UNIVERSITEIT GENT

FACULTY OF SCIENCES

The cell biology of cell death:

Using aleurone and stigmatic papilla cells to study programmed cell death in *Arabidopsis thaliana*

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belspo

Plant Systems Biology

Introduction

Programmed cell death (PCD) is an actively controlled, genetically encoded mechanism of cellular self-destruction. Whereas many forms of PCD have been described and molecularly dissected in animals, there is only little known about the control of PCD processes in plants (Van Doorn, 2011). Nevertheless, PCD processes are an essential part of a plant's reaction to the biotic and abiotic environment, as well as a crucial component of many plant developmental processes (REF). Especially in plant reproductive development, developmentally controlled cell death processes are ubiquitous and precise initiation and execution of cell death is essential for successful reproduction (Olvera-Carillo et al, 2011, in press). In our laboratory at the VIB PSB department in Gent, we are studying the molecular basis of developmentally controlled PCD during reproductive development in the reference species *Arabidopsis thaliana.* In my research projects, I am focusing on the development of a model system that allows the investigation of the cell biological processes that control and execute cell death. I started with the investigation of PCD in the aleurone of germinating seeds, and recently added a new system looking at PCD in stigmatic papilla cells of unfertilized flowers.

PCD in endospermic aleurone cells (AL) during seed germination

In *Arabidopsis* the aleurone, a typically single-celled outer endosperm layer that escapes endosperm cell death during mid-seed development undergoes a form of developmentally controlled programmed cell death (PCD) after seed germinates (Bethke et al, 2007). The morphological simplicity and easy accessibility makes the aleurone layer an attractive cellular model system to study PCD in plants. So far only studied in cereals and in Rizinus (Schmid et al, 1999; Gietl and Schmit, 2001), but here limitation of genetic and cell biological analysis due to the lack of easy transformation and incomplete genome information. In Arabidopsis, aleurone layer is formed analogous to cereals, and undergoes similar morphological and ultrastructural modifications upon germination (Bethke, 2007). So here investigation whether Arabidopsis aleurone cells show a similar PCD program during germination as aleurone cells in cereals and whether we can use this system to investigate genetic and cell biology of PCD during developmentally controlled cell death.

PCD in stigmatic papilla cells (SPC) in unpollinated flowers

Similarly to the aleurone, Arabidopsis stigmatic papilla cells are ideally suited to investigate the genetics and cell biology of developmentally controlled PCD.
Stigma together with style, and ovary are the main parts of the pistil, which is the female receptive organ in pollination through which the pollen tube travels to deliver the sperm cells to the egg. The stigma, as the entry into the pistil's specialized transmitting tract tissue, provides a receptive surface for compatible pollen to adhere, hydrate, germinate, and grow. The epidermal layer of the stigma of Cruciferae consists of papilla-like cells covered with a cuticle (Christ, 1959). Papilla cells have a species-specific life span, after which they undergo developmentally controlled programmed cell death (PCD). The control of PCD in the stigma is one of the major determinants of the genetically fixed receptive period of a flower. Once stigma senescence is

initiated the reproductive capacity of a flower is irrevocably lost. Long, protruding papillae cells are easy to isolate and readily amenable to cell biological investigations.

Aims

The aim of the present project is to assess the aleurone and stigmatic papillae cells as the model systems to study developmentally controlled PCD during plant reproduction.

The project was set out to fulfill three main objectives:

1. To basic analysis of the entire process of aleurone and stigmatic papillae cell (SPC) death on a cell biological scale. The knowledge on the progression of aleurone and SPC degeneration is an important foundation for the investigation of the molecular mechanisms of these tissues cell death.
2. To study transcriptome changes during aleurone/stigmatic papillae cell death to uncover candidate genes that can be functionally analyzed.
3. To investigate promising candidate genes in detail, including gain- and loss-of-function approaches, expression and protein localization studies in aleurone and SPC.

Results

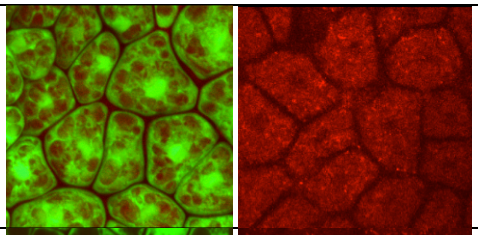
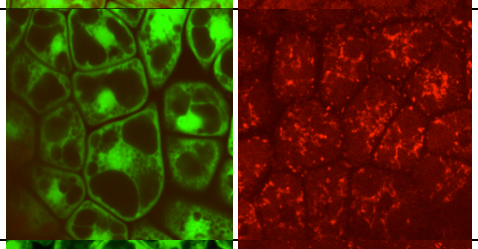
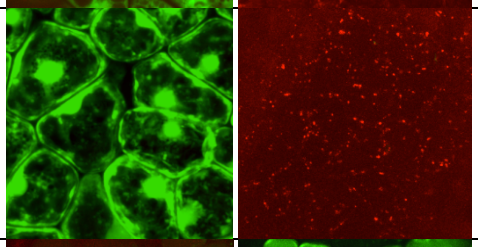
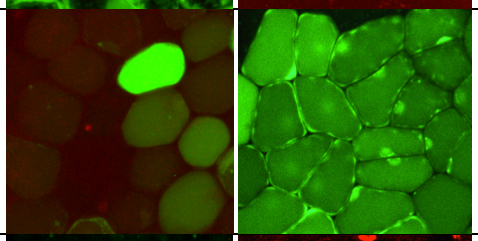
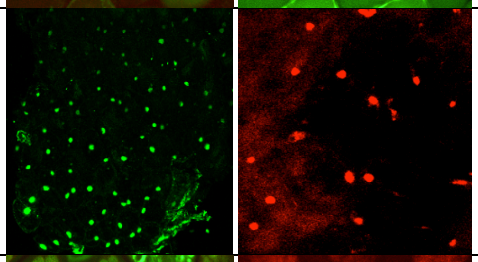
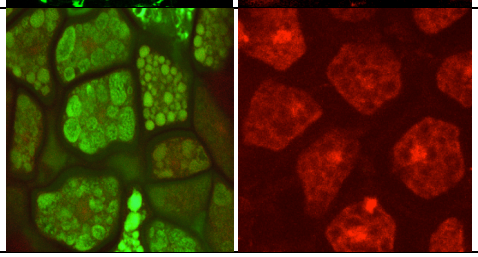
Aleurone programmed cell death

a) Cell biological events in senescing non-pollinated papilla cells

To study PCD in aleurone cells we initially had to establish the system allowing both the manipulation and the progression of cell death in a natural manner. I explored two major experimental setups: In *setup 1* I used aleurone layers isolated from shortly imbibed Arabidopsis seeds with subsequent incubation on solid agar or in $CaCl_2$ - containing liquid medium. In *setup 2* entire Arabidopsis seeds were germinated on solid agar or $CaCl_2$ - containing liquid medium and PCD was studied in AL upon seed germination. The programmed cell death progression was evaluated based on propidium iodide/FDA staining upon the observation of the samples with confocal laser scanning microscope (CLSM). The cell death was found to be happening more uniformly in aleurone in liquid medium compared to agar. We observed that aleurone cells from entire seed were dying progressively via vacuolation while cell death of isolated aleurone was not coupled with vacuolation process, in fact the vacuolation of dying aleurone cells was arrested (Fig. 1). Since a formation of one single vacuole (vacuolation) in aleurone cells was reported to be the hallmark of PCD in aleurone (Bethke el al, 1999), our observations suggest that the entire seed germinated in liquid medium should be used for further study of PCD in aleurone tissue.

