

Master projects in plant molecular biology

Faggruppe Genetikk og plantebiologi, IPM

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Foto: E. Fløystad

Selection and characterisation of abscission-related genes from poinsettia and their function during development of floral abscission

Abscission is the shedding of a plant organ which is no longer of use to the plant, a process that leads to considerable losses in horticulture and agriculture. The quality of flowers (in ornamentals) and yield of harvest (in fruit, berries or cereals/seeds) could be controlled with a better understanding of this important physiological process. During the NFR funded Strategic Programme “Fundamental Studies on Post harvest Physiology and Plant Health” (2002-2005) we have identified many interesting gene sequences involved in flower abscission in poinsettia. These proposed thesis will make use of the competence and resources gained in the above project and will also make use of the Functional Genomics (FUGE) platforms to achieve a broader knowledge of the complex abscission process. We will use the Norwegian Arabidopsis Research Centre (NARC) platform at UMB to study knock-down mutants (RNAi or T-DNA) and over-expression in *Arabidopsis*. This will complement the research we do in our own laboratory on characterising cloned genes from the newly performed Differential Display (DD). We will use RNA *in situ* hybridisation, immunolocalisation using monoclonal antibodies to define any alteration in cell wall polymers, and transformations in both poinsettia and *Arabidopsis* to study effect of promoters and genes. The University of Oslo (UiO) is connected to some of the projects through the NFR project “Elucidation of mechanisms of abscission using the model plant *Arabidopsis* and the ornamental plant poinsettia” (2004-2006).

Our research on flower abscission in poinsettia

Although it has been recognized that low light conditions might trigger cyathia abscission in poinsettia (Bailey and Miller, 1991; Moe et al., 1992), the environmental regulation as well as the biological background of their abscission has not been investigated. Generally abscission is heavily based on inter-organ signalling events, but it is not clear at present how these signals co-ordinate the events. Abscission takes place in the few cell layers “forming” the AZ. The breakdown of the cell wall is likely to be a complex process involving highly co-ordinated series of changes to cell wall structure, cell physiology and gene expression. We are in the process of elucidating these events in poinsettia by using physiological, cellular and molecular tools, as well as transgenic plants. We are beginning to better understand the processes involved, we hope to be able to improve the quality of poinsettia (and other plants) by engineering either the timing of abscission or the site where it occurs.

The abscission zone cells

The cyathia (flowers) abscise at the base of the pedicel, and the AZ is usually seen as a lighter, yellow-green ring around the stem prior to abscission (Figure 2, arrow). The AZ in poinsettia consists of smaller, densely cytoplasmic cells in a layer near the base of the pedicel and can be quite distinct from the surrounding cells (Lee et al., manuscript in preparation).

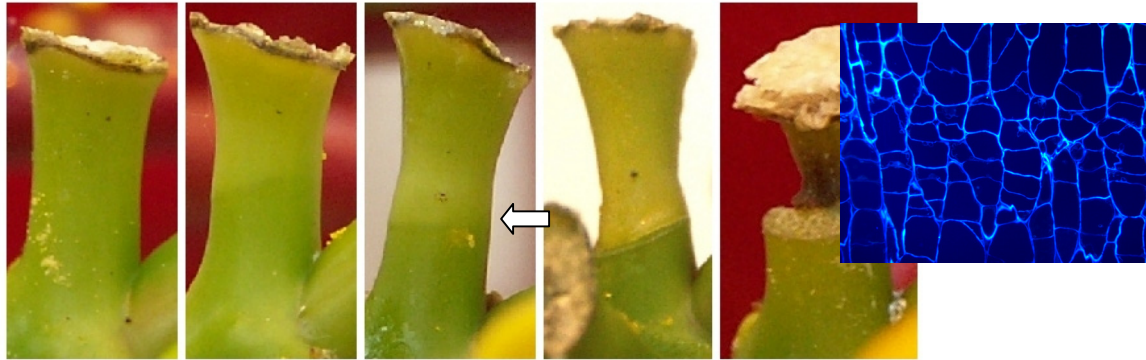


Figure 1. Induction of flower abscission in poinsettia, from left to right; 4, 5, 6, 7 and 8 days after decapitation. After approx 5 days an abscission zone is clearly visible near the bottom of the pedicel (arrow). To the far right is a microsection of an AZ at day 5 stained with calcofluor with a remarkable separation zone in the tissue and cell division occurring at the same time.

