Developing a RNA *in situ* hybridization protocol to study initial events of turnip formation in *Brassica rapa*

Author: Thijs Bouten

Registration nr: 910306-113-080

Study: MSc Plant Sciences

Supervisors: Dr. Ir. Guusje Bonnema and Johan Bucher

Chair group: Laboratory of Plant Breeding

Date: May – August 2016

WAGENINGEN UR
For quality of life

Abstract

The genus *Brassica* is one of most diverse among vegetable crops. There is enormous phenotypic variation present in the different Brassica species and researching this variation is a field of great scientific interest. One interesting morphotype within the *Brassica* rapa species is the turnip morphotype. The genetic background of the different morphotypes in this species is still unknown. Interesting is to investigate the genes underlaying the formation of turnip. One way to do this is *in situ* hybridization and in this thesis a protocol was developed to embed and section hypocotyl and root samples of turnip and pak choi genotypes. The different embedding resins Technovit 8100 and 9100 were tested for embedding quality and sectioning. Furthermore, the morphology between Pak choi and turnip were compared with the new embedded samples. Technovit 8100 was found to be best suitable for embedding root and hypocotyl samples, because of the reliability and easy protocol. This embedding resin was also more save and less reactive compared to Technovit 9100. Section quality was also good for Technovit 8100. However, no results about gene expression was obtained after *in situ* hybridization.

Acknowledgement

This thesis is part of my Master program Plant Sciences with the specialization Plant Breeding and Genetic Resources. I chose this thesis, because I did already a lot of genetic work and I wanted to broaden my horizon and do some morphology and genomic work. Unfortunately the end result was less than expected, but still I learned a lot the last four months.

I want to thank first of all Guusje Bonnema for providing this research. She was always available for me to ask questions and discuss the topic even in difficult times. Also she took care to feedback my report in order to be finished in time.

Also great thank to Johan Bucher. With his endless amount of positive energy it was always a pleasure to work together. He was a great support in de lab and greenhouse.

Further I want to thank Christian Bachem for reviewing and examine my thesis.

Table of contents

1. Introduction

The genus Brassica is extremely diverse encompassing many diverse vegetable crops. There is enormous phenotypic variation present in the different Brassica species comprising heading types such as cabbages, cauliflower, broccoli, but also tuber forming types such as kohlrabi and turnips. Furthermore there are also brassica species for oil production like rapeseed(B. napus and B. rapa). This variation within the species is interesting for breeding, but also for scientific research to unravel the mechanisms behind these morphological differences. It is thought that the large variation in morphology is due to recent genome triplication of a common ancestor with *Arabidopsis thaliana.* The triangle of U (Figure 1.1) shows the different Brassica species. This triangle shows the relationships between the different Brassica species, where there are three diploid species, *Brassica rapa, Brassica nigra* and *Brassica oleracea* and three tetraploid/amphidiploid species, namely *Brassica juncea, Brassica carinata* and *Brassica napus* (Cheng et al. 2014).

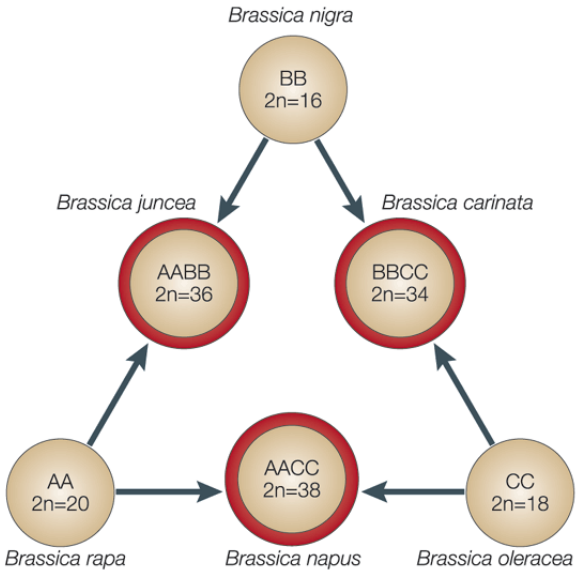


Figure 1.1: Genomic relationships among six crop species of Brassica (Triangle of U).
The Figure shows the three diploid species *B. rapa, B. nigra* and *B. oleracea* with the A, B and C genome respectively. The other three species, *B. juncea, B. carinata* and *B. napus* are the result of intraspecific hybridization between two diploid Brassica species (Stewart et al. 2003).

In this thesis the focus is on *B. rapa* which is the Brassica species which is thought to be the oldest species which was domesticated (Prakash et al. 2011). The origin of *B. rapa* is thought to be in the area of Iran/Iraq and from there evolved independently at different periods in time and at different geographical locations (Song et al. 1990; Zhao et al. 2005). By breeding, different morphotypes have been formed as can be seen in Table 1.1 (Bonnema et al. 2011), with the heading, turnip and oilseed types as the most important ones. This diversity in phenotypes makes the crop an ideal candidate to study genetics and mechanisms resulting in these different developments. Another advantage is that *B. rapa* is related to *Arabidopsis thaliana* which is the model plant for this type of research. *B. rapa* is a diploid species (AA=2n=20) with a genome of 485 Mb. The genome of *B. rapa* has been sequenced in 2011 which allows genomic studies related to the different morphotypes (Wang et al. 2011).

Table 1.1: Different morphotypes present within the *B. rapa* species. Adjusted from (Bonnema et al. 2011)

Morphotype	***B. rapa* species**
Leafy type – heading	Chinese cabbage (*pekinensis*)
Leafy type – loose leaves	Pak choi (*chinensis*)
	Wutacai (*narinosa*)
	Komatsuna (*perviridis*)
Leafy type – Tilling	Mizuna & Mibuna (*nipposinica* & *japonica*)
Swollen stem/ Inflorescence	Caixin or Caitai (*parachinensis*)
	Zicaitai (*purpuraria*)
	Broccoletto, Broccoli raab, Cima di rapa (*ruvo*)
Swollen hypocotyl/root	Turnip (*rapa*)
Oil seed	Turnip rape (*oleifera*)
	Brown and yellow sarson (*dichotoma* & *tricolaris*)

There are many plant species which have tuberous storage organs. Among them the most important for food consumption are potato, sweet potato, beet and cassava. These tubers have a high nutritional value and can be stored for long time. Especially for people in third world countries this is the main source of food and gives food security. Within the Brassica species there is kohlrabi (*B. oleracea*), turnip (*B. rapa*) and rutabaga (*B. napus*) which produce tubers (Prakash et al. 2011). This thesis focusses on turnip formation in *B. rapa* which consist of mainly hypocotyl, with partly stem as well as root tissue (Zhang et al. 2014). Hypocotyl tissue can easily be distinct from sections of root tissue because it has a pith. Visual initiation of tuber formation start around 3 to 4 weeks old plants, however the morphology of the different morphotypes, turnip and non-turnip was visualized under microscope resulting in comparable morphology between the different morphotypes until initiation of turnip formation starts. It suggests that turnip initiation occurs approximately two or three weeks after germination, which is visualized by a larger diameter and higher proportion of vascular tissue in the turnip morphotypes compared to the non-turnip morphotypes (Petrasch 2016a).

Little is understood about the underlying mechanisms of turnip formation and only a recent genomic and transcriptomic study was performed to investigate the turnip formation at transcript level (Bassetti 2015). Most research has been conducted based on morphology changes in turnip formation and classical genetic approaches. This resulted in the identification of several QTLs related to turnip formation(Lou et al. 2007; Lu et al. 2008; Kubo et al. 2010; Zhang et al. 2014). Furthermore, A study was conducted to investigate so called Selective Sweeps in the turnip genomes resulting in the identification of 31 Selective Sweeps on all chromosomes (Cheng et al. 2016). A Selective Sweep is a genomic region which harbours allelic variation that relates to interesting traits for human selection during domestication and as a result shows low variability compared to other regions in the genome. The information of two QTL studies together with a list of turnip-Selective Sweeps and the data of two genome wide expression analyses were used and compared with literature information to come to a selection of 60 genes. The expression pattern of these genes were investigated by collecting RNA from turnip and non-turnip genotypes and measure expression during the first 6

weeks of plant development. From this data the most promising genes were selected based on differences in expression of a certain gene between the turnip and non-turnip genotypes. These select candidate genes (Table 1.2) which are possibly involved in the initiation of turnip formation (Petrasch 2016b, 2016a).

- Bra038700 (gene 3) is an orthologue of the *A. thaliana FLOR1* gene which is involved in flowering time and it has been found that this gene is only expressed in floral meristems and tissues (Torti et al. 2012).
- Bra034022 (gene 7) is an orthologue of the *A. thaliana CYP35A2* gene and has been chosen as candidate gene because it is involved in the cytokinin pathway. It is investigated that this gene is involved in the biosynthesis of the cytokinin trans-zeatin which inhibits growth, but induces shoot growth (Kiba et al. 2013; Ramireddy et al. 2014).
- The third gene Bra003665 (gene 20) is also related to a hormone pathway. It is an orthologue of the *ARF17* gene from *A. thaliana* which is an auxin response factor related to the transcription of several auxin response genes. This gene is reported to be involved in several development processes of the plant. The gene itself is regulated by the microRNA miR-160 (Bustos-Sanmamed et al. 2013; Gutierrez et al. 2009). Research in radish shows that this gene is upregulated during tuber formation which gives more indications that this can be an interesting candidate gene for turnip formation in *B. rapa* (Sun et al. 2015).
- The most promising gene is Bra022954 (gene 34) which is an orthologue of the *A. thaliana SPL3* gene. This gene is like the *ARF17* gene regulated by miR156 and is involved in floral transition and vegetative phase change (Cardon et al. 1997; Yu et al. 2015). Interesting is that in turnip this gene is highly expressed long before flowering starts which can indicate a potential role in turnip formation. Furthermore, it has been found that the *SPL3* gene is plays a role in the tuberization of potato and tomato were it is expressed highly during tuberization (Eviatar-Ribak et al. 2013). This in contrary to radish where recent research showed a decrease of miR156 during tuber formation (Sun et al. 2015). This is in line with expression levels in turnip where increasing levels of *SPL3* indicate decreasing levels of miR156.
- The final selected gene is Bra030232 which is an orthologue of the *A. thaliana* Cyclin D2;1 (*CYCD2;1)* gene. This gene is chosen because it is reported that it is involved in increased cell division rate of the meristemic cells together with an increase of periclinal cell division (Sanz et al. 2011). Another argument is that *CYCD2;1* is induced by the presence of sucrose which is the main sugar found in fully developed turnips (Gupta et al. 2001; Nishijima et al. 2005).

Table 1.2: candidate genes related to the initiation of turnip formation and their expression pattern.

Gene Number[1]	Gene Name	BraID	Expression pattern
3	*FLOR1*	Bra038700	Higher expressed in turnips at all time points
7	*CYP735A2*	Bra034022	Higher expressed in turnips after week 3
20	*ARF17*	Bra003665	Higher expressed in turnips after week 3
34	*SPL3*	Bra022954	Higher expressed in turnips at all time points[2]
35	*CYCD2;1*	Bra030232	Higher expressed in turnips after week 3

[1] Number used in previous work (Petrasch 2016b, 2016a)

[2] In one turnip, the expression decreased when the turnip started to develop

In order to further characterize these candidate genes *in situ* hybridization is a good option to do this. *In situ* hybridization is a technique to visualize the location of an expressed gene in plant tissue. The steps for this procedure are:

1. The first thing is to decide which gene you want to visualize. The lines in figure 1.2 represent mRNAs in a cell with the red colored line representing the gene of interest (fig 1.2A). There are a huge amount of mRNAs present in one cell, so the mRNAs in the figure represent only a small amount of the total mRNAs present in the cell.
2. Synthesis of RNA "antisense" , which is a single stranded RNA molecule complementary to the gene of interest.
3. Label antisense RNA probe with digoxigenin (DIG) molecules (fig 1.2B).
4. Fixing plant material in which you like to see the expression of your gene of interest.
5. Use an embedding in order to be able to make nice slices with a microtome of your samples.
6. Hybridization of the material and detection expression under the microscope. The "antisense" RNA probe binds to the gene of interest. After Hybridization the unbound probe is washed of so only strongly hybridized RNA remains. In the next step anti-DIG antibody will be added which binds to DIG molecules (fig 1.2C). To the antibody the enzyme alkaline phosphatase (AP) was chemically attached. Visualization is performed by adding BCIP, a substrate for AP and NBT a color enhancer. The coloring happens when AP cleaves the phosphate group off of the BCIP which results in a local blue staining (fig 1.2D).

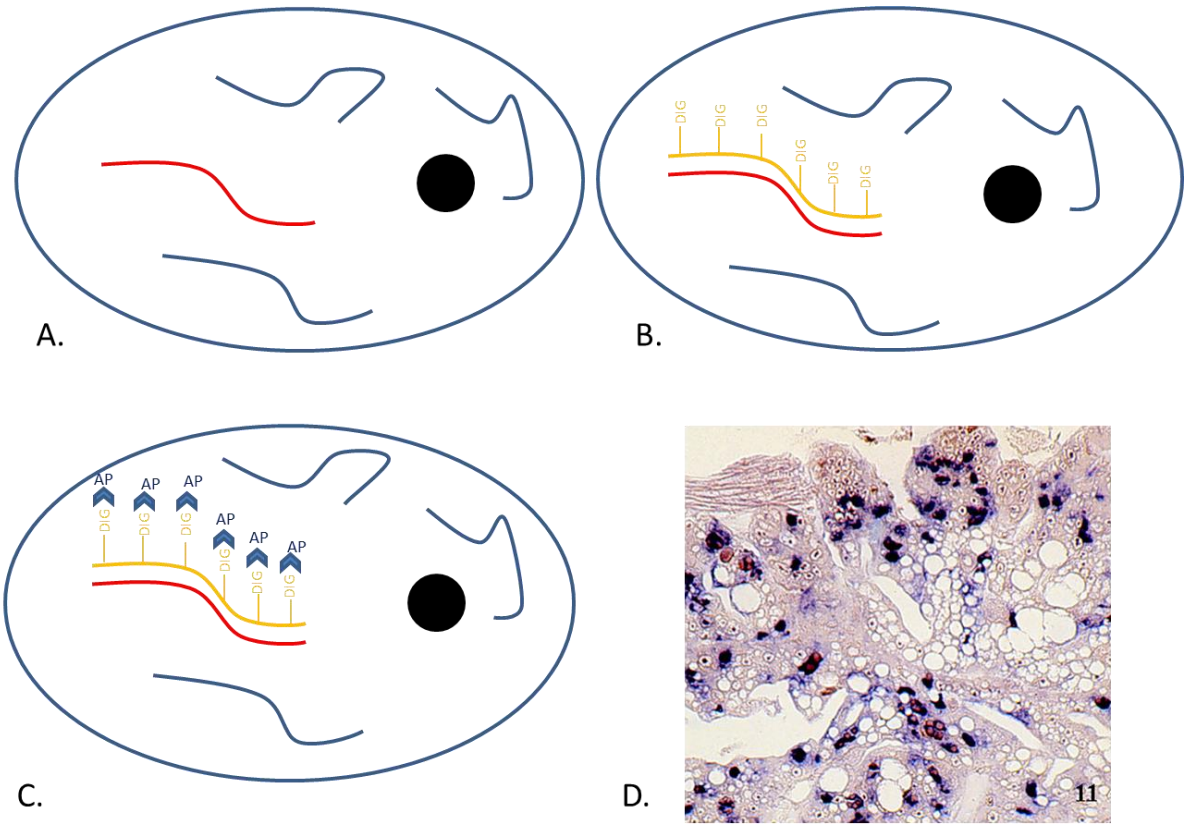


Figure 1.2: Hybridization and detection of expression of a candidate gene in fixed plant material. A) mRNA molecules with the gene of interest in red. B) Binding of DIG labelled probe to the gene of interest. C) Adding of anti-DIG antibody with the enzyme alkaline phosphatase (AP) attached. D. Result of staining under the microscope. Blue colour means presence of the gene of interest.