Using CLSM we observed that Arabidopsis aleurone cell death includes several distinctive steps and starts with transformation of protein storage vacuoles (PSV) into lytic vacuoles (LV) and culminates in a loss of plasma membrane integrity and shrinkage of the cell corpse. The more detailed description of PCD progression in aleurone layer of *Arabidopsis* is performed in Table 1.

Table 1. Programmed cell death in *Arabidopsis* aleurone cells

	Features	Detection methods	CLSM image
Stage 1 (24h)	• 20 or more PSV • nucleus in the center • cytoplasm distributed uniformly • round active mitochondrias distributed uniformly in cytoplasm	FDA, Mitotracker Red stains	
Stage 2 (48h)	• 5 -2 large PSV • nucleus in the center • cytoplasm volume decreased • active mitochondrias accumulated in cytoplasm and around the nucleus	FDA, Mitotracker Red stains	
Stage 3 (72-96h)	• large vacuole • nucleus at the cell wall • thin cytoplasm layer between PM and tonoplast • active mitochindrias • secondary lytic vacuoles in cytoplasm	FDA, Mitotracker Red, BCECF	
Stage 4 (120-144h)	• tonoplast permealization and rupture • PM still intact	FDA background uniformly distributed in the cell	
Stage 5 (terminal)	• clearance of cell protoplast • PM integrity lost • turgor lost • nucleus condensation • mitochondrion disappereance	PI and Cytox penetrates cells and staines nucleus FDA staining disappers No staining with Mitotracker Red	
Necrosis (1mM H_2O_2, isolated aleurone)	• PM integrity lost • protoplast shrinkage • unprocessed cell corpse remnants	FDA/PI	

Our observations suggest that programmed cell death of Arabidopsis aleurone could be classified as a 'vacuolar' cell death (van Doorn, 2011; Hara-Nishimura and Hatsugai, 2011). Notably, the cell death after application of H_2O_2 in concentrations 1mM and higher and in isolated aleurones was accompanied by early plasma membrane rupture, protoplast shrinkage

and unprocessed cell corpse remained. This type of cell death differs from developmentally programmed cell death of *Arabidopsis* aleurone and rather could be defined as a necrosis. H_2O_2 in concentration of 3.25 mM and 325 mM were previously used to trigger programmed cell death in aleurone cells of barley (Bethke and Jones, 2001), however the way aleurone cells die was not described. More detailed analysis of cell ultrastructure of *Arabidopsis* necrotic cells with TEM showed that an entire volume of dead cell was packed with unprocessed lipid bodies.

Plant hormones and ROS control PCD in aleurone

In the cereal aleurone, PCD follows the vacuolation process and is as well tightly regulated by GA and ABA. While GA induces vacuolation and onset of aleurone PCD, ABA delays vacuolation and cell death execution (Kuo et al. 1996; Wang et al. 1996; Bethke et al. 1999; Bethke et al. 2007). We found that in *Arabidopsis* aleurone cells complete vacuolation (PSV fused into one single vacuole) happens at 72 hours after seed imbibitions, while the cell execution occurs much later at 7-8 days after imbibition. Treatment of aleurone layers with GA (25uM) caused earlier vacuolation completed at 48 hours after imbibitions but did not affect the time of cell execution. Fusion of protein storage vacuoles was not observed in isolated aleurone layers treated with ABA (25uM), unlike to aleurone of the entire seed, where fusion took place but at much slower rate compared to non-treated control. In ABA-contained medium isolated aleurone layers remained non-vacuolated and alive for 2 months. Therefore in *Arabidopsis* aleurone ABA arrests vacuolation and inhibits cell death, while GA facilitates vacuolation but does not affect the visual rate of cell death.

ROS play an important role in the PCD regulation of aleurone cells. ROS can act in two ways: on the one hand, high levels of ROS directly damage proteins, nucleic acids and membrane systems. On the other hand, ROS (especially the long-lived H_2O_2) are known to act as signaling molecules, causing the expression of genes involved in PCD. We studied the effect of two ROS inducers, hydrogen peroxide and methyl viologen (paraquat) on PCD in Arabidopsis aleurone. Upon the application of 1mM H_2O_2 aleurone cells showed the hallmarks of degeneration after 96h after treatment. Mainly the plasma membrane integrity of aleurone cells was affected which was indicated by leakage of FDA from the cells and staining of the nuclei with plasma membrane impermeable dyes (Sytox Green, PI). After 120h of H_2O_2 treatment all aleurone cells were dead, nuclei looked fragmented and stained with PI. Treatment of aleurone layers with paraquat (1uM) caused uniform death of all aleurone cells on 5 day after application. Compared to H_2O_2 treatment the nuclei fragmentation was not observed and PI stained condensed chromatin of the nuclei of dead cells. Therefore, H_2O_2 and paraquat treatments induce PCD in aleurone cells. The triggering of PCD with H_2O_2/paraquat will be later used in order to uncover specific effectors or executioners of cell death in Arabidopsis.

Programmed cell death in unpollinated stigmatic papillae cells of Arabidopsis

Second system I use to study PCD in reproductive organs of *Arabidopsis* is unpollinated stigmatic papillae cells. To study the progression of PCD in stigmatic papillae cells of unpollinated *Arabidopsis* stigmas we used male sterile mutants (*ms 1-2*) or emasculated plants of Col-0. A novel, completely male sterile ms1 null mutant allele has been complemented with a proMS1::MS1:GR fusion construct. Upon treatment with dexamethasone, the MS1:GR fusion

protein is translocated to the nucleus and rescues the ms1 mutant phenotype, restoring pollen development and male fertility. In this way, homozygous ms1 mutant seeds can be generated that do not require additional selection after sowing. This male sterile mutant is a convenient tool to study non-pollinated pistil senescence and PCD, as all stages of senescence are present on a single stem, and no manual removal of anthers to prevent pollination is required. The obtained results provide an experimental framework of senescence and PCD progression in stigmatic papilla cells: After full elongation, papilla cells remain receptive and alive for about 2 days. First signs of PCD include a tonoplast rupture, a hallmark of „vacuolar PCD" in plants (Hara-Nishimura and Hatsugai 2011; van Doorn 2011). Shortly afterwards, nuclear degradation occurs, closely followed by plasma-membrane disruption, turgor loss and collapse of the papilla cell. The entire sequence from first signs of PCD to the collapse of the papilla cells takes about 12 hours. This sequence has been divided in 5 distinct stages that correspond to particular stages of non-pollinated flower development (Fig 1)