At present our hypothesis is that in poinsettia, the cells in the AZ are not predestined to form an abscission zone since we can shift AZ to a different place by ethylene exposure to decapitated buds (Munster et al., manuscript in preparation). We would also like to confirm our observations that the abscission process involves cell division before cell wall degradation (Fig. 1, far right).

The signalling hormones

We have developed a system to induce abscission in poinsettia; the flower bud is decapitated and left to abscise (Mathiesen et al., manuscript in preparation). Normally the AZ is visible after four to five days (Fig. 2, arrow) and the whole pedicel abscises after 7-10 days depending on time of year. The correct positioning of the cut is of vital importance for reliable induction. The fact that the cut position is essential for induction of abscission made us hypothesise that signals (hormones?) from the floral organ must be involved in a positive and/or negative regulation of the abscission process. We have done extensive studies on the effect of plant hormones on induced abscission in poinsettia (Mathiesen et al., manuscript in preparation; Munster et al., manuscript in preparation).

Our results suggest that floral organ abscission in poinsettia is regulated in a similar manner to the abscission processes described for other plants (Addicott, 1982), and thus is a good model for studying abscission. Since we get exciting varying results depending on position of the cut, we will continue the hormone studies on inducible abscission system in poinsettia using a variety of tools; monoclonal antibodies against hormones to see hormone distribution in the bud and RNA *in situ* hybridisation with e.g. probes for known ethylene and GA receptors to localize these in the flower bud.

The genes involved

As mentioned, few details are known about the abscission process, but cell wall degrading enzymes like glucanase and polygalacturonase are often involved (Roberts et al., 2002). This seems to be the case also in poinsettia (Hvoslef-Eide et al., 2003). DD is a powerful tool to show variations in gene expression at the mRNA level. We have used random primers to identify novel genes not known to be involved in the abscission process. In these studies abscission is induced as described above, and mRNA is prepared from tissue containing the

AZ. Then the mRNA is transcribed to cDNA. The DDs are run with cDNA from non-induced pedicels as control and compared with cDNA from abscission zones with 24 h intervals until abscission on day seven. Real-time reverse transcriptase (RT)-PCR and mRNA *in situ* hybridisation are methods used to verify the genes picked up as differentially expressed. We have at present obtained 30 interesting clones containing gene sequences related to flower abscission in poinsettia. Eleven of these are homologous to known genes. One example is immunophilin, thought to be involved in endoreduplication, a process where the cell replicates chromosomal DNA without intervening mitoses (Sugimoto-Shirasu and Roberts, 2003). We also have additional clones from the DD that need further analyses to verify link to the abscission process. Some of these clones were too short for the initial screen, but are expressed in such an interesting manner (e.g. only in day 1, 24 h after decapitation) that we intend to do 5'RACE to enable another database search to find more candidates.

Cell wall polymers and enzymes involved in abscission

The breakdown of the middle lamella between cells in the abscission zone is thought to be the crucial step in abscission. Pectinases, cellulases, chitinases and β -1,3-glucanases have been shown to be involved in the abscission process. Using a time series of the induced abscission process and immunocytochemistry with monoclonal antibodies to a series of pectic epitopes (from Paul Knox, University of Leeds, UK), we have generated data suggesting that rhamnogalacturonan-I and homogalacturonan polymers are modified during the abscission process in poinsettia (Lee et al., manuscript in preparation). This will direct research towards specific enzyme activities. The role of the different enzymes in the abscission process has been difficult to ascertain. The separation zone is only a few cell layers thick, making biochemical analysis difficult (Jinn et al., 2000). Studies using Fourier Transform Infrared Micro spectroscopy (Sene et al., 1994; Chen et al., 1998) to biochemically characterise the AZ and the adjacent cells, have revealed changes in pectin degradation of and the formation of lignins in the AZ during early stages of the abscission process in poinsettia.

