Studying the role of temperature on pollen germination and tracking stem cell specification during flower development in *Arabidopsis thaliana*

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Certificate of Examination

This is to certify that the dissertation titled "Studying the role of temperature on pollen germination and tracking stem cell specification during flower development in Arabidopsis thaliana" submitted by Mr. Athul R Vijayan (Reg. No. MS12109) for the partial fulfilment of BS-MS dual degree programme of the Institute has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. Ram Yadav (Supervisor) Dr. Rhitoban Ray Choudhury Dr. Kuljeet Sandhu

Dated: April 21st, 2017

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr.Ram Yadav and Late Prof PS Ahuja at the Indian Institute of Science Education and Research Mohali. This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Athul R Vijayan
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(Candidate)

Dated: April 21st, 2017

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr. Ram Yadav
(Supervisor)

Acknowledgement

Above all my heart shall be raised in gratitude to the almighty for his universal grace towards me. I thank Indian Institute of Science Education and Research, Mohali for giving such a nice opportunity to do my final year thesis in one of the labs. It is my pleasure to extend my deep sense of gratitude and indebtedness to my guides Dr. Ram Yadav and Late Prof. P.S. Ahuja, for their guidance, along with benevolent and sustained encouragement bestowed on me during the course of training.

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I gained a lot of knowledge during this work. I acknowledge my sincere gratitude to my parents for their constant support and encouragement. I thank almighty for guiding my hands to success

Athul R Vijayan

List of Figures

NOTATION

APX	Ascorbate peroxidase
At	Arabidopsis thaliana
cDNA	Complementary DNA
CLV3	*CLAVATA3*
CAT	catalase
EtBr	Ethidium bromide
FP	Floral primordia
FM	Floral meristem
GPX	Glutathione peroxidase
GFP	Green fluorescent protein
LB	Luria broth
MCT	Micro centrifuge tube
MS	Muraschine Schoog
OD	Optical density
PCR	Polymerase chain reaction
ROS	Reactive oxygen species
RTqPCR	Reverse transcription semi quantitative polymerase chain reaction
SAM	Shoot apical meristem
SOD	Superoxide dismutase
TAE	Tris acetate EDTA
TF	Transcription factor
YFP	Yellow fluorescent protein
WUS	WUSCHEL
βME	β-Mercaptoethanol

Contents

Abstract

Arabidopsis offers advantage in studying various developmental mechanisms in plants. Herein we are studying two different projects. We used pollen as a system to understand the oxidative stress mechanism in one of the project. The other project is focused on stem cell specification in developing flower primordia.

Environmental stress has profound impact on plant the growth and development. Plants respond to environment by activating various stress responses which leads to increased reactive oxygen species (ROS) production. Nature has its own antioxidant mechanisms for ROS scavenging. The high altitude plants have better evolutionary adaptation for antioxidant enzymes to resist various climate changes. The present study is focused on pollen level stress characteristics of Cu/Zn superoxide dismutase (Cu/Zn-SOD) isolated from *Potentilla atrosanguinea* and ascorbate peroxidase (APX) isolated from *Rheum australe* at high altitudes of Indian Himalayas. Pollen germination and tube length at high temperature for SOD, APX and SOD'APX double transgene plants were studied to understand how the pollen cells respond to temperature stress in the presence of ROS scavenging enzymes. My findings suggests that APX overexpressing plant pollen grains are able to germinate efficiently than wild type.

Spatiotemporal regulation of gene expression is the key phenomenon in multicellular organisms to specify fate of distinct cell types. This regulation of gene expression is achieved by various mechanisms such as regulation of transcription, cell signaling etc. Shoot apical meristem of Arabidopsis thaliana harbor a set of stem cells from which the various cell types of plant arise. How stem cells differentiate into different cell types of the shoot and how organs form from these differentiated cells is still far from our understanding. To investigate the events leading to specification of stem cells we studied the flower organ primordia development as a model. The niche establishes first in the growing primordia this follows the specification of stem cells in the epidermal layer of flower meristem. When the carpel primordia form in the centre of the flower, stem cell fate terminates. Thus the present study gives a spatiotemporal understanding of stem cell specification in the developing flower primordia. Furthermore, on the lines of live-cell imaging, gene expression studies can be carried out to understand the regulatory mechanisms underlying fate specification and termination of stem cells in the dynamic environment of flower primordia formation.