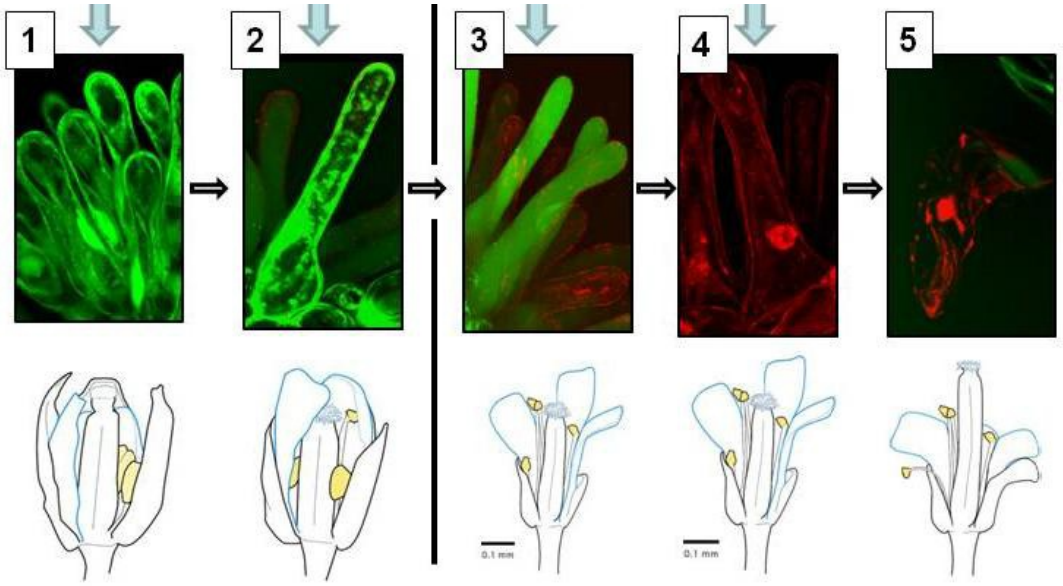


Figure 1. Five stages of papilla cell senescence with drawings of the associated flower development of the ms1 mutant. 1. Sepals level with style, the longest stamens are shorter than sepals; PC are not fully elongated. 2. Petals longer than sepals, anthers level with stigma, anthers in close proximity with stigma; PC are fully extended and rigid, nucleus pushed against a cell membrane (parietal nucleus), thin cytoplasm layer are between PM and tonoplast. 3. Lond anthers are situated above stigma; PC are fully extended but less rigid, tonoplast ruptures but PM are still intact, secondary lytic vacuoles appear in cytoplasm. 4. Lond anthers are below stigma, petals senescing; PC are bending, clearance of cell protoplast, PM integrity lost nucleus round positioned in the center. 5. Stigma above anthers, petals senescing or fallen; PC are crumbled deformed, turgor lost, nuclei is condensed.

a) Cell biological events in senescing non-pollinated papilla cells

We used confocal (CLSM) and transmission electron microscopy (TEM) to reveal the ultrastructural features of senescence and cell death, which are very valuable to understand the mechanisms of PCD (Hara-Nishimura and Hatsugai 2011; van Doorn et al. 2011). Our confocal and

TEM observation allowed us to track a consistent sequence of events in dyeing papilla cells and describe the changes on the level of a single organelle.

Tonoplast

The start of PC senescence programmed could be announced from the moment when nuclei is displaced and pushed against the PM (Fig 2 b). This was followed by tonoplast rupture and vacuole collapse (Fig 2 c) with cell protoplast clearance in the end (Fig 2 d).

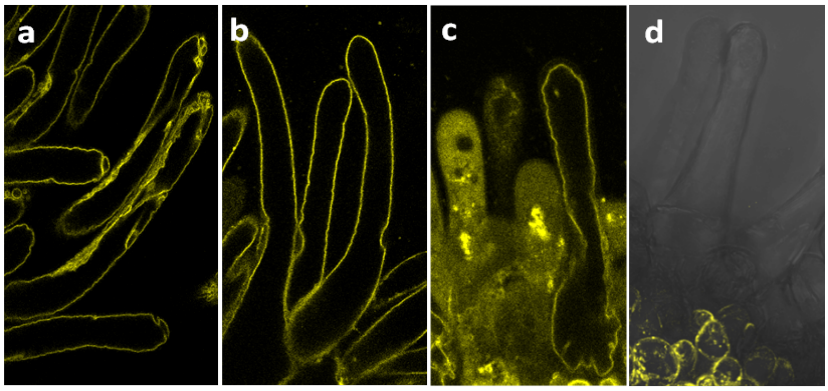


Figure 2. Tonoplast faith during PCD of PC with SYP22-YFP used as tonoplast marker.

Nucleus

Nucleus degradation starts in fully elongated cells and includes lobbing of parietal nuclei and subsequent formation of a big vesicle (vacuole) containing chromatin (Fig 3 upper B C). At the terminal stage of PCD lobbed nuclei disappears (Fig 3 upper D). On TEM images we did not observe any additional membrane surrounding the degrading nucleus which could confirm out confocal data. Nevertheless, the presence of membrane-enclosed DNA material leave a place for possibility of macroautophagy-mediated degradation of whole nuclei which was ones reported in Aspergillus oryzae. TEM showed that disintegration of nuclear membrane happens on certain stage of PC PCD (Fig 3 lower A-D)

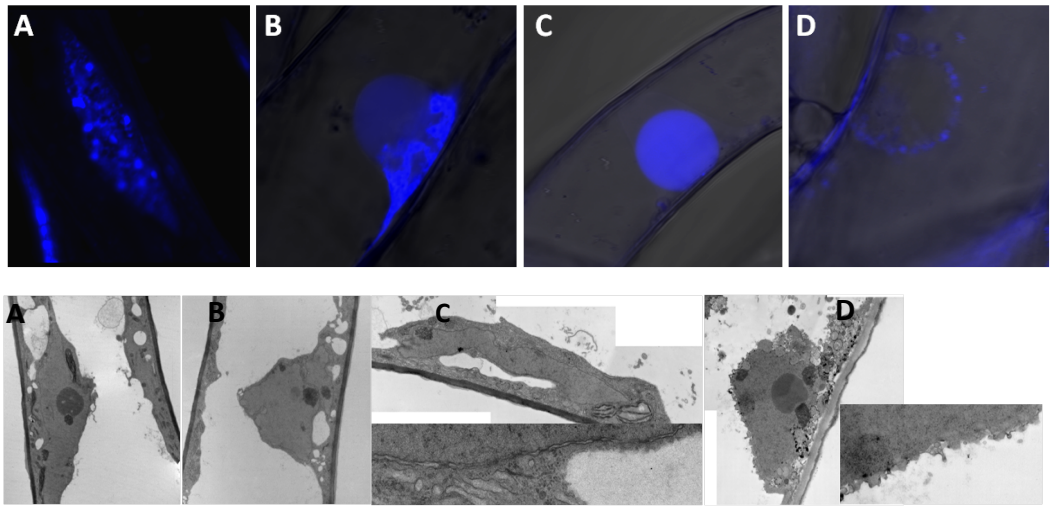


Figure 3. Nuclei faith during PCD of PC. DAPI stained (upper row), TEM (lower row).