References

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The candidates:

The successful candidates should have interest and background in plant biology and have taken courses using molecular biology techniques.

By working in one of these projects, the candidate will achieve good skills working with DNA and RNA and will have improved knowledge of modern (plant) molecular biology. He or she will also be well introduced to the field of bioinformatics.

Supervisor: Ass. Prof. Trine Hvoslef-Eide
Co supervisor: Dr. Cecilie Mathiesen or
Dr. YeonKyeong Lee

These projects can be both 60 or 30 points for Master in Plant Sciences or 60 points for Master in Biotechnology.



Poinsettia on a plate published in Curtis' Botanical Magazine in 1836.

A. Screening, cloning and characterisation of genes important in shedding of floral organs (60 points)

This master project will start with RNA *in situ* hybridisation screening of genes that has been identified as differentially expressed during the process of shedding floral organs (abscission). The model plant is Poinsettia, or Christmas star, an economically important pot plant that has flower abscission as a quality problem. Then one or two of the positive sequences/genes will be picked for further characterisation via whole gene sequencing (RACE), homology analyses (bioinformatics), over expression and/or knock-down (RNAi) studies in *Arabidopsis thaliana*. In more detail the work will have the following steps:

1. Sub cloning of cloned sequences to a vector suitable for RNA *in situ* hybridisation.
A very good introduction to traditional molecular biology!
2. RNA *in situ* hybridisation
In situ hybridisation is a technique that allows specific nucleic acid sequences to be detected in morphologically preserved tissue sections, cells or chromosomes. Combined with immunocytochemistry, *in situ* hybridisation can relate gene activity at the DNA, mRNA or protein level to microscopic topological information. Positive hybridisation with an anti-sense mRNA probe in the abscission zone of poinsettia is the ultimate proof of a gene specifically active in this region. We use digoxigenin (DIG) labelling of probes generated with specific primers from cDNA template and use indirect detection by anti-DIG alkaline phosphatase conjugate.
3. One or two genes with a positive *in situ* result will be fully cloned using 5'RACE-technology and further characterized via
 - i) Bioinformatical analyses (PSI-BLAST homology, 2D/3D topology, signal sequences, domains,.....).
 - ii) Making of constructs for over expression and transformation to *A. thaliana*. Phenotype characterisation.
 - iii) Knock-down (RNAi) analysis in *A. thaliana*.
ii) and iii) will be done in collaboration with the Norwegian Arabidopsis Research Centre (NARC) at UMB.

B. Sequence and real-time RT-PCR analysis and RNA *in situ* hybridisation screening of differentially expressed genes during shedding of floral organs (60 points)

This master project will start with 5'RACE analysis of genes that has been identified as differentially expressed during the process of shedding floral organs (abscission). The model plant is Poinsettia, or Christmas star, an economically important pot plant that has flower abscission as a quality problem. By RACE more of the gene sequence is achieved and it will be cloned and used for real-time RT-PCR analysis. The differential expression will be confirmed by RNA *in situ* hybridisation screening. The cloned sequences will also be analysed for homology (bioinformatics).