MSc student Stefan Petrasch has already started developing a protocol for *in situ* hybridization with root/hypocotyl tissue from *B. rapa (Petrasch 2016a).* In his research several turnip and non-turnip *B. rapa* morphotypes were selected to investigate the gene expression of the candidate genes that are listed in Table 1.2. In his research paraplast was used as embedding resin. This resulted in a minimum cutting thickness of 20 µm which is not thin enough to get nice pictures under the light microscope due to several cell layers. Furthermore, Technovit 7100 was tested as embedding resin. This resulted in better sections of 10 µm compared to paraplast embedded samples and had good RNA maintenance. The thinner samples are due to the harder structure of Technovit. In situ hybridization was attempted with the Technovit 7100 samples. However, there was still a problem with the adherence to the microscope slides of tissue embedded in Technovit 7100 when adding the hybridization buffer to the samples. This should first be improved before the real *in situ* hybridization can be performed.

The aim of this research is to first develop a reliable *in situ* hybridization protocol for turnip and hypocotyl tissues of young one to three week old plants of turnip and non-turnip genotypes and from there investigate the four candidate genes for their expression pattern in the different tissue at the time points when turnip formation is initiating. This will be done by means of RNA *in situ* hybridization. This new information about the location of the expression of certain genes and their expression pattern can give more information about the possible function regarding turnip formation and can be a start to further characterize the genes in more detail.

The research questions which will be answered are:

1. What is the best method for embedding fixed hypocotyl/root samples of *B. rapa* when the purpose is *in situ* hybridization?
2. At what time points are the selected genes expressed in turnip and non-turnip samples?
3. At what tissue or in which cell types are the selected genes expressed in turnip and non-turnip samples?
4. What is the difference in expression pattern for the different selected genes between turnip and non-turnip morphotypes?

2. Material and Methods

2.1 Plant material

For the in situ hybridization experiments, several different double haploid (DH) *Brassica rapa* genotypes representing different morphologies were used (Table 2.1). The four different Turnip genotypes are visualized in Figure 2.1 (Zhang et al. 2014) and were selected for their seed availability and geographic origin. Two Turnips are originating from Japan and two turnips are originating from Europe. From each line a total of 60 seeds was sown in 11 cm pots with two seeds per pot. Only for Turnip VT-009 a total of 48 seeds were sown due to a lack of seeds in the stock. A randomized block design was used where pots were placed in three blocks with 10 pots of each line per block, except for VT-009 with 8 pots per block.

Table 2.1: Different morphotypes with the names, genebank ID, accession number WUR and origin

Morphotype	Name	Genebank ID	Accession number WUR	Origin
Turnip (Japanese)	VT-123	CGN15220	BrDFS_A_120	Japan
Turnip (Japanese)	VT-009	CGN06717	BrDFS_A_098	Japan
Turnip (European)	VT-053	CGN07167	BrDFS_A_145	Germany
Turnip (European)	FT-005	CGN06688	BrDFS_A_088	Germany
Broccoletto	BRO-030	CGN06829	BrDFS_A_127	Italy
Pak Choi	PC-175	VO2B0226	BrDFS_A_055	China
Pak Choi	PC-183	VO2B0655	BrDFS_A_057	China

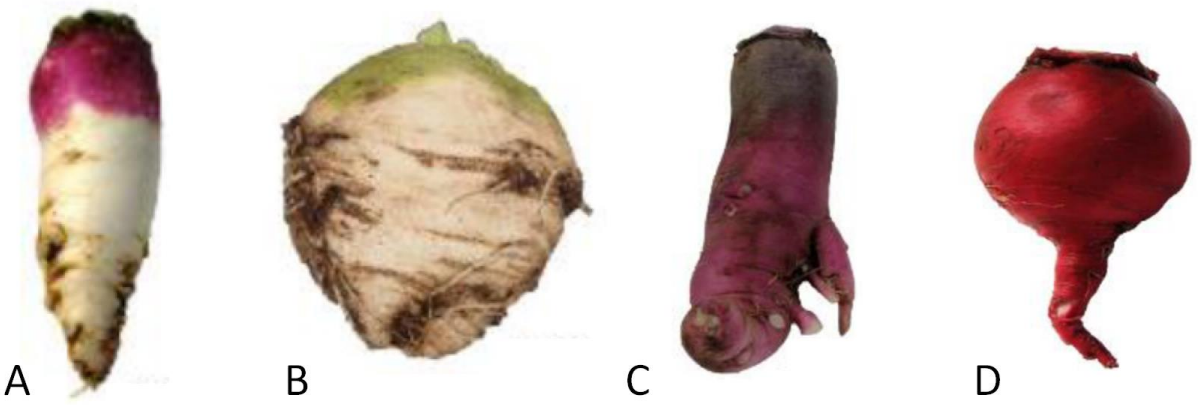


Figure 2.1: Turnip morphotype from the different DH lines: A. FT-005, B. VT-053, C. VT-123`and D. VT-009. Adjusted figure Zhang et al. 2014.

2.2 Sample preparation

Plants were growing under greenhouse conditions in the same department. There was no extra heating and lighting in the greenhouse. Hypocotyl/root tissue was harvested at seven different time points, namely 7, 10, 13, 17, 19 and 21, 24 days after sowing. At each time point three plants per line were harvested, one from each block. First a picture was made from the plant and after that three approximately 5-10 mm large samples per plant were cut with a surgical blade from the hypocotyl, transition zone and root part respectively (Figure 2.2).

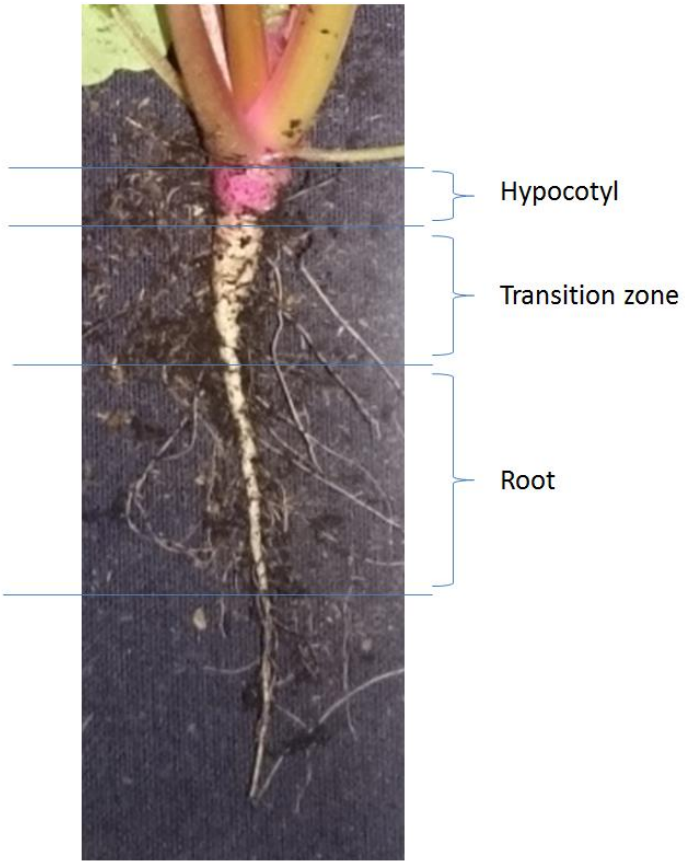


Figure 2.2: cutting sites of hypocotyl, transition zone and root zones. Approximately 5 mm large samples were cut.

These samples were immediately placed in 4% Paraformaldehyde (PFA) on ice and after collection of all the samples placed in a vacuum for three times 10 minutes. After the vacuum, samples were placed in new fresh fixative and stored overnight at 4 °C. After that the samples were placed in the following ethanol series.

- 1x PBS (30 minutes)
- 1x PBS (30 minutes)
- 30% EtOH (60 minutes)
- 40% EtOH (60 minutes)
- 50% EtOH (60 minutes)
- 60% EtOH (60 minutes)
- 70% EtOH (60 minutes)

After the samples were at 70% EtOH they were stored at 4 °C and 70% EtOH was once replaced after 2 days. At this stage the samples can be stored for several months.

2.3 Technovit 9100

2.3.1 Embedding samples

Technovit 9100 was used as polymerisation system which is based on methyl methacrylate (MMA). The embedding procedure was performed according to the technovit 9100 protocol. All solutions were prepared before starting the protocol (Table 2.2). The first step was to destabilise the basic solution by filling a chromatography column with 12,5 g aluminium oxide and allow first 40 ml of the Technovit 9100 basic solution to flow through. After that another 200 ml basic solution was added which flowed through in about 10 hours. The destabilised basic solution was collected in a glass bottle covered with aluminium foil and stored at 4 °C. Stock solution A and B were made with destabilized basic solution. Furthermore, infiltration and Stock solution A were made by adding the PMMA powder to 2/3 of the basic solution and this was stirred for 2 to 3 hours to obtain a homogenous solution. After all PMMA powder was dissolved, Hardener 1 and the rest of the basic solution was added. This final solution was stirred for 1 h and stored at 4 °C.

Table 2.2 Preparation of the different Technovit 9100 solutions

Solutions	xylene	Stabelised basic solution	destabilised basic solution	PMMA	Hardener 1	Hardener 2	regulator
Preinfiltration 1	50 ml	50 ml					
Preinfiltration 2		100 ml			0,5 g		
Preinfiltration 3			100 ml		0,5 g		
Infiltration			100 ml	8 g	0,4 g		
Stock solution A*			50 ml	8 g	0,3 g		
Stock solution B*			10 ml			0,8 ml	0,2 ml

*Stock solution A and B must be mixed in a ratio 9:1 immediately before use. This is the polymerization mixture.

Samples in the 70% ethanol were dehydrated through a gradient ethanol series followed by placing the samples two times in a xylene solution. before going to the preinfiltration steps. After the preinfiltration steps, the samples were placed in the infiltration solution for 48 hours (Table 2.3). Polymerization was performed by adding 9 ml stock solution A to 1 ml Stock solution B and mix well. First the sample was placed in the right orientation. About 2 ml was added to each well of an embedding mold and if necessary the sample was replaced in the proper orientation. Finally the well was covered by a round specimen holder. The whole mold was sealed very good in a plastic container to avoid air contact and placed in a -18 freezer overnight.

Table 2.3:Dehydration, preinfiltration, infiltration and polymerisation steps of the embedding protocol.

Step	Solution	Concentration	Time (h)	Temperature
Dehydration 1	Ethanol	70%	1	Room
Dehydration 2	Ethanol	80%	1	Room
Dehydration 3	Ethanol	96%	1	Room
Dehydration 4	Ethanol	96%	1	Room
Dehydration 5	Ethanol	Absolute	1	Room
Dehydration 6	Ethanol	Absolute	1	Room
Dehydration 7	Ethanol	Absolute	1	Room
Intermediate 1	Xylene		1	Room

Intermediate 2	Xylene		1	Room
Preinfiltration 1	Xylene/Technovit 9100 NEW Basic (Stabilized)	50%	1	Room
Preinfiltration 2	Technovit 9100 NEW Basic (Stabilized) + Hardener-1		1	Room
Preinfiltration 3	Technovit 9100 NEW Basic (Destabilized) + Hardener-1		1	4 °C
Infiltration	Technovit 9100 NEW Basic (Destabilized) + Hardener-1 + PMMA-Powder		48	4 °C
Polymerization	Stock solution A + Stock solution B (9:1)		18-24	-20°C

2.3.2 sectioning and *in situ* hybridisation

A 24 day old hypocotyl sample from DH-line VT-053 was used for sectioning and *in situ* hybridization. Sectioning of the samples was done by using a Leica/Reichert 2055 Autocut microtome with a Tungsten Carbide knife with a D profile. The microtome was set to 9˚ and thin section of 5 and 10 µm were cut. These sections were mounted on normal and coated Superfrost Ultra Plus microscopy glasses using 96% ethanol. The coating was done by dipping the slides in 0,1 Aurion BSA-c solution and let it air dry. After adding the slices to the slides they were placed at 37 ˚C for 2 days. After these 2 days, the slides were ready for the *in situ* hybridization.

Prior to the start of the *in situ* hybridization the samples were deplastified at room temperature by placing the slides in the following solutions according to the protocol of the Technovit 9100 instructions.

- Xylene 2x 20 min
- 2-methoxyethyl acetate (2-MEA) 1x 20 min
- High-purity acetone 2x 5 min
- Sterile Milli-Q water 2x 5 min

Slides were dried and the *in situ* hybridisation was performed using the following protocol which is partly based on the Technovit 9100 instructions and partly based on the Dolf Weijers protocol. Slides were placed in a slide holder in the following solutions:

- 3% H_2O_2 in methanol 30 min
- Sterile Milli-Q 2x 5 min
- Proteinase K buffer 10 min
- Sterile Milli-Q minimum 10 min
- Fresh sterile Milli-Q overnight

After the last Milli-Q step the slides were dried and from this point the protocol of the Dolf Weijers lab was used starting from Day 3- Hybridization. Slides were examined under the light-microscope and the maintenance of RNA was tested with Acridine Orange staining.