Mitochondria

The changes in mitochondrial morphology were closely linked to the progression of PCD in PC. The mitochondria in not-fully expanded and expanded turgescent PC appeared to have intact membranes and well organized cristae apparatus (Fig 4 a b). On the later stages (Fig 4 c d) mitochondria appeared to be misshapen (rounded) and swollen and displayed degraded cristae and electron dense outer membrane. Similar changes in mitochondria morphology together with changes in mitochondrial permeability have been demonstrated to be involved very early in the series of events leading to death resulting from the execution of various types of PCD in both animals and in plants (Logan, 2008).

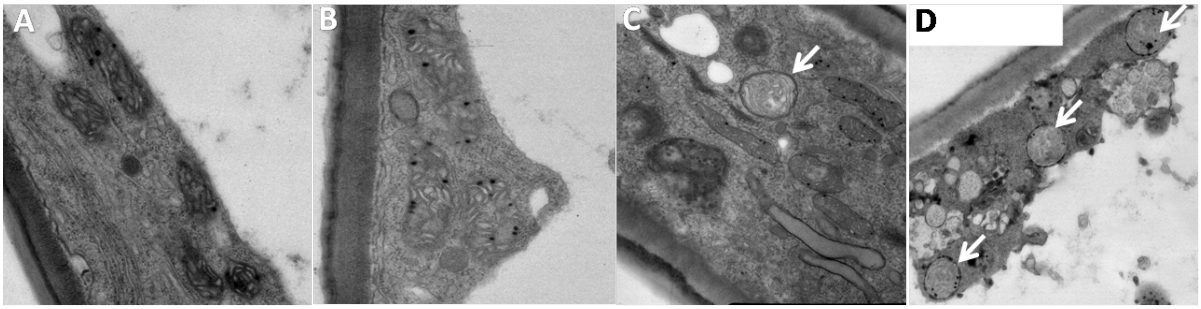


Figure 4. TEM- captured mitochondrial morphology dynamics in senescing PC.

Chloroplast

Apart from being essential to survival, chloroplasts and other plastids have also been implicated in ageing and death (van Doorn and Yoshimoto, 2010). During developmental cell death of plant organs the chloroplasts undergo gradual degradation which starts with chlorophyll loss and transformation into so-called gerontoplasts (van Doorn, 2006), indicating that chloroplast at least passively are involved in senescence and cell death. Moreover, it has been shown that chloroplast could actively promote the symptoms of cell death by altering levels of ROS (Zapata et al, 2005). The finding of acid phosphatase activity in plastids of some species gave rise to a hypothesis that plastids in plants can function in a way similar to both autophagosomes and autolysosomes in animal cells (van Doorn, et al 2011). In our study using confocal imaging we established sequential events of chloroplast transformation and degradation along the course of PCD in papilla cells. During PCD of PC we noted the disappearance of chlorophyll pigment significant reduction in both chloroplast size and number. At early PCD stage in papillae cell chlorophyll positive (autofluorescent) chloroplasts were accumulating around the nucleus (Fig 5 upper A) in a ring-like structure. Later on it was commonly to see misshapen chloroplasts aggregated together (Fig 5 upper B). Upon cell death progression chloroplasts were losing chlorophyll and were enclosed in MDC positive vesicle, suggesting the place for chlorophagy (macro-autophagy of plastids) in PCD of stigmatic papilla cells. TEM images of papilla cells at different stages of PCD indicated the accumulation of plastoglobuli in stroma of senescing chloroplasts (Fig 5 lower B, C, D arrows). We observed entire chloroplasts in the vacuolar space at the terminal stage of papilla PCD meaning that chloroplast degradation happens after tonoplast rupture. Therefore, it is unclear if chloroplasts are indeed transported to the vacuole for degradation via macro-autophagy or they are digested as a cytoplasmic entity by vacuolar hydrolases after tonoplast rupture. We do not exclude that both steps take place in PCD of papilla cells.

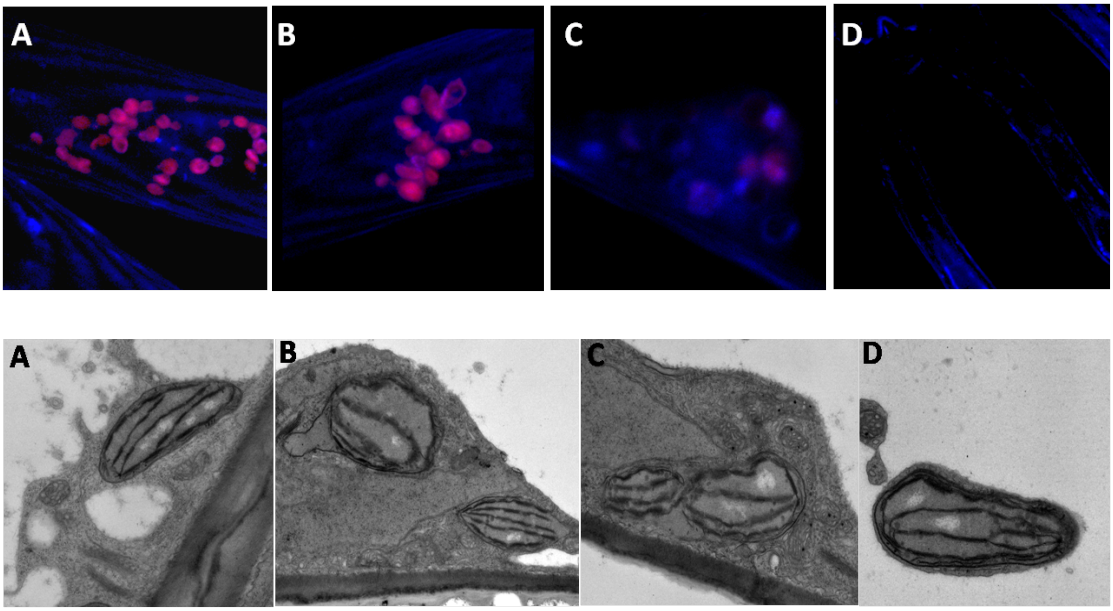


Figure 5. Plastid morphology changes in senescing PC. Confocal (upper row) and TEM (lower row) observations.

Lytic vacuoles/autophagasomes

The cases of the formation of secondary vacuoles with proteolytic activity coinciding with senescence of plant tissue were reported by Schmid et al (1999; 2001), Swanson et al (1998), Otegui et al (2005), Hayashi et al (2001), Matsushima et al (2003). It was suggested that highly acidic compartments with protease activity are involved in degradation of proteins during senescence.