1. 5'RACE analysis on short sequences to achieve longer sequences to clone into a vector suitable for RNA *in situ* hybridisation.
A very good introduction to traditional molecular biology!
2. Real-time RT-PCR analysis for a first confirmation of the differential expression during abscission. Involves total RNA preparations from poinsettia abscission zones, cDNA production and real-time PCR technology. The latter analysis will be done at Bioforsk.
3. RNA *in situ* hybridisation
In situ hybridisation is a technique that allows specific nucleic acid sequences to be detected in morphologically preserved tissue sections, cells or chromosomes. Combined with immunocytochemistry, *in situ* hybridisation can relate gene activity at the DNA, mRNA or protein level to microscopic topological information. Positive hybridisation with an anti-sense mRNA probe in the abscission zone of poinsettia is the ultimate proof of a gene specifically active in this region. We use digoxigenin (DIG) labelling of probes generated with specific primers from cDNA template and use indirect detection by anti-DIG alkaline phosphatase conjugate.
4. One or two genes with a positive *in situ* result will be fully cloned using 5'RACE-technology (if not achieved at point 1.) and further characterized via bioinformatical analyses (PSI-BLAST homology, 2D/3D topology, signal sequences, domains,.....).

C. Transformation of poinsettia with *IDA* gene using RNAi techniques

This project is connected to some of the projects through the NFR project “Elucidation of mechanisms of abscission using the model plant *Arabidopsis* and the ornamental plant poinsettia” (2004-2006). Floral abscission mutants of *Arabidopsis*, and the affected genes, as well as gene constructs designed to investigate and manipulate abscission in poinsettia will be employed. The project is a collaboration between the University of Oslo (UiO), Norwegian University of Life Sciences (UMB), John Innes Centre (JIC), Norwich, UK and the University of Madison (UoM), Wisconsin, USA.

This master project will start with RACE analysis with *IDA* gene primer that has been identified as abscission involved gene in *Arabidopsis*. Transformation of poinsettia will be done with RNAi technology. Characterization of abscission in *ida* mutant in poinsettia will be searched.

1. RNA isolation in abscission and RACE analysis using *IDA* gene primer to identify homology in poinsettia.
A basic introduction to traditional molecular biology.
2. RNAi construction: RNAi allows the quick and easy reduction in the expression of a particular gene in living cells, often by 90% or greater in mammalian system, allowing the analysis of the effect that gene has on cellular function. RNAi (RNA interference) refers to the introduction of homologous double stranded RNA (dsRNA) to specifically target a gene's product, resulting in null or hypomorphic phenotypes. The most interesting aspects of RNAi are the following:
 - dsRNA, rather than single-stranded antisense RNA, is the interfering agent
 - it is highly specific
 - it is remarkably potent (only a few dsRNA molecules per cell are required for effective interference)
 - the interfering activity (and presumably the dsRNA) can cause interference in cells and tissues far removed from the site of introduction

Such a new technique to study gene function in plant system and the candidate could obtain advanced technique in molecular biology.

3. Transformation of abscission involved gene in poinsettia.
In our group, we have nicely invented method to make transformant in poinsettia and using this method, produce successful mutant.
4. Characterization of *ida* mutant in poinsettia: investigate whether the *IDA* gene can be used to modify the floral abscission process in poinsettia, a commercially important plant species.

D. Cell wall alteration during abscission of ida mutant in poinsettia using FT-IR and FT-Raman analysis

Abscission is accompanied with cell wall alteration such as wall degradation by several enzymes and accumulation of additional cell wall components. However, the separation zone is very limited area which has only a few cell layers thick and it makes biochemical analysis difficult. Using Fourier Transform Infrared Micro spectroscopy (Sene et al., 1994; Chen et al., 1998) FT-IR) and Fourier Transform Raman spectroscopy enable us to probe directly the structure of the plant cell wall.

FT-IR and FT-Raman spectroscopy permits the study of localized changes in cell wall composition and structure of individual cells and the comparison of these with different and distant tissues. This work is in collaboration with IFR (institute of Food Research) in Norwich, England.

1. Cryo-section of abscission zone in poinsettia
2. Scan the abscission zone to obtain the FT-IR and FT-Raman spectra and analysis of changes in chemical composition of abscission zone.

The FT-IR microscope was invented in 1949 but remained a curiosity until the addition of Fourier transform techniques in 1982. Its use then became widespread throughout forensic and material science. In contrast, the application of this technique to plant sciences has been relatively limited and is only now becoming more widespread. The master candidate can have a new insight obtained through the use of infrared and Raman spectroscopy in plant science research.