2.4 Technovit 7100 and 8100

2.4.1: embedding Technovit 8100

Infiltration solution was prepared by adding 1 bag of Hardener 1 to 100 ml of Technovit 8100 basic solution and placed at 4 ˚C. This infiltration solution was used to make the infiltration steps 1-4 of the right composition. Fixed samples where dehydrated with an ethanol series followed by two absolute aceton steps. Samples were than infiltrated gradually with an acetone/infiltration solution 2/1, 1/1 and 1/2 before going to 100% infiltration solution (Table 2.4). After the first 100% acetone step and the last infiltration step, the samples were placed shortly in vacuum.

Table 2.4: Dehydration and infiltration steps prior to polymerisation.

phase	solution	concentration	time/temp
dehydration 1	ethanol	80%	1 h/ 4 ˚C
dehydration 2	ethanol	90%	1 h/ 4 ˚C
dehydration 3	ethanol	100%	1 h/ 4 ˚C
dehydration 4	acetone	100%	1 h/ 4 ˚C
short vacuum (low boiling point so pay attention)			
dehydration 5	acetone	100%	1 h/ 4 ˚C
infiltration 1	acetone/ infiltration solution	2/1	overnight / 4 ˚C
infiltration 2	acetone/ infiltration solution	1/1	8-10 h / 4 ˚C
infiltration 3	acetone/ infiltration solution	1/2	overnight / 4 ˚C
infiltration 4	infiltration solution	100%	8-10 h / 4 ˚C
short vacuum before embedding (low boiling point so pay attention)			

The final embedding steps were performed according to the protocol from Yeung and Chan,2015 with some small adjustments as described below.

1. Molding trays (Figure 2.3A) were used and filled halfway with infiltration solution. Then, the samples were placed into the cup of the mold (Figure 2.3B). Important is to not expose the tissue to air, so completely cover it with infiltration solution. No Hardener 2 is added yet, so this step was done slowly. After all samples were in place, they were briefly vacuumed ensuring no air was in the solution.
2. Embedding solution was prepared by adding 0,5 ml Hardener 2 to 15 ml infiltration solution. As soon as Hardener 2 is added the reaction started so it was used immediately. Infiltration solution was removed with a disposable pipette and the sample was rinsed once with embedding solution before adding new embedding solution to all cups (Figure 2.3C). The right orientation of the samples was checked and reoriented if necessary. Finally a plastic specimen holder was placed on top of the cup (Figure 2.3D). It was taken care of that enough embedding solution (2 ml) was added to fill the whole cup (Figure 2.3E).
3. The entire cup was polymerized at room temperature overnight. Proper embedded samples had a clear background with the samples good visible (Figure 2.3F).

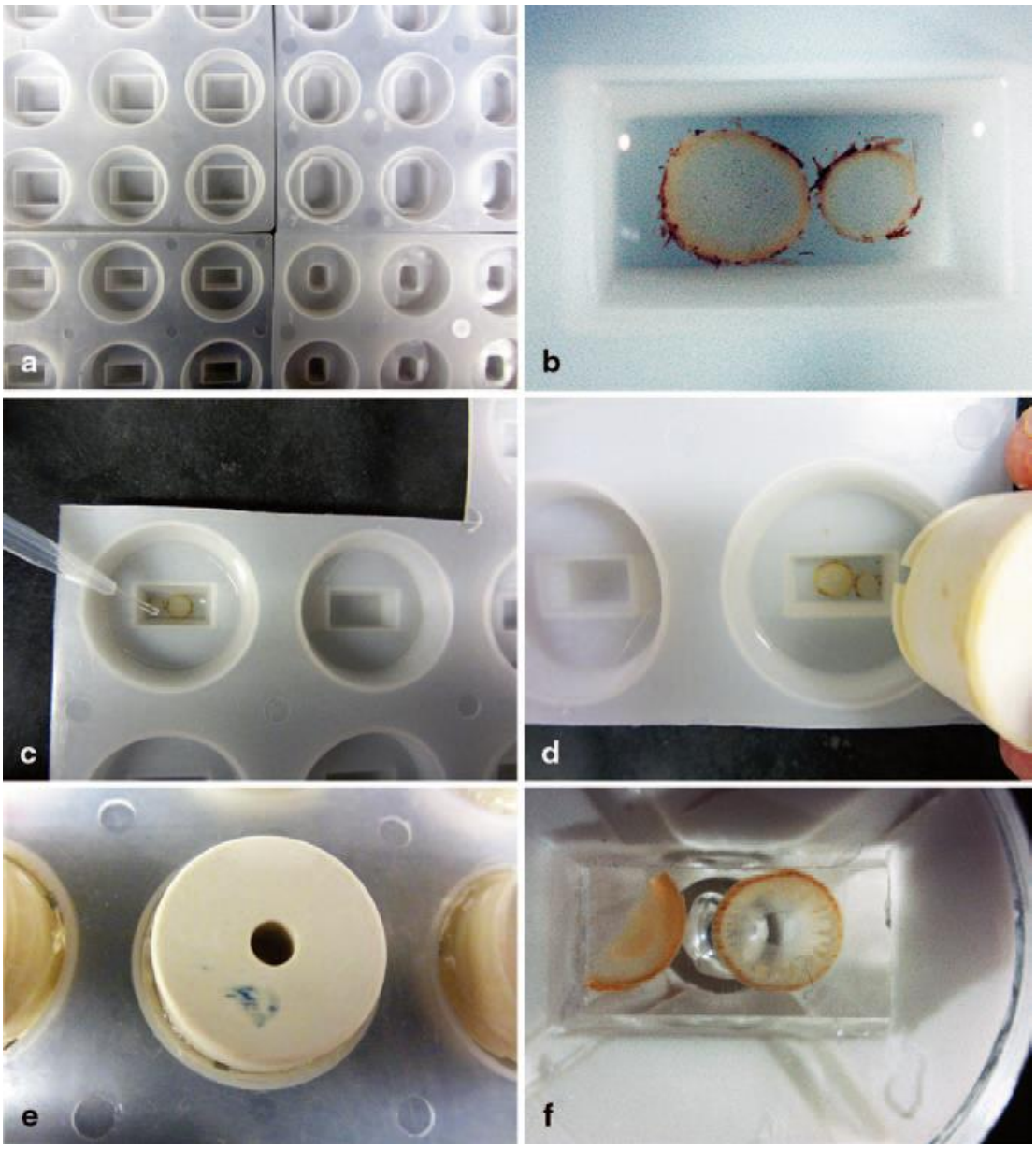


Fig. 2.3 Embedding procedures. A. Different sizes of embedding mold. B. Tissue pieces are transferred into a molding cup with infiltration solution. C. Once the tissues are properly arranged, the infiltration solution is removed using a disposable pipette and the specimens are rinsed once with the embedding solution before refilling with embedding solution. D. Apply a round specimen holder to the molding cup. E. Ensure that the embedding solution is filled to the rim, allow the solution to polymerize for at least 2 h before sectioning. F. A proper polymerized block is relatively clear and the tissues are visible. (Figure from Yeung and Chan, 2015).

2.4.2 sectioning Technovit 7100 and 8100

Embedded samples of Technovit 7100 (Petrasch 2016a) and the new embedded Technovit 8100 were used for sectioning. Sectioning of the samples was done by using a Leica/Reichert 2055 Autocut microtome with a Tungsten Carbide knife with a D profile. The microtome was set to 9˚ and thin section of 7 µm were cut. These sections were mounted on Superfrost Ultra Plus microscopy glasses by placing the slices on a microscopy glass with a droplet of sterile water. After adding the slices to the slides they were placed at 37 ˚C. Technovit 7100 samples were incubated for 3 days and Technovit 8100 samples for 1 day before the slides were ready for the *in situ* hybridisation. Furthermore extra slides of Technovit 8100 samples were prepared. With these samples the

morphological differences of turnip and pak choi were investigated and these samples were also used to check the RNA quality by staining with acridine orange after fixation and embedding.

2.4.3 *in situ* hybridization Technovit 7100 and 8100

The *in situ* hybridization was performed with three samples embedded in Technovit 7100 and six samples embedded in Technovit 8100. While for Technovit 9100 embedded samples, the Technovit itself needs to be removed prior to the hybridization steps, this is not the case for the Technovit 7100 and 8100 embedded samples.

The RNA *in situ* hybridization protocol for Arabidopsis embryos from the Dolf Weijers laboratorium was followed (appendix 1). In this protocol samples were embedded in paraplast. Therefore, in that protocol, the first pre-hybridization step was removal of paraplast by washing with xylene, which is not needed in our case. So the procedure directly started with the ethanol series, that follow this xylem washing steps.

Probes: Stefan Petrasch (former MSc student) had cloned the genes into pGEM®-T Easy vectors, and had incorporated the DIG labels. This was quality checked on gel and was used as staring material in this research. The next steps were performed during the hybridization steps as described in the protocol. For this *in situ* hybridization experiment a DIG labelled probe containing the reference *Cyp* gene was used in the reaction. Slides were checked for the blue hybridization signal under the microscope.

3 Results

3.1 Plant development

Seed germination was good for all selected turnip and non-turnip DH lines, so that plants could be harvested on all planned time points. Germination was fast and uniform. From each turnip DH line, four to five plants were left in the greenhouse to fully mature. After 70 days these plants were harvested (Figure 3.1). Turnips VT-053, VT-123 and VT-009 are more or less similar to the phenotype as described in Zhang et al. 2014. However, Turnip FT-005 did not show a turnip like phenotype except for one plant which had a thickening, but not as big as expected. Therefore, VT-053, VT-123 and VT-009 were selected for the *in situ* experiments and FT-005 was excluded.

Figure 3.1: Mature turnips 70 days after sowing. European turnips FT-005 (A) and VT-053 (B) together with Japanese turnips VT-123 (C) and VT-009 (D).

Furthermore, the plants looked very uniform. After 13 days plant started to grow faster and differences between genotypes became more clear (Figure 3.2). Visual turnip formation for VT-009, VT-053 and VT-123 was first seen at measuring point 6 which is 21 days after sowing. At 24 days after sowing this thickening was even better visible with clear difference between turnip and non-turnip plants.

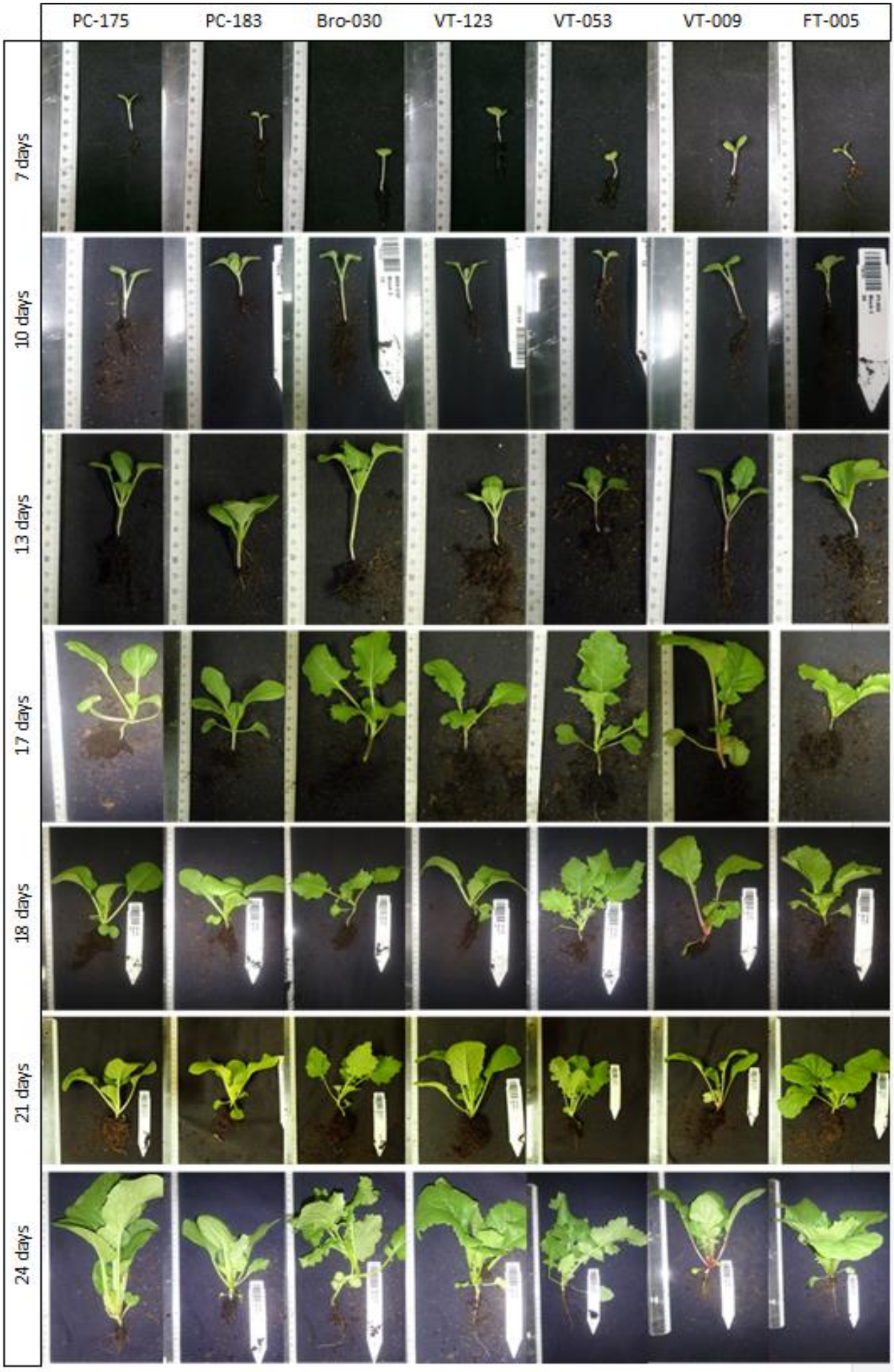


Figure 3.2: representative plants of each DH line at all harvest time points.

3.2 Technovit 9100

3.2.1 Embedding with Technovit 9100

No reliable method has been found to embed samples in Technovit 9100 resin. The first attempt was done by mixing destabilised stock solution A (3g Hardener 1) with destabilised stock solution B 9:1 (v/v). Three test samples were used for this procedure. This mixture was poured in molds and the samples were placed at the right position. Than the mixture was covered by a round specimen holder sealed and placed in the -18 ˚C freezer for 3 days. It turned out that the Technovit was reacting with the plastic specimen holders and that the embedding solution was not solid yet. The specimen holders were removed and the samples were replaced in the freezer. After one week one of the three samples turned out to be embedded properly. This same procedure was repeated, but now without using the round specimen holders. This resulted in no polymerization of the samples, even after more than 1 week in the freezer. The mixture had a rubber like structure, but not like a hard plastic. The assumption was that solution A and B were not mixed well prior to adding it to the mold. With the same mixture, tests were done by mixing solution A and B vigorously before applying it to the mold and also to first vacuum for a few minutes before storing in the freezer. Furthermore all the above mentioned tests were performed with stabilised solution A (4 g hardener 1) and stabilised solution B, however all these tests did not result in proper embedded samples.