Autophagy is also a part of degradation machinery in plants and often is involved in cell execution during PCD. Autophagy (meaning „self-eating“) is a highly conserved cellular process amongst eukaryotes, and highly homologous genes can be found in fungi, animals, and plants. Autophagy generally refers to macro-autophagy, a process during which bulky cellular components are recycled by trapping and transporting them to lytic compartments (lysosomes or lytic vacuoles) for breakdown (Thompson and Vierstra 2005). Autophagy has important functions for turnover and recycling of larger cellular structures that are either damaged or not needed any more; hence blocking autophagy renders cells more sensitive to stress and starvation. Plants that are compromised in autophagy will show respective symptoms of N- or C-starvation sensitivity and early onset of leaf senescence (Bassham 2007). On the other hand, during some forms of PCD strong upregulation of autophagy appears to be used to degrade cellular compartments before – and maybe leading to – cell death. In animals, the category of „autophagic cell death“ has been coined, and also during plant PCD a strong induction of autophagy has been reported (Bassham et al. 2006).

In our study the appearance of acidic vesicles stained with Lysotracker Red in the vacuolar space was observed starting from stage 2 (Fig 6 A). Moreover, at this stage we observed the occurrence of vesicle-like structures stained with monodansylcadaverin (Fig 6 B), a stain frequently used to label autophagosomes (Biederbick et al., 1995). Analysis of ATG8-YFP autophagy reporter revealed accumulation of autophagosomes in later stages of papilla cell death (Fig 6 C).

Furthermore, we observed accumulation of autophagic bodies within the central vacuole of dying papilla cells. Therefore, in our experiments, we observed clear indications that autophagy is induced strongly during papilla senescence and PCD.

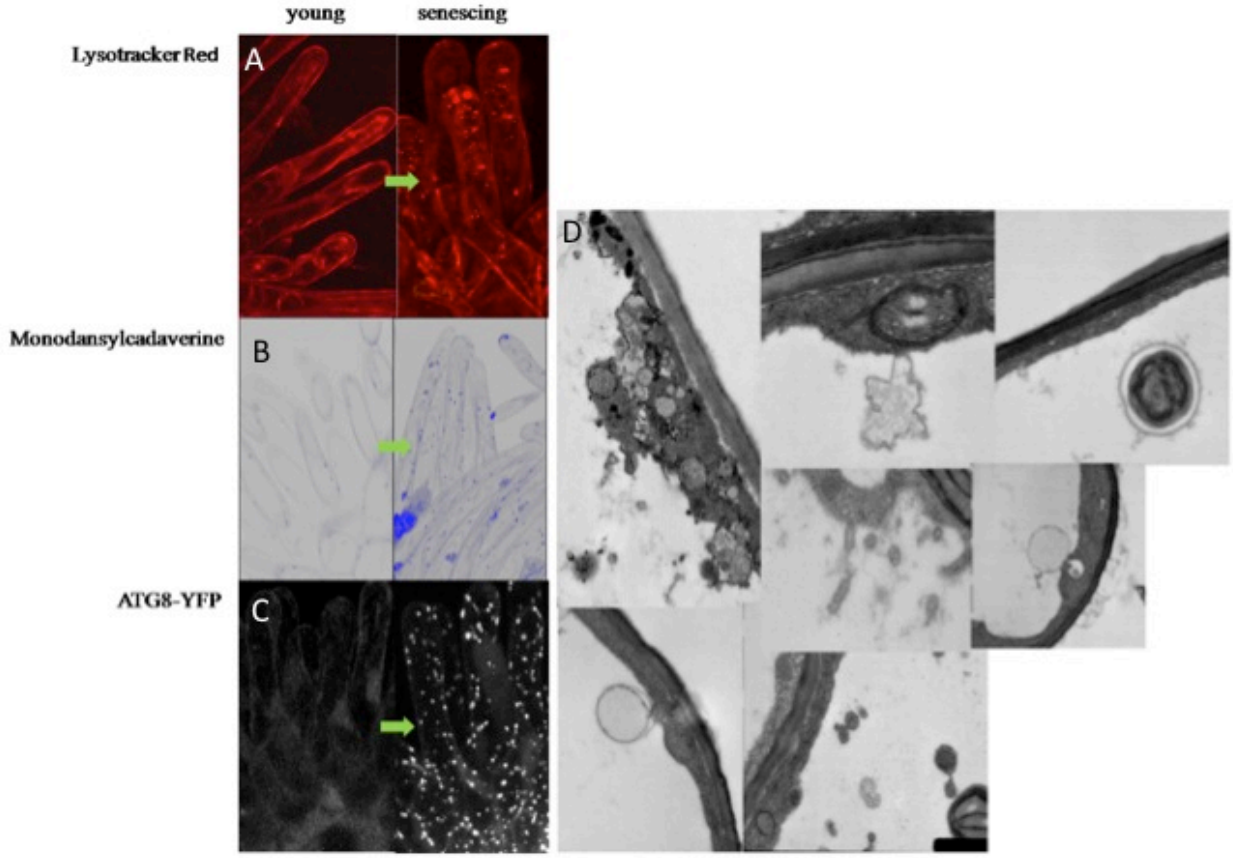


Figure 6. Vacuolar activity during senescence of PC.

TEM allowed us more close examination of the vesicular activity in PC during PCD. We often observed vesiculation at PM, formation of membrane-surrounded bodies in the cytoplasm, membrane-enclosed organelles in vacuolar space and budding of cytoplasmic moiety, double-membraned bodies, sometimes containing organelle material (Figure 6 D). At the terminal stage of PC PCD most of the protoplast that survived vacuole rupture was observed to be enclosed in vesicles.

This study reports a consistent order of events that occur in lace plant cells undergoing PCD, in addition to providing visual evidence of autophagy.

To further test the role of autophagy during non-pollinated papilla senescence, we will phenotype the autophagy-impaired mutant plant lines atg5 and atg7. Both show the typical early senescing leaf phenotype (Doelling et al. 2002; Thompson et al. 2005), but effects on non-pollinated papilla cell development have not been reported yet. If papilla cell senescence is regulated in the same way as leaf senescence, we could expect early senescence of papilla cells in autophagy mutants. If, however, autophagy is instrumental for papilla PCD, we might observe a delayed execution of cell death in these mutants

Analysis of pBFN1and pCEP1 in papillae cells of unpollinated stigmas

The induction of plant nucleases which are likely to be involved in DNA or/and RNA degradation was suggested to be associated with different PCD processes such as hypersensitive response, aleurone cell death, tracheary element formation (Sugiyama et al, 2000; Mittler and Lam, 1997; Fath et al; Aoyagi et al, 1998). In Arabidopsis endonuclease BFN1 has been identified as a senescence- and PCD-associated nuclease (Farage-Barhom et al, 2008). The senescence-activated promoter of the BIFUNCTIONAL NUCEASE 1 gene (*pBFN1*) was previously found to be activated in unfertilized senescing pistils and in the septum and stigma of developing fruits after fertilization (Carbonell-Bejerano et al, 2010). In papillae cells the expression of *pBFN1* was found to be started at stage of fully elongated cells with parietal nuclei before onset of tonoplast rupture (Figure 7 b).