Chapter 1

Introduction

1.1 *Arabidopsis thaliana* – Model organism

Arabidopsis is widely used as a plant model organism; it's an angiosperm, a dicot from the mustard family (Brassicaceae). It has a small genome (114.5 Mb/125 Mb total) with rapid life cycle (about 6 weeks from germination to mature seed). It grows 20-25cm in tall, hence easily grown at high density in glasshouse or culture room. Self-pollination allows easy selfing, thus makes it an attractive model organism for mutagenesis. Single plant can produce thousands of seeds. It has several traits that make it a powerful model for understanding the molecular, genetic and cellular biology of angiosperm. Thus Arabidopsis offers important advantages for basic research in genetics, molecular and developmental biology.

Arabidopsis thaliana life cycle can be divided into two phases, vegetative and reproductive phase. During vegetative phase, the shoot apical meristem produces a compact spiral/ rosette of leaves. They follow a spiral phyllotaxy. These leaves are slight purple or green in colour and these leaves are covered with small, unicellular hair-like structure called trichomes. During the later stages of vegetative phase, the stem elongates rapidly. In the subsequent period of growth, the SAM become a dome like structure and produce flower buttress in the periphery. As the development proceeds, floral buttresses turn into a floral meristem. This phase is known as the reproductive phase. These flowers have both female and male reproductive organs (Fig.1 B); hence, they can self-pollinate and self-fertilize to form seeds. Through artificial means, cross-fertilisation is also possible. Each fruit / pod will contain 50-60 seeds. After forming seeds, the growth is terminated. The seed then undergoes dehydration until it gets into the dormant stage; under favourable conditions the seed germinate and the cycle continues.

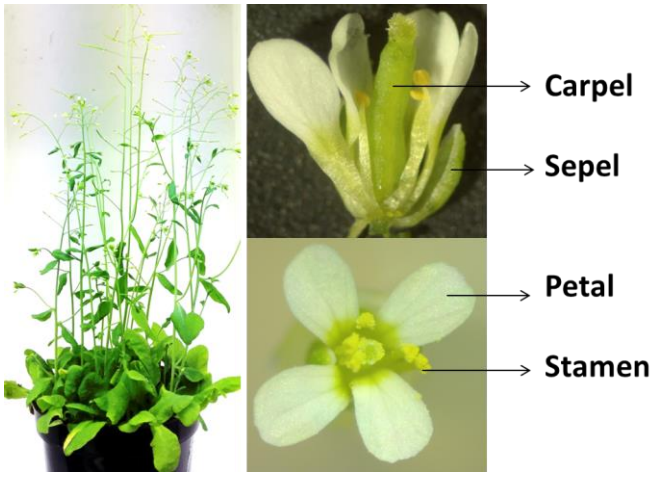


Figure1.**Model organism.**
(A) *Arabidopsis thaliana* (WT col) plant; (B, C) Wild-type flower of Arabidopsis with first whorl sepal, second whorl petal, third whorl stamen and forth whorl carpel.

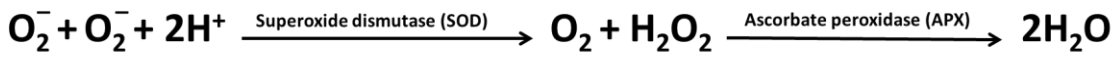
1.2 Antioxidant enzymes SOD (Superoxide dismutase) and APX (Ascorbate peroxidase) in plant oxidative stress defense.

