KOD, for its use in TAP experiments. The pictures were taken 10 days after the induction treatment with dexamethasone and heat shock to 12 day old T2 seedlings. A) NTAP-BAX fusion triggered cell death in the seedlings (right), while the CTAP-BAX fusion was not functional. B) None of the KOD fusions with TAP (NTAP-KOD nor CTAP-KOD) gave a cell death phenotype in the seedlings after the same induction treatment.

VI. Construction of a semiautomatic tissue harvester for RNA sequencing.

One of the difficulties of the tissues we are working with (Arabidopsis ovule and seed) is their very small size. To be able to obtain RNA for deep sequencing experiments with a high purity and in enough amounts, we had to harvest the tissues in a selective and rather fast manner, to avoid contamination from other parts of the reproductive tissues, and in a cool environment to avoid degradation of the RNA. In collaboration with Dr. Jean Phillipe Vielle-Calzada (LANGEBIO, Irapuato) and Dr. Mario Arteaga (UV, Xalapa) from Mexico, we could develop a semiautomatic tissue harvester using a vacuum pump method (Sánchez-León et al., 2012). The device allows for the fast collection of ovules directly in a deep frozen container. The RNA was extracted from emasculated wild type Col-0 plants from a developmental series: from fully receptive ovules (2 days after emasculation, DAE) until the last degeneration step of the unfertilized ovules (5 DAE). For each sample (time point) the ovules from 50 individual pistils (from 50 different emasculated wild type-plants) were pooled. We conducted several test RNA extractions from unfertilized ovules. Our results indicated that the RNA from the ovules of 50 pistils would satisfy the requirements for RNA sequencing (Figure 8).

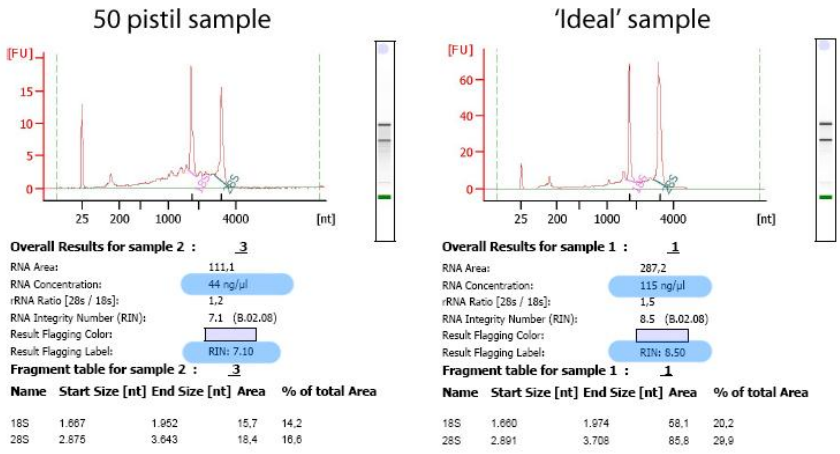

Figure 8 Preliminary results for RNA extraction from isolated ovules. Agilent BioAnalyzer report for two RNA samples. Left: Result of a test extraction performed on the ovules of 50 pistils. Right: The result of an extraction performed on 50 whole pistils. This idealized sample is indicative for the best result that can be obtained when the sampling is done under normal conditions i.e. without the use of a micro-aspirator. The most important values are highlighted in blue. RIN is a measure for the quality of the RNA and should preferably be over 7, the quantity/concentration is also indicated.

The different stages of degeneration that we have sampled for the RNA extraction have been described as part of the Master thesis of the member of the Seed Development laboratory, Matthias Van Durme. With these developmental series, we will be able to track down the transcriptome changes from early stages of ovule abortion, to define the key genes which are involved in the degeneration process of the unfertilized ovules. In addition, this tissue harvester will be useful for the extraction of seeds and for other purposes, such as protein extraction from both ovules and developing seeds.

VII. Other academic activities

Additional academic activities of the first term period of the fellowship included the writing and publication of a book chapter in collaboration with members of the Seed development lab, an up to date review of the programmed cell death responses during reproductive development in plants. Also, a student from the Biochemistry and Biotechnology Master Program was received during a 6-week internship period to cover the Practicum of its first Master Project. Helena de Bleser was taught 4 techniques of common use in laboratory practices during this time period corresponding to 96 hours of laboratory work. A bachelor student (Kevin Goslin) was received in a summer stay during the second term of the fellowship for a period of ten weeks, during which he received training for common use techniques in plant molecular biology and biochemistry.

CONCLUSIONS AND PESRSPECTIVES

The in depth morphological characterization of the degeneration process of unfertilized ovules indicated that the senescence process is an ordered event. It starts at the synergid cells, to disable over aged ovules from attracting pollen tubes, followed by the decay of the integuments, while the egg cell and the central cell are the last ones to degenerate.
A common toolbox of tissue specific promoters and cell death effectors in a transactivation system was developed. These allowed us to study diverse subcellular markers, driven by ovule specific promoters, and enabled us to follow the ovule senescence process using live cell imaging techniques. With the tools we could characterize the subcellular processes that take place during the degeneration of unfertilized ovules in specific organelles which are known to be involved in the cell death process.
The optimization of ovule harvesting using a micro suction device has enabled us to obtain high purity and high quality RNA to perform a comparative transcriptomic analysis of the degeneration stages in unfertilized ovules, to find those transcripts that are regulated during the senescence process. Functional analysis of the candidate genes will follow.
The mechanism of action of the functional BAX-TAP fusion protein can be studied with the purification of protein complexes from the seedlings that express this bait protein in the inducible system. The identification of putative interacting proteins during the cell death process can be corroborated with other techniques to unravel the mechanism of action of this cell death trigger.
Additionally, in close collaboration with a member of the Seed Development group, Matthias van Durme, we are setting up a mutant screen for altered ovule longevity. For this purpose I have created the molecular tools necessary for the screen, namely, a conditional male sterile mutant (conditional *ms-1*) has been established and transformed with ovule cell death markers (pCEP1, pBFN1, pSAG12) carrying a reporter gene (GUS-GFP or Luciferase) to find mutants with a delay in the ovule senescence process. Map based cloning will reveal the genes with key functions in the degeneration of unfertilized pistils.

Literature cited

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