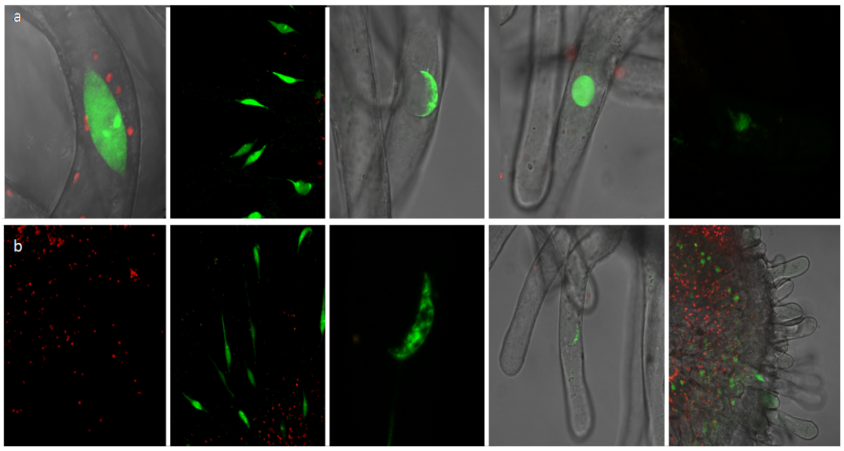


Figure 7. Expression pattern of the PCD markers *pBFN1* (b) and *pCEP1* (a) during papilla PCD

At later stages pBFN1-GFP was not observed in papilla cells which could be associated with acidification of the cell lumen due to tonoplast rupture and subsequent quenching of GFP fluorescence. Alternatively DNA degradation could cause absence of GFP signal. Plant PCD was shown to be effected by a group of papain-like cystein endopeptidases (CysEP) with a C-terminal KDEL endoplasmic reticulum (ER) retention signal (Gietl et al., 2000). CysEP is synthesized as a prepro-enzyme and transported from the ER to the cytosol of the senescing cells in the form of vesicles. The CysEP-containing bodies occur at final stage of PCD coincide with nucleus degradation, vacuole rupture, cytoplasm acidification. Rupture of CysEP-containing vesicles, subsequent CysEP release and its activation upon N-terminal propeptide and the C-terminal KDEL cleavage culminates in degradation of cytosolic remnants by CysEP. In Arabidopsis three KDEL-tailed cysteine endopeptidases were identified (Helm *et al*, 2008).). The expression of the KDEL-tailed cysteine endopeptidase CEP1 is tightly associated with developmentally regulated PCD of generative tissues and with distinct stages of flower development. *CEP1* promoter activity was found in unpollinated, degrading ovules (Helm *et al*, 2008). The expression of *pCEP1* in stigmatic papilla cells starts at early stage of not fully elongated papillae cells and disappears at later stages of papillae cell death progression (Fig 7 a). The differential expression of *pBFN1* and *pCEP1* could be explained by the function and activation of its gene product. As CEP1 is synthesized as a pre-enzyme, stored and transported in

specialized vesicles until the final stages of PCD when it cleans up the cell remnants it is relevant to expect the earlier activation of *pCEP1* compared to *pBFN1*.

Analysis of the bfn1-1 and cep1-1 mutants

The activation of *pBFN1* and *pCEP1* prior to execution of papillae cells suggests its putative function in PCD. We aimed to study BFN1 and CEP1 function in PCD by using its loss-of-function mutants which have not been described in literature so far. In the CLSM analysis the *bfn1-1 and cep1-1* mutant behaved similar to the wild type but in papillae cells of *cep1-1* we noticed the absence or less abundant amount of lytic vacuoles compared to the wild type. The absence of profound phenotype could be explained by genetic redundancy. The further experiments will be carried out to evaluate the involvement of BFN1 and CEP1 in stigmatic papilla cells PCD.

Creation of activator lines

For over-expression of gene of interest we clone the gene together with tissue specific promoter and then transform into the plant by using a GAL4VP16 transactivation system. In this system, the promoter driving the gene of interest is activated indirectly (*in trans*) by a transcriptional activator (GAL4VP16) that is produced only in specific tissues as it is under the control of a tissue specific promoter. For that we cross two different lines: an activator line which produces a transcriptional activator only in specific tissues and an effector line in which the gene of interest is cloned with a promoter which is activated by the transcriptional activator of first line (Fig 8).

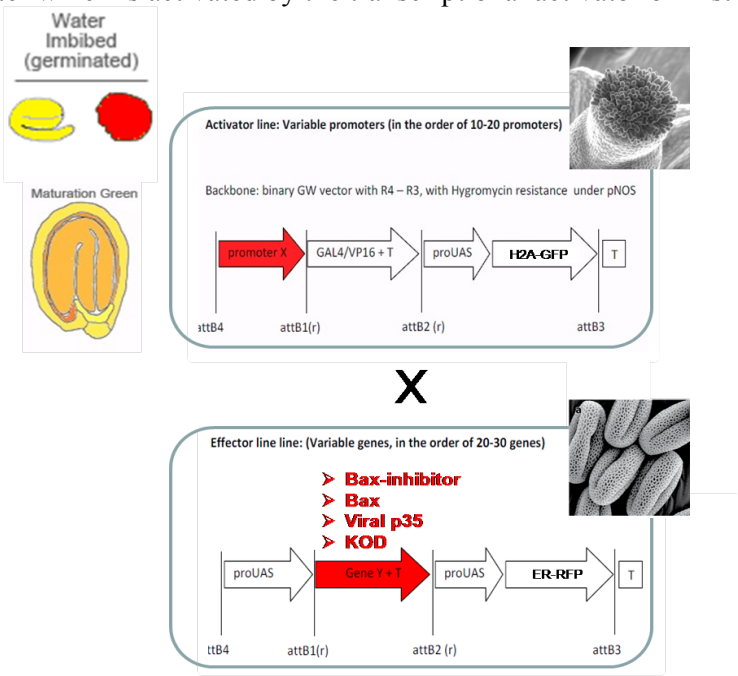


Figure 8. GAL4VP16 transactivation system used to over-express cell death effector genes under stigma-specific promoter.

For generation of activators lines several genes were chosen based on their specific expression or putative role in PCD in mature aleurone or stigmatic cells (Table 2). The promoters of

the following genes were cloned for the creation of mature aleuronic or stigma-specific activator lines.