3.2.2 sectioning and *in situ* hybridisation Technovit 9100

With the one sample which was proper embedded, tests with sectioning were performed. The first step was to check the adherence of the slices to the superfrost ultra plus microscopy slides. Some superfrost ultra plus slides were coated with a 0.1% Aurion BSA-c solution. Coupes of 5 and 10 µm were mounted onto the coated and non-coated slides with 96% ethanol and baked to the slides at 37 °C for two days. Adherence was good for both the coated and non-coated slides. First step of the *in situ* hybridisation was to remove the plastic from the sections which is a special feature of Technovit 9100. This worked well without losing the samples. After the *in situ* hybridisation no signal was found in all the slides.

3.2.3 Sample and RNA quality after *in situ* hybridisation

No real quality differences of the samples were visible between coated and non-coated slides. However, there was a difference in quality between 5 µm and 10 µm sections. The 5 µm sections tend to break and have more damage than the 10 µm sections. RNA maintenance of the slides is good for 10 µm sections and bad for the 5 µm sections. Only in one of the four samples tested, the RNA was still intact as can be seen at the red colour in Figure 3.3. The green colour represents the DNA in the cells.

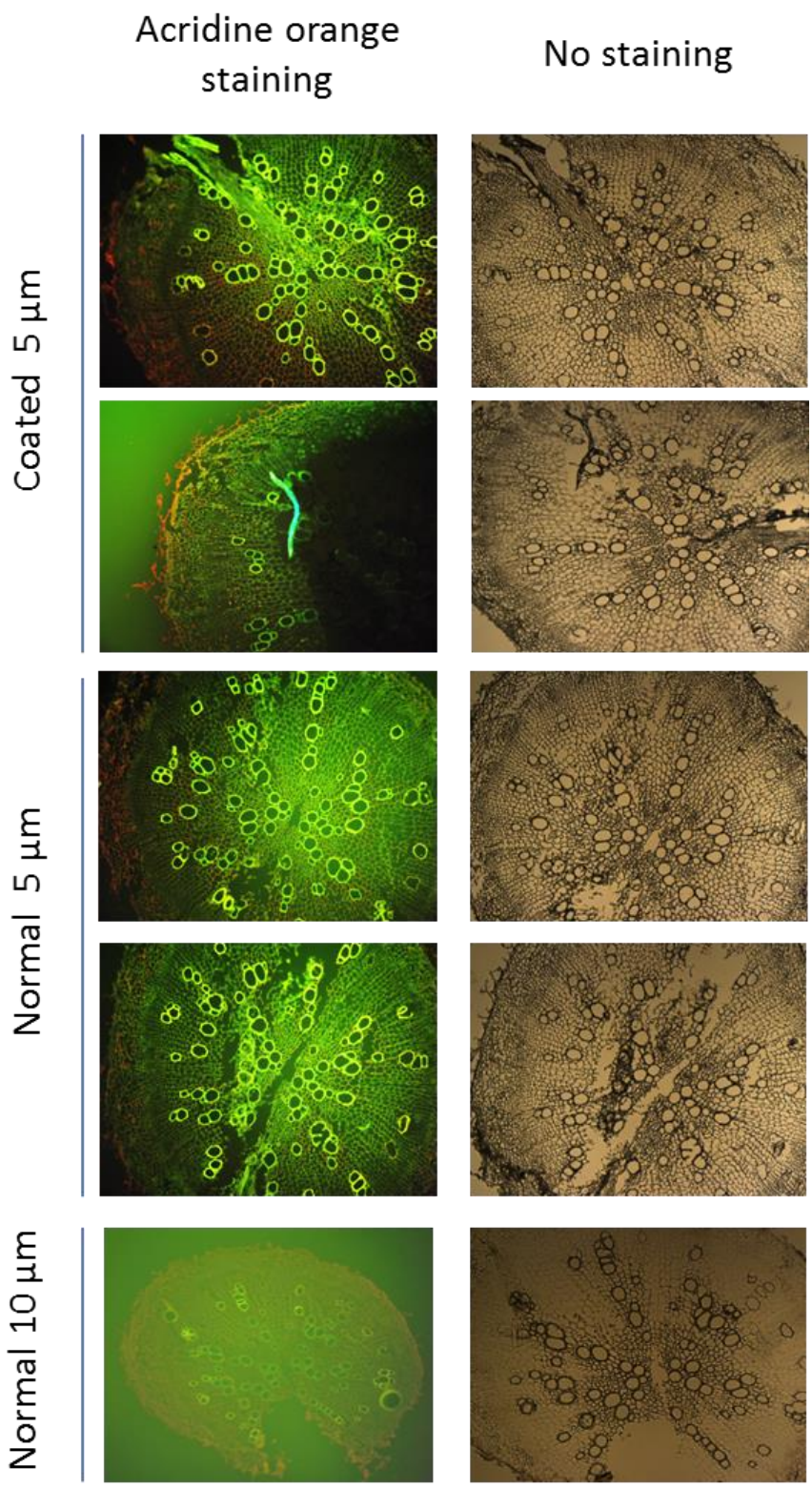


Figure 3.3:Transverse sections of a 24 day old hypocotyl sample of VT-053. First colum is acridine orange staining of sample one of each slide. The second colum is the same sample, but without staining. Two slides are coated and two slides non-coated with 5 µm sections. One slide has no coating with 10 µm sections.

3.3 Technovit 7100 and 8100

3.3.1 Technovit 7100 sections

Sections were made with Technovit 7100 and 8100 embedded samples. Previously embedded samples of Technovit 7100 were used to cut 7 µm samples of hypocotyl, transition zone and root parts. Due to limited choice and time the following four samples were chosen, namely 2 week old pak choi and turnip VT-117 (Figure 3.4A) together with 3 week old turnip VT-117 and 3 week old turnip VT-053 (Figure 3.4B). Sections of 7 µm result in good sections of Technovit 7100 embedded samples. Hypocotyl sections were clearly different from root and transition zone sections, because of the presence of a pit in the middle of the section. No clear differences were visible between 2 week old Pak Choi and VT-117. However, after 3 weeks turnip morphotypes had a strong growth of vascular tissue and also in diameter.

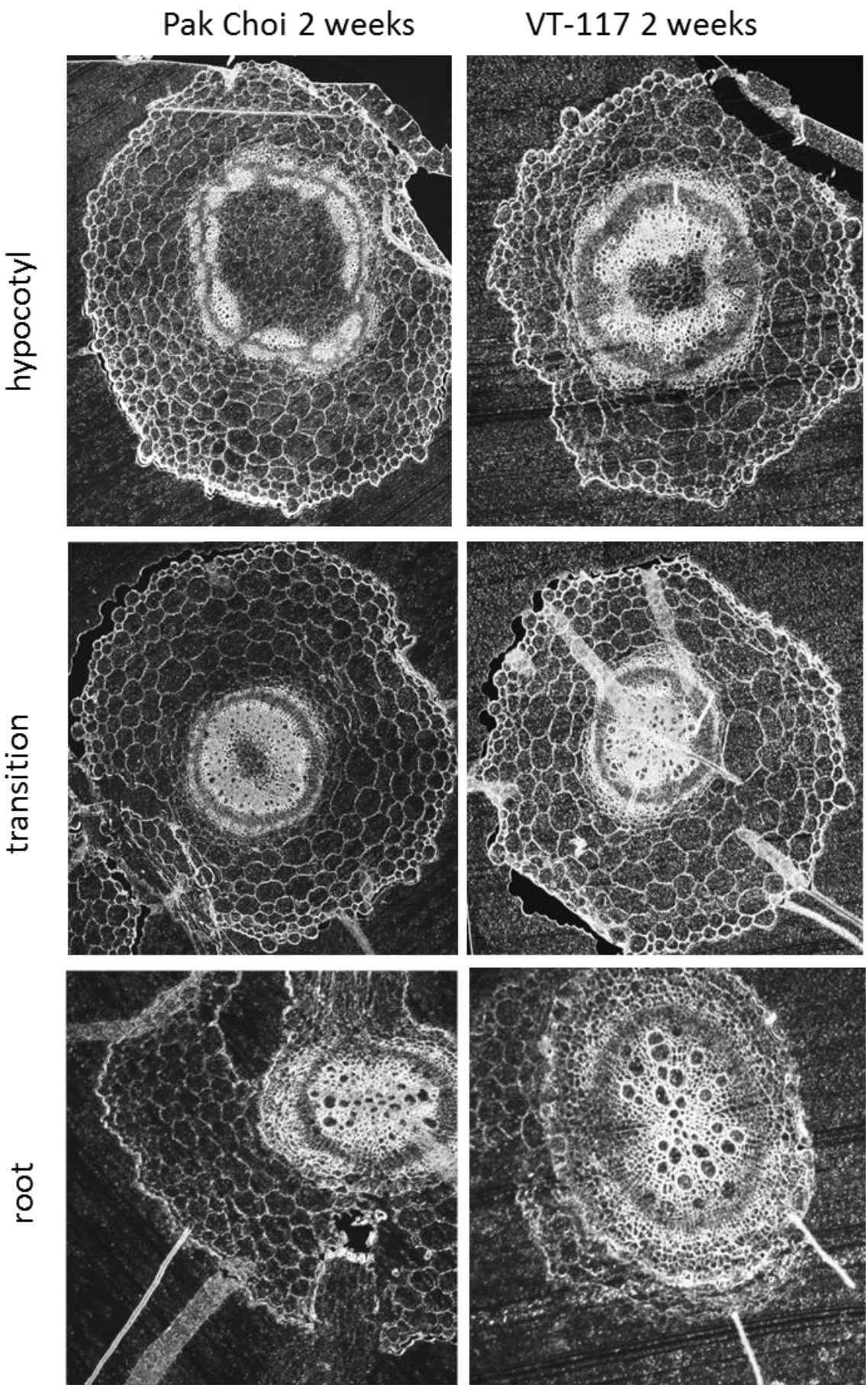


Figure 3.4A: Transverse sections of technovit 7100 embedded samples of hypocotyl, transition zone and root tissue of pak choi DH line PC- 175 and turnip DH lines VT-117 from two week old plants. Thickness of the sections is 7 µm.

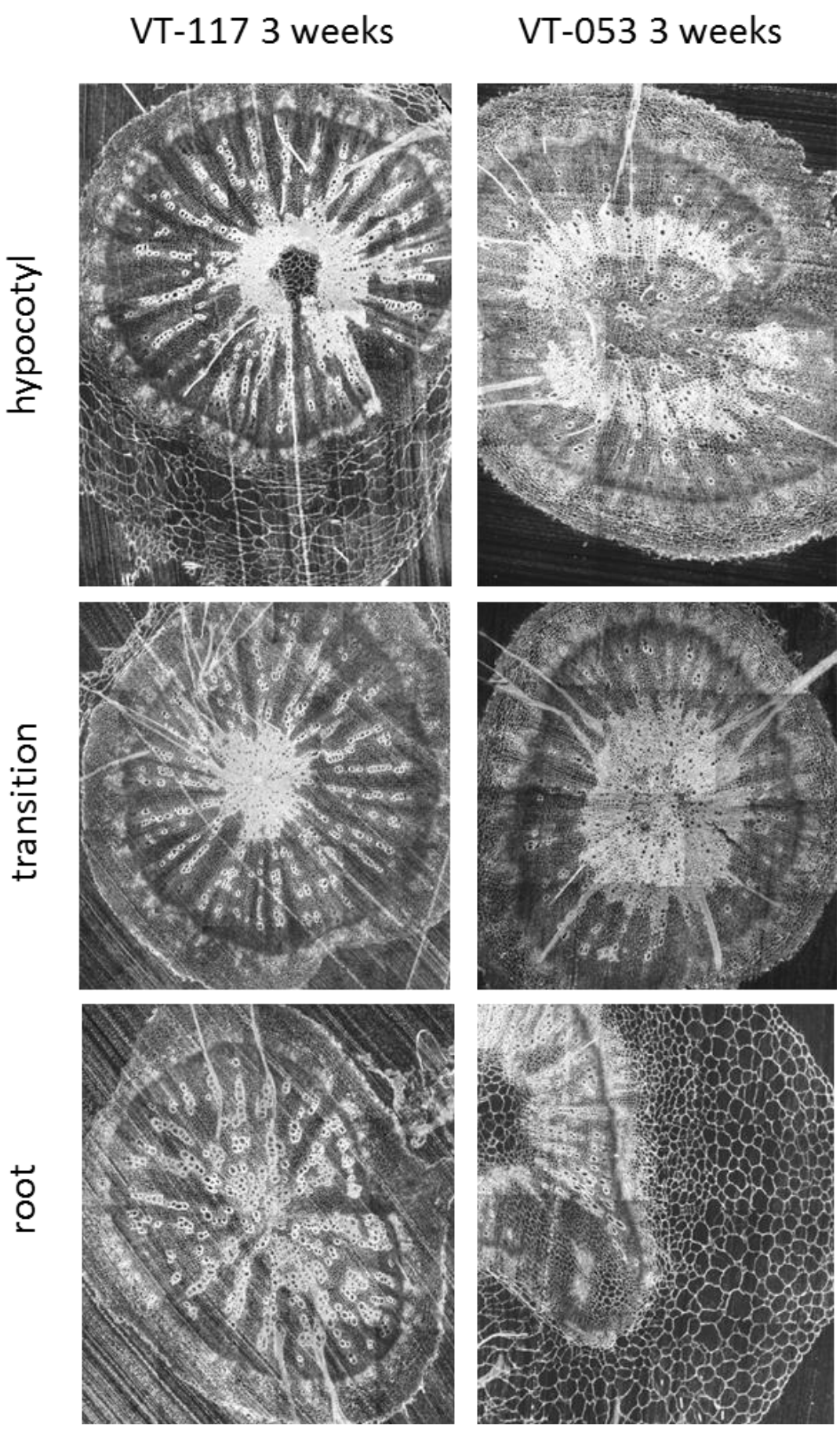


Figure 3.4B: Transverse sections of technovit 7100 embedded samples of hypocotyl, transition zone and root tissue of turnip DH lines VT-117 and VT-053 from three week old plants. Thickness of the sections is 7 µm.