Sexual reproduction in flowering plants involves formation of haploid gametophyte. Pollens are the male gametophyte, it undergo regulated rehydration to initiate germination and polarized pollen tube growth. Pollen tube carries sperm cell to fertilize the female gamete, embryo sac. But how does the egg cell recognize that fertilization occurred and how embryogenesis is initiated on the molecular level is still not clear. Pollen tube growth involves vesicular activity, membrane synthesis and many more molecular processes. The pollens are in direct interaction with the environment and the pollen germination and development are affected by the climate changes. The changing climatic condition due to global warming poses serious challenge to the agriculture in the 21st century. Plants need to get adapted quickly to altered climatic condition so that they can to grow and reproduce viable offspring. Loss in pollen germination and tube growth could occur at high temperature that leads to failure in fertilization and thus productivity of plant reduces rapidly. (Taylor & Hepler, 1997; Zonia, 2010; Takeuchi & Higashiyama, 2011)

Genes that confer properties to survive in extreme environments are valuable for improving the productivity of crops in the face of abiotic and biotic stresses. O_2 is an essential element for the survival of aerobic life; it is present at a concentration of ~21% in the atmosphere. Aerobic metabolism is efficient and necessary for the processes of life. However, the presence of oxygen in the cellular environment poses a constant threat to the cellular structures and also the anabolic processes (Alscher et al, 1997). Like other organisms, Oxygen is also essential for the existence of plant system, but toxic oxygen species or Reactive oxygen species (ROS) is a common aspect of all adverse conditions (Boyer, 1982), which includes the oxygen centered superoxide ($O_2^{\cdot -}$), and hydroxyl ($OH^{\cdot}$) free radicals, as well as hydrogen peroxide (H_2O_2), are generated in all aerobic cells. Production of ROS is an inevitable result of the leakage of electron onto molecular oxygen in chloroplast; mitochondrion and plasma membrane linked electron transport in plant cell (Asada 1994).

ROS damages DNA, proteins and also membranes by lipid peroxidation. Thus, plant cell have highly regulated and controlled mechanisms to modulate their intracellular ROS concentrations to ensure minimum damage and optimal functioning. Under normal conditions, the formation and removal of O_2 are in balance. However, the defense system

can be overwhelmed when confronted with increased ROS formation under stress conditions (Alscher et al., 2002). During normal conditions, the cellular system is in homeostasis where the ROS produced are taken care of by the cellular antioxidant machinery by the mechanism known as the Asada–Halliwall Pathway, however, during stress the cellular homeostasis is perturbed and the increased ROS cause cellular damage. Thus, oxidative stress in plants is inhibitory for the growth.



Enzymatic ROS defense include superoxide dismutase (SOD), catalases (CAT), peroxidases and antioxidant molecules like ascorbate and glutathione. SOD converts the superoxide radical in to H_2O_2, and ascorbate peroxidase (APX) triggers the conversion of H_2O_2 to water and oxygen (Asada, 1999; Fridovich, 1995). Thus, SOD acts as the first line of defense converting O_2^- to H_2O_2 which is then detoxified by APX, glutathione peroxidase (GPX) and CAT. Plant stress tolerance can be increased by increasing the level of antioxidant enzymes (Allen, 1995) as they appear to be a critical component of defense against oxidative stress. It has been estimated that 1% of the oxygen consumed by plants is metabolized to form ROS in different subcellular components (Bartoli et al., 1999). Under non-stressed environmental conditions the inherent antioxidant system in plant cells comprising of SOD, APX and CAT effectively protects the cellular machinery from ROS. However, during environmental stress conditions i.e. cold, high temperature, salinity, drought, flooding, radiations (high intensity of ultra-violet and visible light), chemicals and pollutants (heavy metals, pesticides, and aerosols), ozone, wind, nutrient deprivation as well as biotic stresses like pathogens (viruses, bacteria, and fungi), insects, herbivores, rodents etc., ROS generation increases many folds, thus overwhelming the cellular antioxidant capacity causing oxidative stress (Hernandez et al., 2000).