Table 2. List of selected aleurone and stigmatic papillae-specific genes of *Arabidopsis* for the creation of activator lines

AGI code	Name	Expression	Function
Mature aleurone			
At5g05290	EXP2	Specifically expressed in aleurone layer of imbibed seeds	expansin, cell wall loosening, many diverse functions
At1g01380	ETC1	Specifically expressed in aleurone layer of imbibed seeds	MYB transcription factor - enhancer of TRY and CPC 1 (ETC1) - promoter is targeted by the TTG1 complex
At3g42850	ISA1	Specifically expressed in aleurone layer of imbibed seeds	arabinose/ galactose kinase, putative
At5g55930	OPT1	Expressed during leaf senescence stage in Arabidopsis; Specifically expressed in aleurone layer of imbibed seeds	oligopeptide transporter
At2g32460	MYB101	positive regulation of programmed cell death; Specifically expressed in aleurone layer of imbibed seeds	transcription factor, GA response
At4g30270	SEN4	Expressed during leaf senescence Expressed in aleurone layer of imbibed seeds	xyloglucan endo-1,4-beta-D-glucanase, senescence
At1g56010	NAC1	Expressed in aleurone layer of imbibed seeds	transcription factor involved in auxin mediated lateral root emergence, degraded by miR164
At1g17020	SRG1	Expressed during organ senescence Expressed in aleurone layer of imbibed seeds	Fe(II)/ascorbate oxidase gene family
At4g21980	ATG8A	Expressed during organ senescence Expressed in aleurone layer of imbibed seeds	autophagy related
Stigmatic papillae			
at1g65450		Specifically expressed in stigma	expressed protein, transferase activity, transferring acyl groups other than amino-acyl groups, transferase activity
at2g20340		Expressed during leaf senescence stage in Arabidopsis; Specifically expressed in stigma	putative tyrosine decarboxylase, response to wounding, cellular amino acid metabolic process
at2g17000		Specifically expressed in stigma	hypothetical protein, transmembrane transport
at5g19880		Specifically expressed in stigma and during oxidative stress	Peroxidase, response to ethylene stimulus, oxidation reduction, response to oxidative stress, response to virus, N-terminal protein myristoylation
at3g12000		Specifically expressed in stigma	S-locus related protein SLR1, putative (S1), recognition of pollen

At5g11400		Specifically expressed in stigma	protein kinase activity, kinase activity, ATP binding, protein amino acid phosphorylation
at1g05450		Specifically expressed in stigma	lipid-transfer protein, Encodes a Protease inhibitor/seed storage/LTP family protein
at3g03670		Specifically expressed in stigma	peroxidase, oxidation reduction, response to oxidative stress
at2g33850		Specifically expressed in stigma Expressed during leaf senescence stage in Arabidopsis	unknown protein
at3g01530	MYB57	Specifically expressed in stigma	myb trans factor, DNA binding, DNA-dependent, gibberellic acid mediated signaling pathway

Based on promoter expression profile we chose 2 candidates to be used for transactivation system generation for stigmatic papilla: pSLR1 (at3g12000) described as stigma-specific promoter and pAt2g33850 (unknown protein) specifically expressed in stigma and trichomes.

The effector lines were generated based on putative cell death- related genes: BAX and BAX-inhibitor. BAX is an animal pro-apoptotic Bcl-2-family protein that resides in the cytosol and translocates to mitochondria upon induction of apoptosis, and therefore involved in regulation of cell death in animal cells. It has been shown that Bax induces the release of cytochrome C in conjunction with apoptosis and may play a modulating role in blocking the mammalian cell death machinery by acting upstream of caspase function and upstream or at the mitochondrial level (Finucane et al, 1999). Although there was no BAX gene found in plant genome, an evidence for a function of BCL2-like proteins in plant PCD is accumulating. For example, a BCL2 homologue was detected in plant cells by immunoblotting as well as it was shown that animal BCL2-like proteins are able to modify cell death processes in plants (Dickman et al, 2001; Lam et al, 1999). Finally, homologues of human Bax inhibitor-1 were isolated from Arabidopsis thaliana and rice, cloned in yeast and showed suppression of Bax-induced cell death in yeast (Kawai, 1999; Sanchez). In addition, overexpression of AtBI1 attenuated cell death in plants expressing mammalian Bax (Kawai-Yamada, 2001).

In our study ER-localized mRFP signal in aleurone and papilla cells (Fig 9) confirmed the successful transactivation after crossing of activator and effector lines.

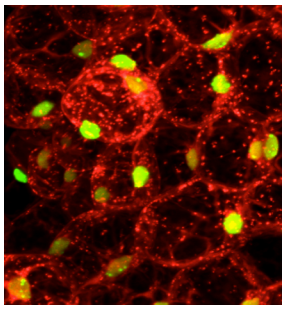


Figure 9. Transactivation of BAX and ATBI using GAL4VP16 transcriptional activator.

The analysis of F1 plants carrying over-expressed BAX and BAX-inhibitor genes under SLR1 (stigma-specific) and EXP2 (aleurone-specific) promoters did not reveal any significant changes in the induction or blockage of aleurone or papilla cell death. In parallel we directly over-expressed BAX and BAX –inhibitor under the same tissue-specific promoters. The phenotypical analysis is currently ongoing. Excitingly, we observed profound phenotype in F1 plants upon over-expression of BAX under pAt2g33850 (a promoter as we established earlier was active in both stigmatic papilla and leave trichomes of Arabidopsis). Strong signal of promoter-derived GFP as well as ER-mRFP was observed in the trichomes of F1 plants which confirm successful transactivation. The plants of this transactivation line had much smaller in size and significantly less amount of leave trichomes. The vast amount of trichomes have aberrant morphology indicating that

overexpession of BAX was able to impair a normal development of trichomes, possibly via triggering a cell death machinery similar to the one in animals. The study of the role of BAX and BAX-inhibitor in trichomes and papilla cells death will be continued in SEEDDEV lab in PSB.

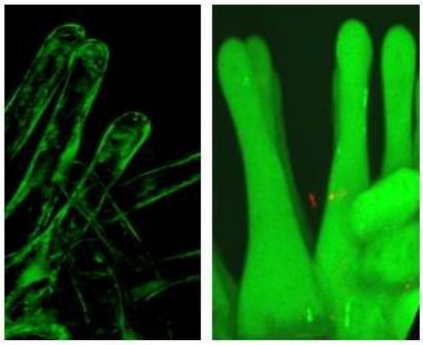
The roles of ethylene, ROS, NO in papilla senescence and PCD

In parallel with the transcriptome profiling and identification of novel candidate genes, we will specifically focus on three pathways that might be functionally involved in papilla senescence and PCD.

Ethylene The plant hormone ethylene has been implicated in many plant physiological and developmental responses, but has first been noted for its ability to induce leaf senescence (Lin et al. 2009). Recently, ethylene has also been associated with the senescence of unfertilized pistils (Carbonell-Bejerano et al. 2011).

To test the role of ethylene in the senescence and PCD of papilla cells we analyzed PCD progression in PC of T-DNA insertion lines carrying the mutations in ETR1, ETO2, ETO3, EIN3 (ethylene synthesis or signaling genes). We observed that unpollinated PC of ein3 line stayed alive for about 24h longer compare to wild type. Though there were no significant differences recorded in eto2, eto3, etr1 lines. In parallel, we used pharmacological approach based on drug application that effect ethylene synthesis or signaling. We applied ACC (a precursor that is rate-limiting to ethylene production), ethephon (a chemical producer of ethylene), aminoethoxyvinyl glycine (AVG) and STS (inhibitor of ethylene synthesis). These experiments showed that ethephon application tremendously promoted papilla cell senescence and PCD suggesting role of ethylene in the process.