3.3.2 Morphology of Technovit 8100 sections

Morphology was well preserved after sectioning for almost all samples (Figure 3.5). Only for the hypocotyl sample of PC-175 it was not possible to cut the sample without damage. Clear differences were visible between pak choi and turnip morphotypes especially in the hypocotyl and transition zone samples. In the root sample the difference was less easy to see. In hypocotyl and transition zone sections of VT-053 growth of secondary xylem was high and star shaped xylem was observed. This was not observed in PC-175 samples where the centre was still dense. Furthermore, the cell walls seem more lignified in PC-175 than VT-053. This can already be observed when cutting the samples. This is more difficult in pak choi than for turnip morphotypes.

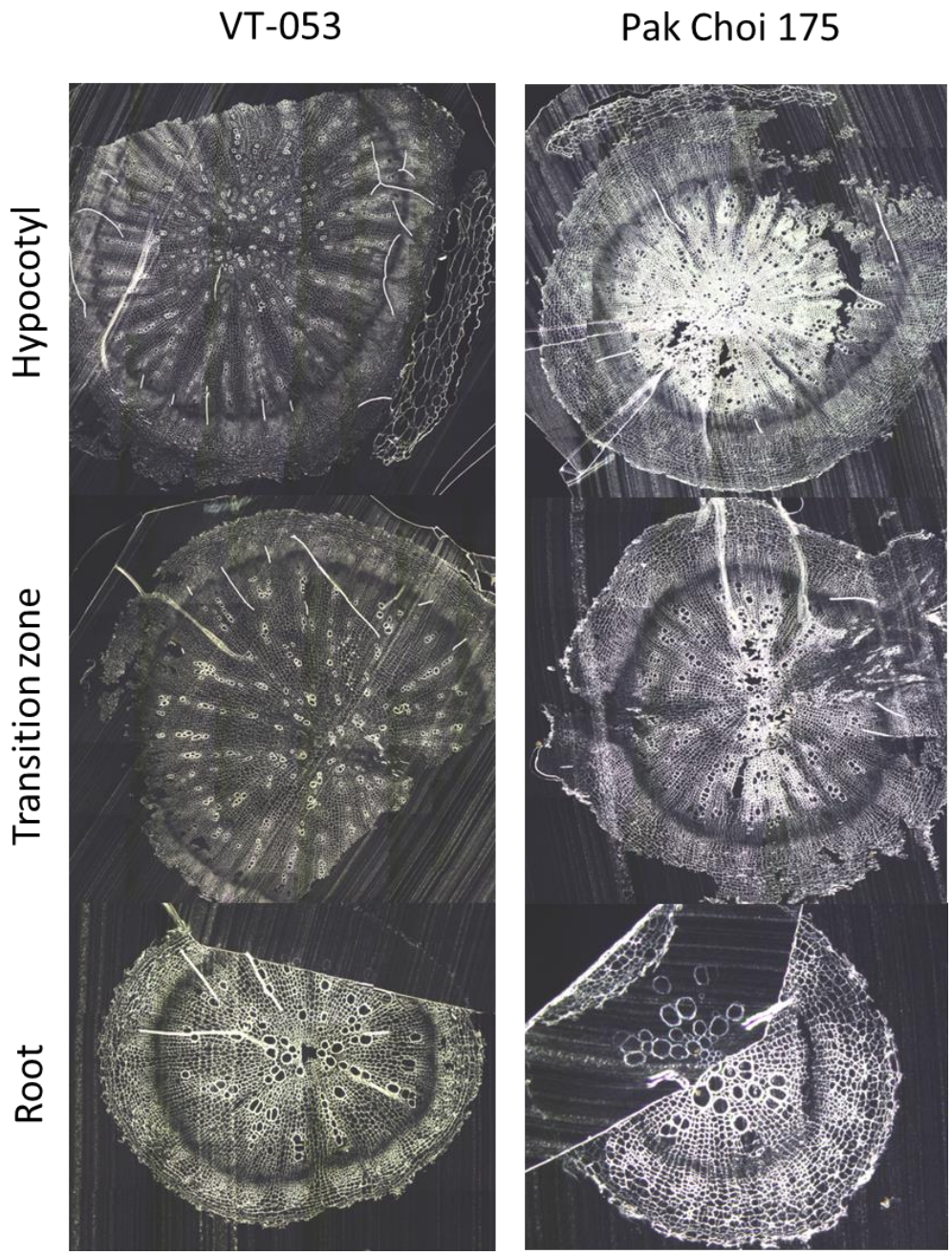


Figure 3.5: transverse sections of technovit 8100 embedded samples of hypocotyl, transition zone and root tissue of turnip DH line VT-053 and pak choi DH line PC-175. Samples are taken 24 days after sowing and sections are made with a thickness of 7 µm.

3.3.3 Quality of Technovit 8100 embedding and RNA maintenance

Embedding of plant samples in Technovit 8100 resin resulted in nice clear embedded samples. Cutting was very easy with a new sharpened D-knife and the samples were nicely attached to the Technovit 8100. No falling out of the samples was observed during cutting. Although it was difficult to stretch sections of 7 µm on the glass slides it was possible with some practise. RNA quality differed between samples (Figure 3.6). Hypocotyl sample of PC-175 seemed not well fixed and showed no intact RNA. In contrary, the transition zone and root sample did have good intact RNA. Samples of VT-053 were all fixated well, resulting in very good RNA maintenance after sectioning of hypocotyl, transition zone and root samples (Figure 3.7).

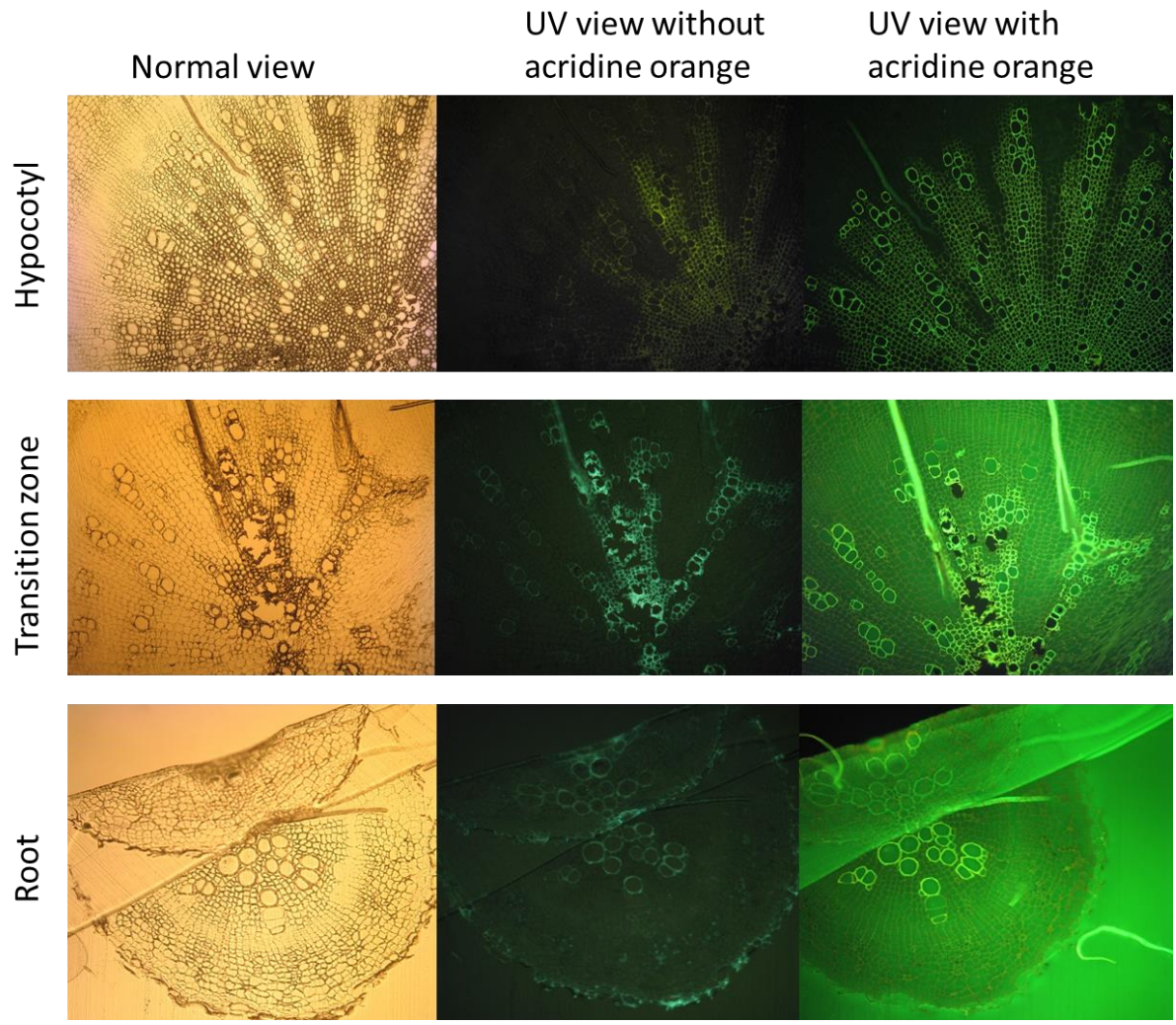


Figure 3.6: RNA maintenance check before in situ hybridization of embedded PC-175 samples of hypocotyl, transition zone and root sections. Samples are taken from plants 24 days after sowing

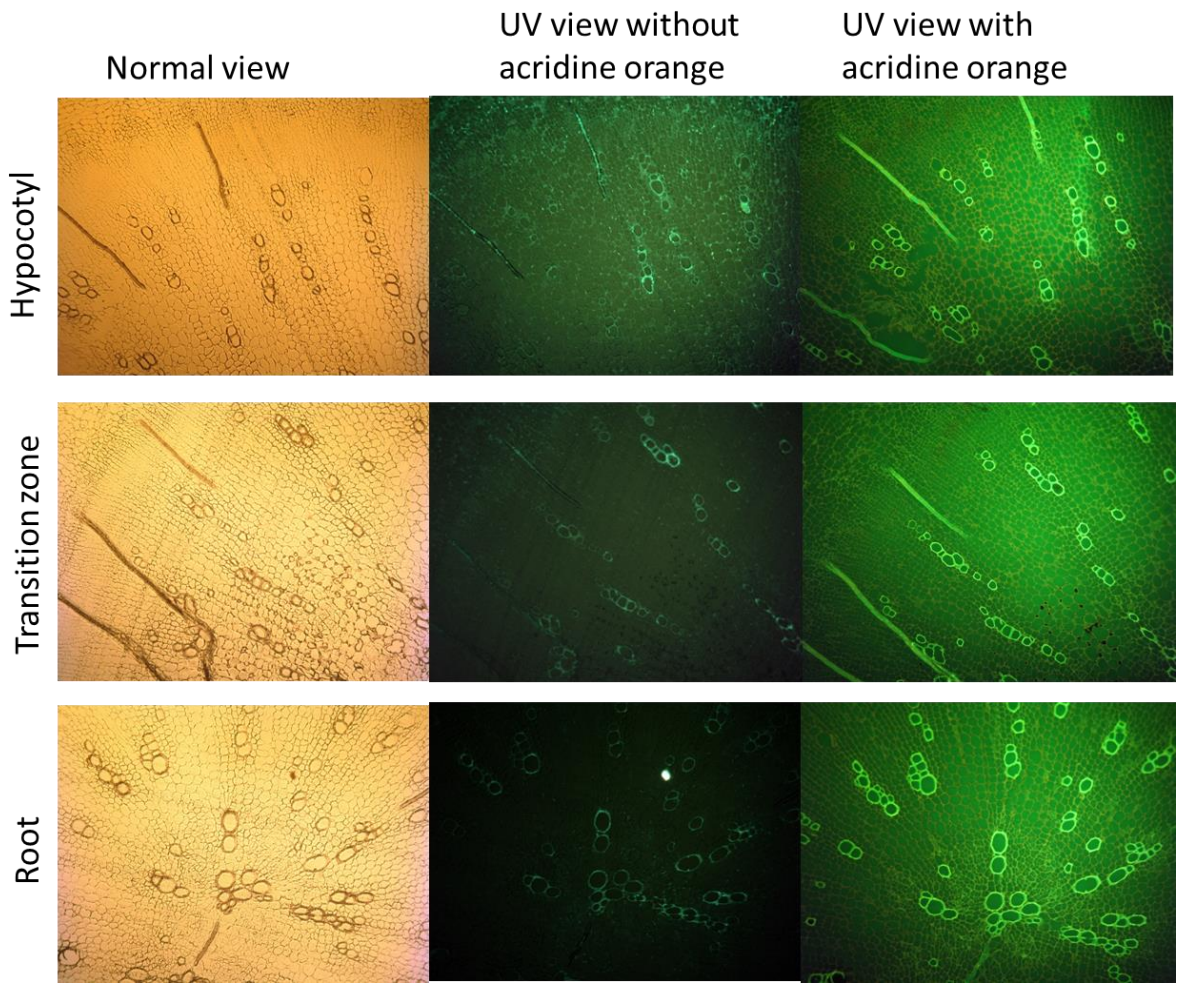


Figure 3.7: RNA maintenance check with acridine orange staining before in situ hybridization of embedded VT-053 samples of hypocotyl, transition zone and root sections. Samples are taken from plants 24 days after sowing. Red is RNA and green is DNA.

3.3.4 *in situ* hybridisation with Technovit 7100 and 8100 embedded samples

During the *in situ* hybridisation it turned out that samples of Technovit 7100 were mounted better to the slides than the Technovit 8100 where some samples were washed of or were shrinking during the washing steps. Therefore only the Technovit 7100 samples were possible to investigate under the microscope after performing the hybridization steps. This resulted in no signal of the probe.

4. Discussion

4.1 Plant development

Within the plant genotypes there was a uniform development during the different time points. Looking at only turnips, they visible turnip initiation started at 21 days after sowing except for fodder turnip FT-005. Only one out of four mature plants showed a little bit of a turnip and therefore this accession can be excluded for further turnip formation studies. The reason why this accession is not forming a turnip is unclear. This was also the reason in a previous research (Petrasch 2016a) where one genotype didn't form a turnip and it was thought to be caused by a to small pot. However, in this experiment bigger pots were used. DH lines were used, so normally this should be very uniform. Turnip formation started at the time point 21 days after sowing which is comparable to turnip development in previous studies (Petrasch 2016a).