E. Elucidation of the cell wall alteration during abscission using immunological methods

Molecular probes such as antibodies enable us to visualize the distribution of the molecular components of cell walls and to map organization features. Immunolocalization techniques using light microscopy can indicate the distribution of antigens/epitopes within tissues, between cells or broad patterns of distribution within a cell, and immunogold electron microscopy can provide an indication of variation in the occurrence of particular epitopes or antigen across a cell wall. We have several types of monoclonal antibodies against plant cell wall and these probes are useful for the study of the localization of structural elements of macromolecules that comprise the dynamic cell wall.

1. Plant sample preparation and anatomical analysis of abscission zone in poinsettia. Using different type of staining methods, examine the structural changes with light microscopy and electron microscopy .
To learn basic techniques in plant cell biology.
2. Immunohistochemistry (Using fluorescent microscopy and confocal microscopy)
 - i) Sections obtained from resin-embedded material (1µm- thick) are incubated in a milk powder in phosphate-buffered saline (MPBS). Sections are then incubated in a solution containing monoclonal antibody in MPBS. The sections are extensively washed in MPBS and then incubate in a solution containing anti-rat monoclonal antibody linked to fluorescein isothiocyanate (FITC) as a secondary antibody. The sections are washed extensively with PBS, mount in a anti-fade solution, and examine with a microscope equipped with epifluorescence.
 - ii) Confocal microscopy : Use confocal microscopy for lamda scanning of abscission zone and neighbor tissues to compare the intensity of labeling/epitope. In addition, confocal microscopy can be used to identify the autofluorescence and subtract autofluorescence from the labeling and show actual distribution of the epitopes.
3. Immunocytochemistry (IEM: immunogold electron microscopy)

Section (50-100nm-thick) are blocked in bovine serum albumin in phosphate-buffered saline (BSA/PBS). Transfer grid to a droplet of primary antibody and wash grids by incubation in PBS. Transfer grid to secondary antibody (goat anti-rat IgG-gold 10nm: Wash in D.W, allow the grid to dry, and then examine in an electron microscope.

Through this project, the master candidate can be trained to work in plant cell biology and have an insight to inter/intra-cellular changes in abscission phenomenon.

F. Histology studies: cell division and developmental events in the abscission zone

We would like to clarify the cellular events going on in a non-predefined AZ. Our investigations indicate that cell division is re-initiated within the AZ just prior to abscission (Fig. 2, far right) and we wish to test whether this is necessary for completion of abscission. The abscission process requires two profound changes in cellular structure that might be mediated by cell cycle related processes. Cell volume increases dramatically, and this could be driven either by vacuole expansion or by endoreduplication. Endoreduplication would require cell cycle re-entry. The other process is modification of cell-cell adhesion and there are at least two distinct mechanisms that might achieve this - either through modification of cell walls between two neighbouring cells (most recognised) or by the de-novo creation of a modified wall following mitosis. First, we will characterise the process in detail by following cellular development along a time-course through the process using both light - and electron microscopic techniques as appropriate. In particular, anti-tubulin antibodies and the DNA specific dye, DAPI, will allow us to characterise any changes in the microtubule cytoskeleton or in nuclear DNA content and thereby determine when either mitosis or endoreduplication has occurred. If we find that mitosis does occur prior to abscission and within the AZ, we will isolate cell cycle genes as markers (i.e. mitotic cyclins for mitosis, histone for S-phase) (Fobert et al., 1994). Using anti-microtubule drugs (i.e. oryzalin, taxol) and anti-cell cycle drugs (aphidicolin, roscovitine) to inhibit cell cycle entry, S-phase, mitosis or cytokinesis, we will test which cell cycle related processes are required for abscission. The drugs will be applied on the cut surface of decapitated buds and their cellular effects monitored using appropriate cytological staining techniques. This project will be in collaboration with John Doonan's research group at John Innes Centre (JIC), Norwich, UK.