As a follow up, we are expressing the mutant eto2, eto3, etr1, acs2 alleles specifically in papilla cells, using the above mentioned driver lines. For example, the eto2, eto3 allele carries a point mutation and overproduces ethylene, making it a convenient tool to cause local ethylene synthesis when expressed under a tissue-specific promoter. Moreover, we are expressing the mutant etr1 allele in papilla cells. The mutant etr1 protein dominantly blocks ethylene signaling, making cells expressing it insensitive to this phytohormone. Additionally, plants with ethylene overproducing or ethylene insensitive papilla cells can later be used as a tool to dissect downstream effects of ethylene signalling, without the pleiotropic effects caused by systemic ethylene mutants.



Reactive oxygen species (ROS) ROS have been identified as important signalling molecules during senescence and PCD in many eukaryotes. In plants, ROS signalling has been correlated to PCD during the hypersensitive response, and during developmental cell death. Using, H2DCFDA, we could observe clear accumulation of ROS during the time course of papilla cell senescence. (Fig 10). Now, we aim to verify these results using genetically encoded redox-sensitive (roGFP) and H2O2-sensing (HyPer) reporter proteins (Meyer et al. 2007; Costa et al. 2010).

Figure 10: Preliminary data indicating a role of ROS and autophagy during papilla senescence and PCD. A-B) Accumulation of ROS indicated by H2DCFDA staining in senescing papilla cells

Transcriptome analysis of stigma senescence and PCD time series

Genome-wide transcriptome profiling is a powerful tool to identify potential regulators of a given biological process. In the case of stigmatic papilla cell senescence and PCD, we extracted total RNA of stigmatic tissue during a time series experiment. Discrete stages were defined according to the progression of PCD defined above, and correlated to distinct floral stages (**Fig 11 left**). Preliminary experiments indicate that we need to harvest the stigmas of 400 flowers per time point and biological replica to extract sufficient total RNA for further processing during transcriptome profiling. To realize this number, we maked use of a conditional male sterile mutant that has been established in the Seed Development lab.

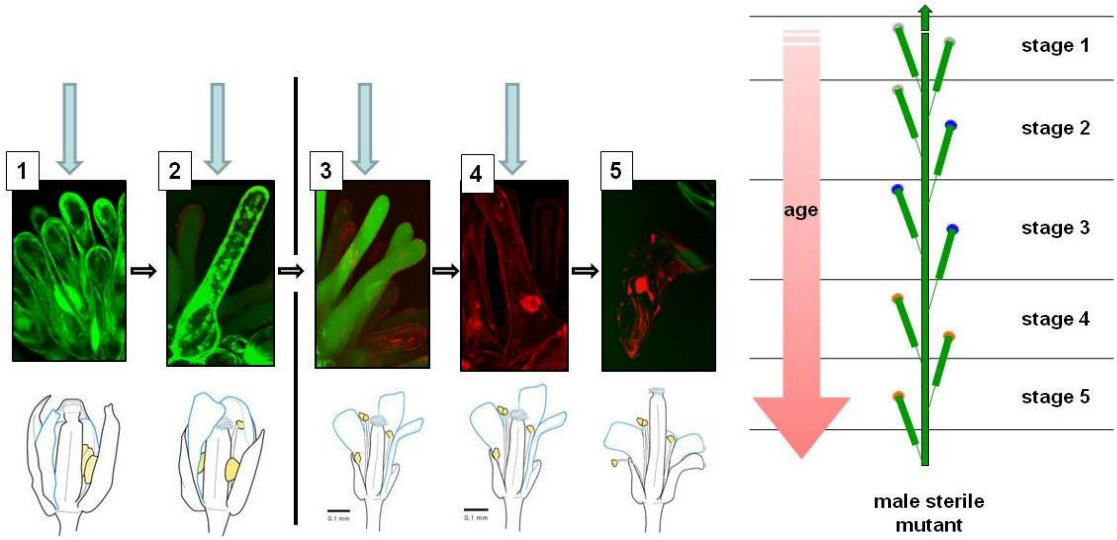


Figure 11: Design of the transcriptome profiling of non-pollinated papilla cell senescence and PCD. On the left: 5 stages of papilla cell senescence with drawings of the associated flower development of the ms1 mutant. Total RNA will be extracted from the stages indicated by arrows. **On the right**: Staging of senescing flowers on a ms1 stem. The younger flowers are closer to the floral meristem on the tip of the stem, and the position on the stem correlates with increasing age of the non-pollinated flowers.

This male sterile mutant is a convenient tool to study non-pollinated pistil senescence and PCD, as all stages of senescence are present on a single stem, and no manual removal of anthers to prevent pollination is required (**Fig 11 right**). As transcriptome analysis platform we will use next-generation RNA sequencing (RNA-seq). This approach will make the data valuable not only for the first analyses, but make them forward-compatible for future updates of genome annotations. We will employ Illumina HiSeq technology present at the Genomics Core at the UZ Leuven. 50-base-pair reads will be produced in a pair-end approach, with approximately 15,000,000 reads per sample. As our samples will need a linear amplification step prior to RNA-seq, RNA amplification and RNA-seq library preparation will be performed prior to RNA-seq by the Nucleomics VIB core facility in Leuven. Statistical analysis and identification of differentially regulated candidate genes will be performed together with experts of the Systems Biology of Yield lab at the PSB in Gent, who have set up an analysis pipeline based on the Galaxy platform. From the pool of differentially regulated genes, candidates will be selected for further functional analysis. Priority will be given to genes that are strongly regulated during the senescence process, especially transcription factors, proteases and protease inhibitors, and regulatory genes such as kinases or F-box proteins. Furthermore, comparison to other transcriptome experiments in the context of senescence and PCD will be used to reduce the candidate pool to a small number of highly promising candidate genes.

Conclusions:

The current work was set up to answer the question if aleurone layer of Arabidopsis seed and stigmatic papilla could be used as model systems for studying developmentally regulated PCD during plant reproductive development. The simple structure and accessibility of these tissues allowed easy manipulation, visualization of tissues during cell biological studies which allowed us to gain insight in the cell biological and some molecular mechanisms of PCD in these two model systems.

First, we established the cellular basis of senescence and PCD based on analysis of the subcellular and cell biological processes that accompany and steer this process. We described PCD processes in aleurone and stigmatic papilla cells as highly ordered events that are controlled by diverse subcellular compartment, e.g. the nucleus, plastids, mitochondria, vacuole and other compartments of the endomembrane system.

Second, we cloned several candidate genes in Arabidopsis and performed the functional analyze of these candidate genes which further will allow to discover the pathways and functional factors that can prolong the functional life span of stigma tissues in Arabidopsis.

Third, functional analysis of mutants together with cell biological studies allowed us to narrow down the pathways involved in PCD of aleurone and papilla cells. Therefore, in the future investigations the priory will be given to ethylene signaling, autophagy, and reactive oxygen species (ROS).

Forth, we developed the experimental platform for RNA-seq and a comparative transcriptome time series analysis of senescing stigma tissue in Arabidopsis is on run. This will help to to identify candidate genes as the first nodes of a genetic network that controls stigma viability and life span.

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