Differences in anatomy between turnip and pak choi was clearly visible in the hypocotyl and transition zone sections of technovit 8100 samples. These sections were taken from 24 day old plants which is in accordance to the expectation. The fact that the root sections look alike between turnip and pak choi could be explained by the function of that part of the plant. Not the complete root will differentiate in turnip tissue, therefore it can be that this part of the root will also be root tissue when the turnip develops. More lignified tissue can be observed in pak choi which is in accordance to the previous embedded samples of Msc student Stefan Petrasch.

4.2 *in situ* hybridization

The aim of this research was to develop a reliable *in situ* hybridization protocol for turnip and hypocotyl tissues of young one to three week old plants of turnip and non-turnip genotypes and from there investigate the four candidate genes for their expression pattern in the different tissue at the time points when turnip formation is initiating. During this research a good reliable method for embedding samples was obtained. However, still no working *in situ* hybridization protocol was established and therefore also the candidate genes were not yet investigated for their expression pattern in turnip and non-turnip genotypes.

4.2.1 embedding and sectioning of the different Technovit resins.

As has been described in the thesis of Petrasch, 2016 Technovit was best suitable for sectioning hypocotyl and root samples of *B. rapa.* The main was that the normally used paraplast embedding was too soft for tough hypocotyl and root tissue and samples smaller than 20 µm where not possible to cut with the microtome. Stefan tested another embedding resin, the Technovit 7100 which resulted in better quality sections. The downside of this embedding resin is that it is only used for morphology studies. There are two other Technovit resins which are known to be better suitable for *in situ* hybridization studies. Therefore in this research two new Technovit resins, Technovit 8100 and Technovit 9100, were tested to find out wat which of these resin was best suitable for embedding hypocotyl and root samples.

First Technovit 9100 was tested as embedding resin. Technovit 9100 is based on methyl methacrylate (MMA) and is especially suitable for *in situ* hybridization (Singhrao et al. 2012). After several tests and different protocols most samples did not polymerize. However only one sample did polymerize properly and was suitable for sectioning. With this sample it was possible to make 5 µm thick slices

and it was possible to mount these slices on the microscope slide with 96% ethanol. Tests were done to investigate the adhesion to coated and non-coated slides. In both methods the quality and adhesion to the slides was good and the slides could be used for *in situ* hybridization. No coating was needed for these sections. There was however a big difference in quality of thickness of the sections. It turned out that 5 µm thick sections were qualitative much lower than 10 µm section both in damage and RNA maintenance/stability. Acridine orange staining showed that RNA quality in the 10 µm sections compared to the 5 µm sections was much higher and visible in the whole section. This means that if Technovit 9100 is used, sections of 10 µm are more suitable for *in situ* hybridization.

Despite the fact that the Technovit embedding of 1 sample was polymerized properly, It was not possible to find a way to repeat this. Looking at other research which uses the same embedding procedure there is no common way to do this. There are different protocols which are all different from each other. The Differences in the protocol are often based on the tissue which is used. This is related to the time of pre-infiltration and infiltration steps. The bigger the samples the longer the steps are. Often bone or teeth tissue is used in combination with Technovit 9100 (Willbold and Witte 2010; Willbold et al. 2013; Bako et al. 2015; Yang et al. 2003), but also brain tissue (Singhrao et al. 2010) and flower buds (Saito et al. 1999). Another difference between the protocols was the polymerization temperature ranges from -20 to +45 °C and the polymerization time ranges from overnight incubation till one week incubation. According to the official Technovit 9100 protocol polymerization time depends on sample size and polymerization temperature, which ranges between -8 and -20 °C. Another possible cause of polymerization failure can be oxygen which reacts with the Technovit 9100. Air contact turned out to be a bottle neck and often special airtight molds were used for polymerisation (Willbold and Witte 2010; Saito et al. 1999; Bako et al. 2015). Unfortunately these molds can only be ordered from one company in Australia which will take more than a month before it could be delivered. Therefore it was not possible to test the Technovit 9100 with these special molds. A big disadvantage of Technovit is the aggressive nature of the resin. The solution reacts to almost all materials and the smell is very strong. Handling of the different solutions must be done always in the flow cabinet and even after polymerization the smell is still present when making sections.

Technovit 8100 which is based on Glycol methyl methacrylate (GMA), the same resin system as the Technovit 7100 was also tested as embedding resin. The difference between Technovit 7100 and 8100 is that according to Takechi et al., 1999 the Technovit 8100 is suitable for *in situ* hybridization and Technovit 7100 is not because of the dense structure of the resin. In contrary to the Technovit 9100 the protocol of Technovit 8100 is more straight forward and easy to perform. Samples of hypocotyl, transition zone and root tissue were properly embedded with uniform quality. Also there is no strong smell and although the polymerization must be performed in the flow cabinet, the solution is less toxic and reactive.

Slices of Technovit 8100 need to be mounted on the microscopy slides with sterile water in order to proper stretch the samples. If ethanol is used no good samples can be obtained. Stretching will go more easily with water on room temperature compared to warm water. Also it is very important to secure the samples very well to the microtome block by turning the block till the sample cannot move anymore. otherwise the chance of breaking the embedded sample is very high. Best samples were obtained by slowly rotating the microtome manually. Slices need to be placed on the slide as soon as possible before it gets rolled up completely. The use of a new sharpened D-knife is

recommendable. Together with slides containing the newly embedded Technovit 8100 samples also slides with Technovit 7100 samples from Stefan Petrasch were made. This time all slices were 7 µm thick resulting in good quality slices for both the Technovit 7100 as the Technovit 8100 samples. This is 3 µm thinner than previous research (Petrasch 2016a)which is better for *in situ* hybridization, because of the smaller number of cell layers. Acridine orange staining showed that the RNA is intact in almost all samples tested. Only in hypocotyl samples of PC-175 the RNA was not intact in all three replicates which can mean that there was not a proper fixation. It is better to exclude these samples from the experiment, because without intact RNA there will never be a signal.

4.2.3 hybridization

The *in situ* hybridization protocol was conducted with the Technovit 9100 sample that was polymerized properly. The first step was to remove the plastic/technovit leaving only the turnip sections on the slide. This worked very well using the standard Technovit 9100 protocol. After the washing steps only the samples were attached to the microscope slide and all the plastic was washed off. Unfortunately there was no signal after the complete *in situ* hybridization process. It is difficult to pin point the steps which went wrong. It is very likely that there is something wrong with the hybridization of the probe to the gene of interest, because a Dot blot analysis was performed with the probes resulting in no signal at all, even for the control labelled probe from the company itself. The first thing that must be solved is getting a signal with the Dot blot analysis and from there continuing the *in situ* hybridization protocol.

A second *in situ* hybridization was performed with Technovit 7100 and 8100 samples. This resulted in shrinking or floating off of all Technovit 8100 which indicates no proper adherence to the slides. This was not the case for Technovit 7100 samples. This difference could be explained by the baking time of the slices to the slides. The Technovit 7100 samples were placed at 37 ˚C for three days whereas the Technovit 8100 samples were only placed at 37 ˚C overnight. Because it seemed that adherence to the normal Superfrost Ultra Plus slides was sufficient no coating was used. In future experiment it is interesting to investigate the adherence with 0.1% BSA coating. This can be done by dipping the slides in the 0,1 BSA solution and let it air dry. No results were obtained with the remaining Technovit 7100 slides. Comparing this with the Technovit 9100 *in situ* hybridization experiment also here the probe could be the causal problem. The *in situ* hybridization protocol of the Dolf Weijers laboratory (appendix 1) was used. This protocol is made for paraffin embedded samples and is very detailed. There is also a less detailed protocol based specifically for Technovit 8100 embedded samples (Takechi et al. 1999).

4.3 Conclusion and recommendations

The best method for embedding fixed hypocotyl and root samples of *B. rapa* was done with Technovit 8100. This method turned out to be most easy with good embedding results. It is also more save and less aggressive and toxic compared to the Technovit 9100 resin. Unfortunately, no reliable protocol regarding the *in situ* hybridization was conducted, so studying the expression pattern of the different candidate genes was not possible. Still some aspects need to be improved before results can be obtained. The first and most important thing is to check whether the probes are of good quality or not using the Dot blot test. Secondly, the adherence of the slides must be improved. This can be done by elongate the baking time at 37 ˚C or by coating the slides with 0,1% BSA solution. Because Technovit 7100 slices were proper mounted to the slides and Technovit 8100 is based on the same plastic polymere this problem should be solved. Another recommendation is to

use the protocol of Takechi et al. 1999 after sectioning. The signal detection should than be done using the Roche DIG DNA Labeling and Detection kit protocol. If this will lead to no results another possibility is to order the special Technovit 9100 embedding molds and try to avoid air contact and obtain nice embedded samples. This is likely the reason that the samples were not proper embedded.

Literature

Bako, P., M. Bassiouni, A. Eckhard, I. Gerlinger, C. Frick, H. Löwenheim, and M. Müller. 2015. Methyl methacrylate embedding to study the morphology and immunohistochemistry of adult guinea pig and mouse cochleae. *Journal of Neuroscience Methods* 254: 86-93. http://dx.doi.org/10.1016/j.jneumeth.2015.07.017.

Bassetti, N. 2015. *Genome-wide transcriptomic profiling of turnip (Brassica rapa ssp. rapa) during tuber development*. [S.l.: s.n.].

Bonnema, A. B., D. Pino del Carpio, and J. Jianjun Zhao. 2011. "Diversity analysis and molecular taxonomy of Brassica vegetable crops." In *Genetics, Genomics and Breeding of Vegetable Brassicas*, 81-124. Enfield, USA: Science Publishers.

Bustos-Sanmamed, P., G. Mao, Y. Deng, M. Elouet, G. A. Khan, J. Bazin, M. Turner, et al. 2013. Overexpression of miR160 affects root growth and nitrogen-fixing nodule number in Medicago truncatula. *Functional Plant Biology* 40 (12): 1208-1220. http://dx.doi.org/10.1071/fp13123.

Cardon, G. H., S. Höhmann, K. Nettesheim, H. Saedler, and P. Huijser. 1997. Functional analysis of the Arabidopsis thaliana SBP-box gene SPL3: A novel gene involved in the floral transition. *Plant Journal* 12 (2): 367-377. http://dx.doi.org/10.1046/j.1365-313X.1997.12020367.x.

Cheng, F., R. Sun, X. Hou, H. Zheng, F. Zhang, Y. Zhang, B. Liu, et al. 2016. Subgenome parallel selection is associated with morphotype diversification and convergent crop domestication in Brassica rapa and Brassica oleracea. advance online publication.

Cheng, F., J. Wu, and X. Wang. 2014. Genome triplication drove the diversification of Brassica plants. 1: 14024.

Eviatar-Ribak, T., A. Shalit-Kaneh, L. Chappell-Maor, Z. Amsellem, Y. Eshed, and E. Lifschitz. 2013. A cytokinin-activating enzyme promotes tuber formation in tomato. *Current Biology* 23 (12): 1057-1064. http://dx.doi.org/10.1016/j.cub.2013.04.061.

Gupta, A. K., J. Singh, and N. Kaur. 2001. Sink development, sucrose metabolising enzymes and carbohydrate status in turnip (Brassica rapa L.). *Acta Physiologiae Plantarum* 23 (1): 31-36.

Gutierrez, L., J. D. Bussell, D. I. Păcurar, J. Schwambach, M. Păcurar, and C. Bellini. 2009. Phenotypic plasticity of adventitious rooting in arabidopsis is controlled by complex regulation of AUXIN RESPONSE FACTOR transcripts and microRNA abundance. *Plant Cell* 21 (10): 3119-3132. http://dx.doi.org/10.1105/tpc.108.064758.

Kiba, T., K. Takei, M. Kojima, and H. Sakakibara. 2013. Side-Chain Modification of Cytokinins Controls Shoot Growth in Arabidopsis. *Developmental Cell* 27 (4): 452-461. http://dx.doi.org/10.1016/j.devcel.2013.10.004.

Kubo, N., M. Saito, H. Tsukazaki, T. Kondo, S. Matsumoto, and M. Hirai. 2010. Detection of quantitative trait loci controlling morphological traits in Brassica rapa L. *Breeding Science* 60 (2): 164-171. http://dx.doi.org/10.1270/jsbbs.60.164.

Lou, P., J. Zhao, J. S. Kim, S. Shen, D. P. Del Carpio, X. Song, M. Jin, et al. 2007. Quantitative trait loci for flowering time and morphological traits in multiple populations of Brassica rapa. *Journal of Experimental Botany* 58 (14): 4005-4016. http://dx.doi.org/10.1093/jxb/erm255.

Lu, G., J. Cao, X. Yu, X. Xiang, and H. Chen. 2008. Mapping QTLs for root morphological traits in Brassica rapa L. based on AFLP and RAPD markers. *Journal of Applied Genetics* 49 (1): 23-31.

Nishijima, T., H. Sugii, N. Fukino, and T. Mochizuki. 2005. Aerial tubers induced in turnip (Brassica rapa L. var. rapa (L.) Hartm.) by gibberellin treatment. *Scientia Horticulturae* 105 (4): 423-433. http://dx.doi.org/10.1016/j.scienta.2005.02.005.

Petrasch, S. 2016a. *development of RNA in situ hybridization and grafting methods in brassica rapa to facilitate studies of early events in turnip formation*. Minor thesis.

———. 2016b. *Temporal and spatial gene expression during Turnip Formation in Brassica rapa*. Master thesis.

Prakash, S., X. M. Wu, and S. R. Bhat. 2011. History, evolution, and domestication of Brassica crops. In *Plant Breeding Reviews*.

Ramireddy, E., L. Chang, and T. Schmülling. 2014. Cytokinin as a mediator for regulating root system architecture in response to environmental cues. *Plant Signaling and Behavior* 9 (FEB). http://dx.doi.org/10.4161/psb.27771.

Saito, C., M. Hayashi, A. Sakai, M. Fujie, H. Kuroiwa, and T. Kuroiwa. 1999. Improved sensitivity for high resolution in situ hybridization using resin extraction of methyl methacrylate embedded material. *Biotechnic and Histochemistry* 74 (1): 40-48.

Sanz, L., W. Dewitte, C. Forzani, F. Patell, J. Nieuwland, B. Wen, P. Quelhas, et al. 2011. The Arabidopsis D-Type Cyclin CYCD2;1 and the Inhibitor ICK2/KRP2 Modulate Auxin-Induced Lateral Root Formation. *Plant Cell* 23 (2): 641-660. http://dx.doi.org/10.1105/tpc.110.080002.

Singhrao, S. K., K. Nicholson, and S. Crean. 2012. Informed choices for challenging specimens when choosing methacrylate resin systems for histology. *Microscopy Research and Technique* 75 (5): 576-585. http://dx.doi.org/10.1002/jemt.21094.

Singhrao, S. K., A. J. Sloan, E. L. Smith, and C. W. Archer. 2010. Technical advances in the sectioning of dental tissue and of on-section cross-linked collagen detection in mineralized teeth. *Microscopy Research and Technique* 73 (8): 741-745. http://dx.doi.org/10.1002/jemt.20815.

Song, K., T. C. Osborn, and P. H. Williams. 1990. Brassica taxonomy based on nuclear restriction fragment length polymorphisms (RFLPs) - 3. Genome relationships in Brassica and related genera and the origin of B. oleracea and B. rapa (syn. campestns). *Theoretical and Applied Genetics* 79 (4): 497-506. http://dx.doi.org/10.1007/bf00226159.

Stewart, C. N., M. D. Halfhill, and S. I. Warwick. 2003. Transgene introgression from genetically modified crops to their wild relatives. 4 (10): 806-817.

Sun, Y., Y. Qiu, X. Zhang, X. Chen, D. Shen, H. Wang, and X. Li. 2015. Genome-wide identification of microRNAs associated with taproot development in radish (Raphanus sativus L.). *Gene* 569 (1): 118-126. http://dx.doi.org/10.1016/j.gene.2015.05.044.

Takechi, K., W. Sakamoto, M. Katsuhara, M. Murata, and F. Motoyoshi. 1999. In situ RNA hybridization using Technovit resin in Arabidopsis thaliana. *Plant Molecular Biology Reporter* 17 (1): 43-51.

Torti, S., F. Fornara, C. Vincent, F. Andrés, K. Nordström, U. Göbel, D. Knoll, H. Schoof, and G. Coupland. 2012. Analysis of the Arabidopsis Shoot Meristem Transcriptome during Floral Transition Identifies Distinct Regulatory Patterns and a Leucine-Rich Repeat Protein That Promotes Flowering. *The Plant Cell* 24 (2): 444-462. http://dx.doi.org/10.1105/tpc.111.092791.

Wang, X., H. Wang, J. Wang, R. Sun, J. Wu, S. Liu, Y. Bai, et al. 2011. The genome of the mesopolyploid crop species Brassica rapa. *Nature Genetics* 43 (10): 1035-1040. http://dx.doi.org/10.1038/ng.919.

Willbold, E., M. Reebmann, R. Jeffries, and F. Witte. 2013. Electrochemical removal of metallic implants from Technovit 9100 New embedded hard and soft tissues prior to histological sectioning. *Histochemistry and Cell Biology* 140 (5): 585-593. http://dx.doi.org/10.1007/s00418-013-1089-6.

Willbold, E., and F. Witte. 2010. Histology and research at the hard tissue-implant interface using Technovit 9100 New embedding technique. *Acta Biomaterialia* 6 (11): 4447-4455. http://dx.doi.org/10.1016/j.actbio.2010.06.022.

Yang, R., C. M. Davies, C. W. Archer, R. G. Richards, H. Plenk, P. Rooney, and J. Gasser. 2003. Immunohistochemistry of matrix markers in Technovit 9100 New®-embedded undecalcified bone sections. *European Cells and Materials* 6: 57-71.

Yu, N., Q. W. Niu, K. H. Ng, and N. H. Chua. 2015. The role of miR156/SPLs modules in Arabidopsis lateral root development. *Plant Journal* 83 (4): 673-685. http://dx.doi.org/10.1111/tpj.12919.

Zhang, N., J. Zhao, F. Lens, J. De Visser, T. Menamo, W. Fang, D. Xiao, et al. 2014. Morphology, carbohydrate composition and vernalization response in a genetically diverse collection of

Asian and European turnips (Brassica rapa subsp. rapa). *PLoS ONE* 9 (12). http://dx.doi.org/10.1371/journal.pone.0114241.

Zhao, J., X. Wang, B. Deng, P. Lou, J. Wu, R. Sun, Z. Xu, et al. 2005. Genetic relationships within Brassica rapa as inferred from AFLP fingerprints. *Theoretical and Applied Genetics* 110 (7): 1301-1314. http://dx.doi.org/10.1007/s00122-005-1967-y.

Appendix 1: RNA in situ hybridization protocol for Arabidopsis embryos

Based on the Jeff Long *in situ* protocol and adapted by Shunsuke Saiga. Reference for this protocol:

SAIGA, S., FURUMIZU, C., YOKOYAMA, R., KURATA, T., SATO, S., KATO, T., TABATA, S., SUZUKI, M. & KOMEDA, Y. 2008. The Arabidopsis OBERON1 and OBERON2 genes encode plant homeodomain finger proteins and are required for apical meristem maintenance. *Development,* 135**,** 1751-1759.

Jeff Long protocol: http://pbio.salk.edu/pbiol/in_situ_protocol.html

Fixating and sectioning of tissue

Fixation of tissue

1. Collect siliques at the stage required from the desired plants.
2. Prepare the fixation (4% PFA) solution:

Fixation solution	50 ml tube	
PFA	2 g	Dissolve at 70°C
Sterilized MQ	Fill to 43 ml	
1M NaOH	50 µl	
10xPBS	5 ml	Add after cooling down
Dimethyl sulfoxide (DMSO)	2 ml	Add after cooling down

3. Cut the siliques in pieces of about 1 cm. Cut both sides of the siliques open to allow the fixative to easily enter the tissue. Place the cut pieces in a sterilized vial on ice with about 10 ml of ice cold fixative.
4. Place the glass containers, while still on ice, in a near vacuum for 15-30 minutes.
5. Repeat the previous step if the siliques have not sunk to the bottom.
6. Replace the fixation solution with fresh fixative and place overnight at 4 °C.
7. Replace the fixation solution with subsequently, all at 4 °C
    a. 1xPBS (30 minutes)
    b. 1xPBS (30 minutes)
    c. 30%EtOH (60 minutes)
    d. 40% EtOH (60 minutes)

e. 50% EtOH (60 minutes)

f. 60% EtOH (60 minutes)

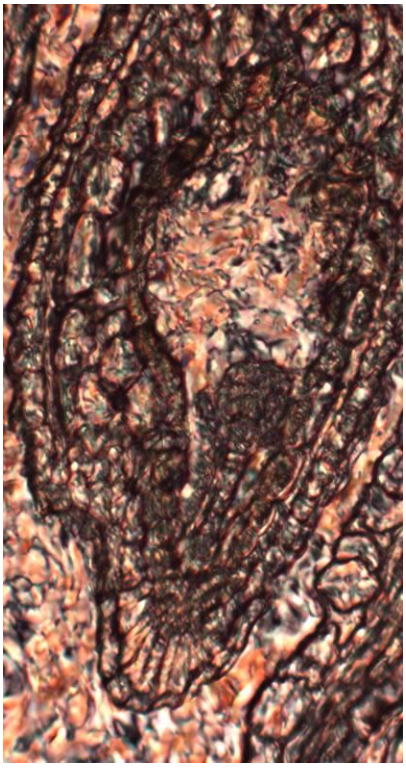
g. 70% EtOH (60 minutes)

8. *Note: t*he tissue can be stored in 70% EtOH for several months at 4 °C.
9. Replace the 70% EtOH with subsequently:
    a. 85% EtOH (60 minutes) at 4 °C
    b. 96% EtOH (overnight) at 4 °C
    c. 100% EtOH (30 minutes) at room temperature
    d. 100% EtOH (30 minutes) at room temperature
    e. 100% EtOH (60 minutes) at room temperature
    f. 100% EtOH (60 minutes) at room temperature
10. Replace the 100% EtOH with subsequently, all at room temperature in the fume hood:
    a. 25% xylene (1 hour)
    b. 50% xylene (1 hour)
    c. 75% xylene (1 hour)
    d. 100% xylene (1 hour)
    e. 100% xylene (1 hour)
    f. 100% xylene with 10 to 20 paraffin chips (overnight)
11. Melt paraffin chips at 63 °C overnight
12. Replace the 100% xylene with melted paraffin. Doing this next to an open flame, in a warm environment will make this easier. Keep at 63 °C.
13. Replace the melted paraffin with fresh after several hours. Repeat this wax change for two more days, twice a day.
14. Preheat the plastic base molds and their covers at 63 °C.
15. Prepare a heat block covered in aluminium foil on which a mold can be placed while it is being handled.
16. Using a glass Pasteur pipette with the end cut off pipet three siliques into the mold. Fill the mold with more melted paraffin.

17. Using a heated needle place the siliques aligned at the bottom of the mold.
18. Place a mold cover on top of the mold, fixate with more paraffin and let slowly set.
19. Store at 4 °C until use.

Sectioning tissue

Make 8 µm sections of siliques and transfer a ribbon of film (several sections attached to each other) into a 45˚C water bath. Put them on a superfrost slide. After each slide (two ribbons per slide) check for the presence of embryos in the sections by DIC optics. At least 3 good embryos should be present per slide. “Bake” the slides overnight at 45°C and store at 4°C until use. It is advisable to use a new section of the microtome blade every one to two blocks.



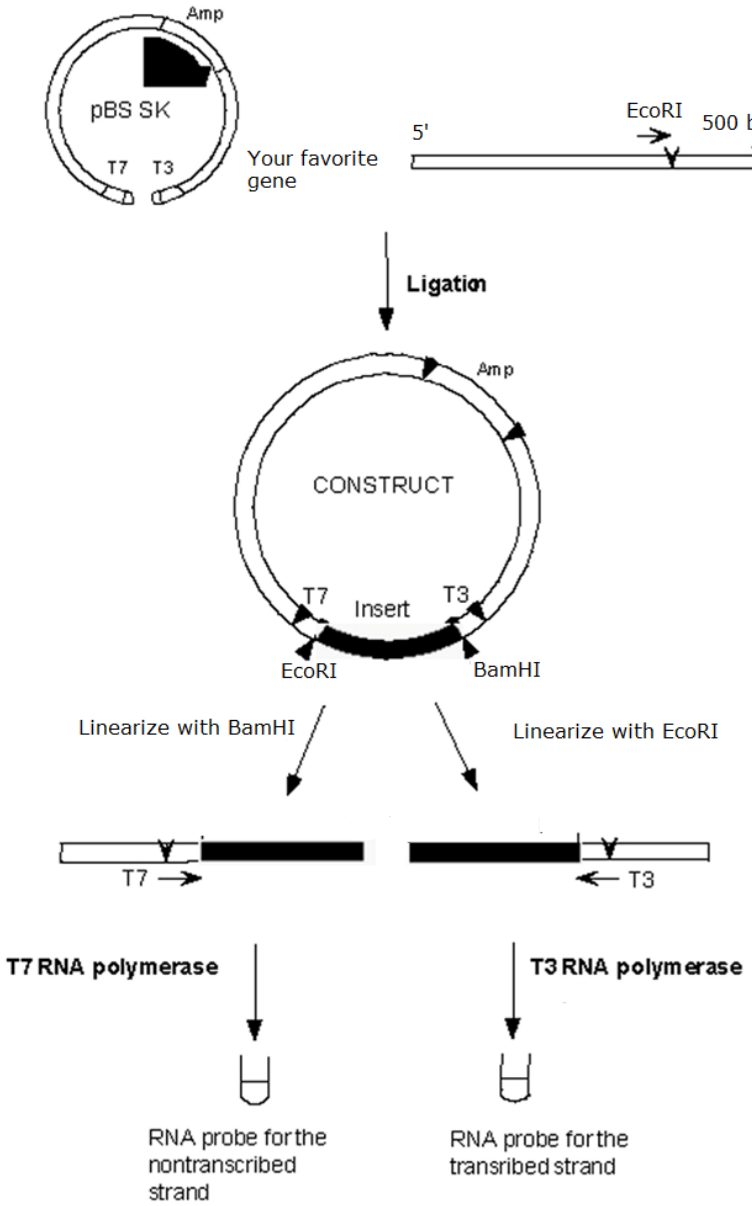
Preparing riboprobe

Probes can be designed for any gene of sufficient length. Some guidelines:

- Design from the cDNA to exclude introns.
- A length of about 500 bp is optimal.
- Check for homology of your probe. RNA probes do not need a 100% basepair match to bind so a close homolog of your gene will interfere.
- Check that no EcoRI or BamHI sites are present within the sequence as this will interfere with the cloning.

Design the primers with an EcoRI site at the start of the forward primer and a BamHI site at the end of the reverse primer. This allows for cloning into the pBS SK(+) vector and later for linearizing the plasmid. You can also use other 3' sticky and blunt end restriction enzyme available within the pBS SK(+) multi-cloning site

After linearizing the riboprobe can be synthesized using the Roche T3 RNA Polymerase by following their protocol. Use a dNTP mix including dig labeled nucleotides. Keep in mind that for detecting the mRNA the reverse complement (anti sense or non transcribed strand) should be synthesized! See also the picture below.



In situ hybridization

General notes and precautions

- The glass containers and their covers are easily broken. Handle them with care.

- Use gloves on any material to prevent contamination with RNAses. The use of filter tips is also advisable. After the washing step of post-hybridization this is no longer necessary.
- When working with Xylene: Always work in the fume hood and wear a double pair of gloves.

Day 1 - Preparations

1. Wash 35 glass containers, their glass covers and the slider holder using the dish washer. Place them tightly within a metal rack to prevent the washing machine from cracking them. Make sure the containers are completely dry before moving on to the next step.
2. Place in an oven at a minimum of 120°C overnight (or a minimum of 4 hours):
    a. 35 glass containers and their covers
    b. 1 slide holder wrapped in foil
    c. 1 glass cylinder of 200ml
    d. 2 magnet stirrers wrapped in foil

Day 2 – Preparations

1. Allow the oven with glassware to cool until the containers can be handled.
2. Cut two parafilm strips per slide of exactly the dimensions of your slides (2.5 cm x 5.7 cm). These are for covering the slides when the probe and antibody is being applied.
3. Prepare a square plastic petri dish per three slides. Put a layer of towel inside and on top two rows of PCR tube lids (1.5 times 8 lids works best). These are for incubating the slides while hybridizing. The lids are for elevating the slides above the towels.
4. Prepare the following solutions and autoclave them. Note that some of the ingredients are not added until after autoclavation or just before use. Ingredients which are added later on are also reminded of in their respective steps in the protocol.
5. Bottles needed: 5x 1 L, 2x 500 mL, 2x 250 mL, 1x 500 mL flask, 1x plastic beaker >300 mL.

2xSSC (300 ml)	300 ml bottle	100 ml is used for 0.2xSSC
NaCl	5.26 g (300 mM)	
Trisodium Citrate Dihydrate	2.65 g (15 mM)	
MQ	Fill to 300 ml	

0.2xSSC (1000ml)	1000 ml bottle	
2xSSC	100 ml	
MQ	900 ml	

Proteinase K buffer (200ml)	250 ml bottle	
1M Tris-HCL (pH 7.5)	20 ml (100mM)	
0.5M EDTA (pH 8.0)	20 ml (50mM)	
Proteinase K (10 mg/ml)	20 µl	Add just before use.
MQ	160 ml	

10xPBS (300ml)	Plastic beaker	All is used for 1XPBS
NaCl	22.79 g (1.3M)	
Na_2HPO_4	2.98 g (70mM)	Do not confuse these two and check for correct hydration
$NaH_2PO_4 \cdot H_2O$	1.24 g (30mM)	
MQ	Fill to 300 ml	

1xPBS (1000ml)	1000 ml bottle	Prepare 3 L
10xPBS	100 ml	
MQ	900 ml	

4% PFA (200 ml)	250 ml bottle	
MQ	180 ml	
1N NaOH	200 µl	Add just before use, keep in 70°C to dissolve (clear solution), cool on ice
Paraformaldehyde (PFA)	8 g	
10xPBS	20 ml	Add after cooling down

Blocking buffer (200 ml)	500 ml Erlenmeyer	
1M Tris-HCL (pH 7.5)	20 ml	
5M NaCl	6 ml	
MQ	174 ml	
Blocking reagent	1 g	Add before use and dissolve in microwave. Solution remains thurbid

buffer A (1000 ml)	1000 ml bottle	
1M Tris-HCl (pH 7.5)	100 ml	Autoclave with stirring magnet
5M NaCl	30 ml	
MQ	857 ml	
BSA	10 g	Add before use, stir
Triton X-100	3 g	

buffer B (400 ml)	500 ml bottle	Can precipitate
1M Tris HCl (pH 9.5)	40 ml	Different pH!
5M NaCl	8 ml	
MQ	332 ml	
1M $MgCl_2$	20 ml	

Day 3 – Pre hybridization

NOTE: start at 12.00-13.00 with step 8 to finish around 18.00.

1. Add PFA and NaOH to the 4% PFA solution and heat the bottle in an oven to 70°C until the solution turns completely clear. A few undissolved PFA particles will remain. Cool down on ice and add 10xPBS.
2. Prepare the wetting agent for hybridization. This is used to wet the towels in the prepared square petri dishes. About 3 to 5 ml is used per petri dish.

Wetting agent	50 ml
Formamide	25 ml
5M NaCl	3 ml
MQ	22 ml

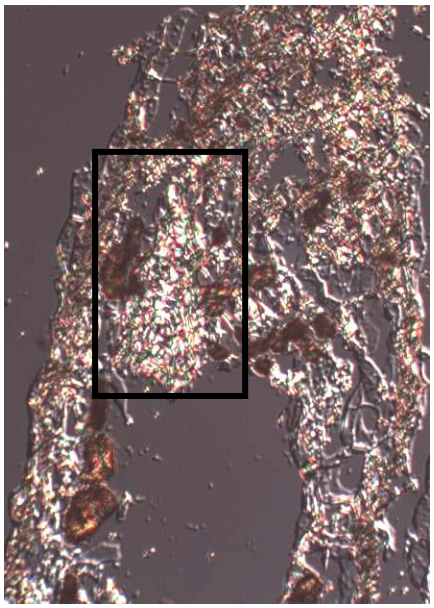
3. If you have not already done so, write names on the slides using pencil and place them in the glass holder with the bottom sides of the slides facing each other (for 20 sildes). *NOTE: do not use a marker or pen as it will wash away in subsequent steps*.
4. Setup the content of the glass containers as in the table below. Each container should contain about 200 ml of the solution. For easy measuring the 2XSSC container can be filled first and used as reference for filling other containers.
5. Use the autoclaved glass cylinder to measure 180 ml of autoclaved MQ for the triethanolamine container and 200 ml of PBS for the glycine/PBS container.
6. Add the autoclaved stirrer bars to the glycine/PBS and triethanolamine container.
7. Place the proteinase K buffer container in a 37 °C stove.
8. Follow the instructions in the table below, moving the slide holder from solution to solution. Let as much of the previous solution drip from the holder as possible before moving to the new solution and move up and down three times.

Solution	**Time**	**Description**
Xylene (1)	10 min	Move holder up and down every 2 minutes. This step removes the paraffin. *Caution: Xylene is toxic; use fume hood. Wear double gloves.*
Xylene (2)	10 min	Move holder up and down every 2 minutes. This step removes the paraffin. *Caution: Xylene is toxic; use fume hood. Wear double gloves.*
100% EtOH (1)	1 min	Still in fume hood.
100% EtOH (2)	1 min	The next steps hydrate the sample.
96% EtOH (1)	1 min	
90% EtOH (1)	1 min	
80% EtOH (1)	1 min	
60% EtOH (1)	1 min	
30% EtOH (1)	1 min	

H_2O (1)	1 min	
2XSSC	15 min	
	Preparations	Thaw the 10 mg/ml Proteinase K stock solution.
H_2O (2)	Wash briefly	
Proteinase K	30 min @ 37°C	Add 20 µl of Proteinase K to the preheated buffer solution. Then add the slides and keep at 37°C. *Caution: Do not contaminate anything with Proteinase K*. **This is a critical step that makes the mRNA accessible for the probe.**
	Preparations	Dissolve 0.4 g of glycine in the Glycine/PBS container using the magnet stirrer. Dissolve 3.7 g of triethanolamine and 1.24ml of 10N NaOH in the triethanolamine container using the magnet stirrer.
PBS (1)	1 min	
Glycine/PBS	2 min	This step stops the proteinase K reaction.
PBS (2)	2 min	
PBS (3)	2 min	
4% PFA	10 min	This step re-fixates the sample to the slide.
PBS (4)	2 min	
PBS (5)	2 min	
Triethanolamine	10 min	While still stirring vigorously using the magnet stirrer add 450 µl of acetic anhydride, wait 6 seconds, stop stirring, and then add the slides. **This is a critical step that acetylates the sample and decreases background signal by decreasing aspecific binding of the probe.**
PBS (6)	2 min	
PBS (7)	2 min	
30% EtOH (1)	1 min	The next steps dehydrate the sample.
60% EtOH (1)	1 min	
80% EtOH (1)	1 min	
90% EtOH (1)	1 min	

96% EtOH (1)	1 min	
100% EtOH (3)	1 min	

9. Take the slide holder from the solution. The steps until the applying of the probe (last step of this day) should be done as quick as possible, preferably in about an hour. This to prevent over drying of the slides.
10. At this point the slides can be evaluated under the microscope quickly for their quality of the embryos present on the slides and the probes to be used for each slide. The embryos can be difficult to recognize at this point.



Day 3 - Hybridization

1. Thaw 50% dextran sulfate and denhardt's solutions. Too cold dextran sulfate is too viscous to pipet. The solution can be placed in the preheating oven at 45°C to make pipetting easier.
2. Preheat two heat blocks at 45°C and 80°C and an oven for overnight use at 45°C.
3. Use the wetting agent to wet the towels in the prepared square plastic petri dishes.
4. Prepare the hybridization solution, mix by vortex, and dispense it over 1.5 ml tubes, one per slide. Keep the tubes at 45°C. Prepare for one extra slide as the solution will be very viscous and thus more will be lost during dispensing. Pipet the viscous solutions as slow as possible.

Hybridization solution	160 µl (1 slide)	1760 µl (10 slides + 1)	3360 µl (20 slides + 1)
Sterile water	16 µl	176 µl	336 µl
50% dextran sulfate	40 µl	440 µl	840 µl
10x in situ salts	20 µl	220 µl	420 µl
Formamide	80 µl	880 µl	1680 µl
50x denhardt's	4 µl	44 µl	84 µl

5. Prepare the probe solution. One 1.5 ml tube per probe. Heat shock the probe solution at 80°C for 2 minute, spin down, and then immediately move to ice.

Probe solution	40 µl (1 slide)	100 µl (2 slides + 0.5)	140 µl (3 slides + 0.5)
100 mg/ml tRNA	2 µl	5 µl	7 µl
Formamide	20 µl	50 µl	70 µl
probe	0.2 µl	0.5 µl	0.7 µl
Sterile water	17.8 µl	44.5 µl	62.3 µl

6. Take 40 µl of probe solution and mix it by pipetting up and down with one tube of hybridization solution. Take the now 200 µl and apply it to the slide without causing bubbles.
7. Take a prepared parafilm strip and cover the slide taking care of any bubbles that may form.
8. Place the slides on top of the PCR tube lids elevated above the wet towels in the square petri dishes. Take care the slides do not touch each other.
9. Close the petri dishes using parafilm to prevent them from drying out.
10. Place the square petri dishes in an oven at 45°C for 16 hrs.
11. Preheat 1L of 0.2x SSC at 55°C

Day 4 – Post hybridization

NOTE: Start at 9.00 with step 5 to start the colouring (step 7) at 16.00.

1. Mix blocking reagent to the blocking buffer by heating it for +- 2 minutes in the microwave, then let it cool down. The solution will remain turbid/cloudy.
2. Mix the BSA and Triton-x-100 in buffer A using the magnet stirrer.
3. Preheat an oven at 55°C.
4. Prepare the content of the glass containers. Each container should be filled with 200 ml of the solution. Prepare:

a. 3 for 0.2 SSC (55°C)
b. 1 for 1 x PBS
c. 1 for blocking solution
d. 4 for buffer A
e. 1 for buffer B. *NOTE:* buffer B can have a precipitate from the MgCl. This is no problem.

5. Place the slides including parafilm strips back on the slide holder. Keep the petri dishes as they will be used later on.

0.2x SSC (1)		Wash off parafilm. *Caution: do not pull the parafilm of the slides as this will damage the embryos, instead move the slider holder up and down until the parafilm floats off.*
0.2x SSC (2)	1 hr @ 55˚C	Move the slide holder up and down every 15 minutes.
0.2x SSC (3)	1 hr @ 55˚C	Move the slide holder up and down every 15 minutes.
1x PBS	5 min	
Blocking solution	45 min	Move the slide holder up and down every 15 minutes (or incubate on a rocking platform ±90 rpm).
Buffer A (1)	45 min	Move the slide holder up and down every 15 minutes (or incubate on a rocking platform).

6. Dilute 19 µl of anti-DIG antibody in 19 ml of buffer A (for 20 slides). Mix by swirling. Apply 900 µl of the solution to each slide. Apply a prepared parafilm strip on each slide. *NOTE*: This will spill. Place the slides in the petri dish boxes with wet towels. (Old petri dishes, new towels, wet with Buffer A) Keep for **1 hour at room temperature**.

7. Place the slides back on the slide holder.

Buffer A (1)	20 min	Wash off parafilm and incubate (on a rocking platform).

Buffer A (2)	20 min	On a rocking platform
Buffer A (3)	20 min	On a rocking platform
Buffer A (4)	20 min	On a rocking platform
Buffer B	Briefly	

8. Dilute 300 µl of NBT/BCIP in 15 ml of buffer B. Prepare 15 ml per 5 slides.
9. Place the slides in a new square petri dish and pour in the NBT/BCIP solution until the slides are just covered. When 5 slides are in petri dish about 15 ml NBT/BCIP solution is required to cover the slides. Wrap the petri dishes in aluminum foil to keep them in the dark. Place at 37°C.
10. Evaluate the coloring of the embryo every hour under the microscope. Be careful not to let the slides dry out while they are under the microscope.
11. The boxes can be placed overnight at 4 °C, however the coloring will continue at this temperature.
12. Stop the reaction by placing the slides on a holder in a container filled with TE buffer. With the container placed in the dark at room temperature the slides can be stored for several days.

Mounting of slides

1. Dehydrate the slides by placing them for 10 seconds in subsequently H_2O (1), 30% EtOH (1), 60% EtOH (1), 80% EtOH (1), 90% EtOH (1), 96% EtOH (1), 100% EtOH (2), 100% EtOH (3).
2. Mix 96% EtOH and xylene 1:1 in a glass container, in the fume hood and wash the slides for 10 seconds.
3. Wash the slides for 10 seconds in Xylene (1) and finally Xylene (2).
4. Let the slides dry for a minute. Apply about 200 µl of mounting solution in small drops on the slide. Cover the slide with a cover glass without making bubbles.
5. After about two hours the slides are ready. The slides can be stored in the dark.

Stock solutions and materials

This is a list of stock solutions and materials.

- 1L of 100% ethanol with molecular sieves
- 1L of 96% ethanol
- 1L of 90% ethanol
    a. 62.5 ml autoclaved MQ
    b. 937.5 ml EtOH

- 1L of 80% ethanol
    a. 166 ml autoclaved MQ
    b. 833 ml EtOH
- 1L of 60% ethanol
    a. 375 ml autoclaved MQ
    b. 625 ml EtOH
- 1L of 30% ethanol
    a. 688 ml autoclaved MQ
    b. 313 ml EtOH
- 2L of autoclaved MQ
- 1L of 1M Tris-HCl (pH 7.5)
- 1L of 1M Tris-HCl (pH 9)
- 1L of 5M NaCl
- Xylene >98% pure for histology
- Proteinase K
    a. 10 mg/ml stock dissolved in sterile water and keep at -20°C
- Acetic anhydride
- 10N NaOH:
    a. 20 g NaOH in 50 ml MQ
- 1N NaOH:
    a. .4 g NaOH in 10 ml MQ
    b. Make fresh every month
- 250 ml 0.5M EDTA pH 8:
    a. 46.53 g Disodium EDTA·$2H_2O$ (MW 372,24)
    b. adjust the pH by 10M NaOH in 250 ml MQ
    c. autoclave
- 1 M Na phosphate (pH 6.8)
- 1M Tris-HCl (pH 8)
- 1L TE:
    a. 10 ml 1M Tris-HCl (pH 8)
    b. 2 ml 0.5 M EDTA
    c. in 1 L MQ
    d. autoclave
- 50% Dextran sulfate dissolved in sterile water

- 10x in situ salts:
    a. 30 ml 5M NaCL
    b. 10 ml 1M Tris-HCl (pH8)
    c. 10 ml 1M Na phosphate (pH 6.8)
    d. 10 ml 0.5M EDTA (pH 8)
    e. in 100 ml MQ
    f. autoclave
- Formamide (deionized)
- 50x denhardt's
- Triton-x-100
- BSA (fraction V)
- Anti-DIG antibody (Roche)
- NBT/BCIP (Roche)
- 100 mg/ml tRNA (dissolved in sterile water)
- Glycine
- Boehringer blocking reagent
- Triethanolamine
- DMSO