At the Institute of Himalayan Bioresource Technology (IHBT), bio prospection studies of high altitude plants of western Himalayas led to the discovery and full length cDNA isolation of a thermostable copper-zinc superoxide dismutase (Cu/Zn-SOD) from Potentilla atrosanguinea (4517m). At the same time, a full length cDNA of ascorbate peroxidase (APX) was isolated from another high altitude plant Rheum australe (4000m). The adaptive features of the above host plants being unique, the present thesis focused on the pollen level functional validation of expression of the above genes alone and in

combination (Cu/Zn-SOD and APX) in the model plant Arabidopsis thaliana ecotype Columbia-0. The analyses of transgenic plants that overexpress these genes provides interesting insights into their relative contribution to oxidative stress tolerance and also show their relation to one another in alleviation of stress. The study was undertaken for identification of pollen level oxidative stress defense mechanisms in transgenic A. thaliana plants at elevated temperature.

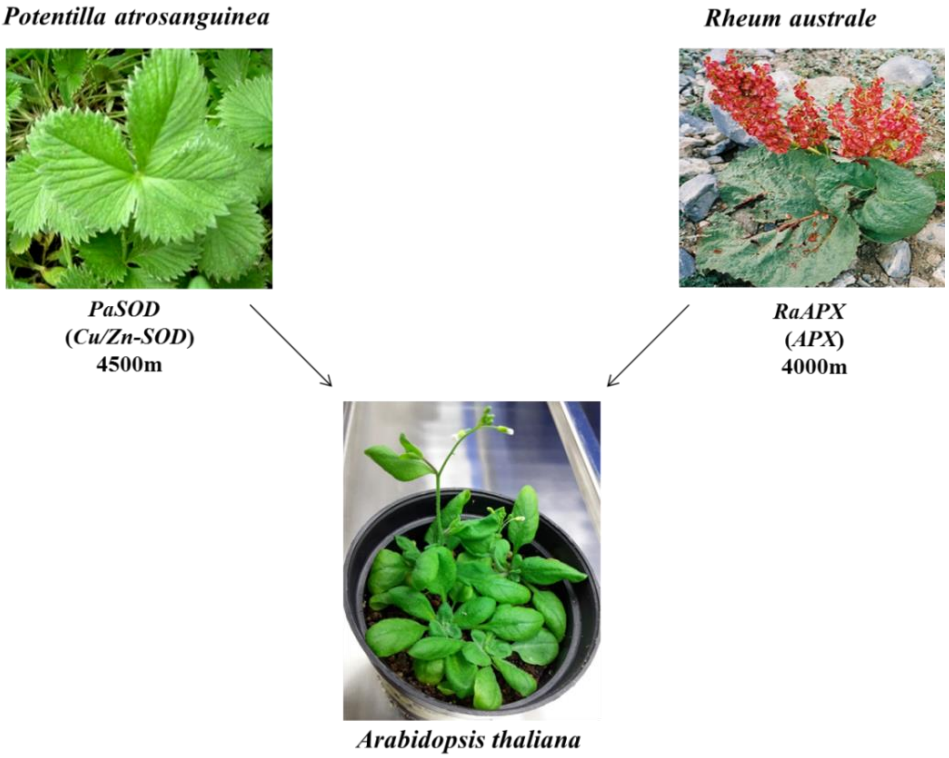


Figure 2. **Plant species from which the genes were isolated for the over expression studies in *Arabidopsis thaliana***

1.3 Stem cells specification during flower development

Shoot apical meristem (SAM) resides at the tip of the plant and contains stem cells that are pluripotent in nature. Stem cells of shoot apical meristem give rise to all post embryonically derived tissues and organs above ground, such as leaf, flower and stem tissues. SAM of higher plants is a highly organized structure and divided in to various cell layers and zones. In Arabidopsis, the SAM is a multilayered structure consisting of approximately 500 cells, organized into three clonally distinct layers of cells. However there are only two distinct layers in monocots. The cell lineages derived from epidermal or L1 cell layer in *Arabidopsis* give rise to pavement cells, guard cells and trichomes in

the leaf. The sub epidermal or L2 cell layer gives rise to the mesophyll, a photosynthetic tissue. The innermost layer is called corpus or L3 cell layer and produces vascular bundles and stem tissues in mature plant. These cells layers comprise most plant organs (Stewart and Derman., 1970).

Contrary to the cell layering, SAM is also subdivided into distinct zones. The central zone or stem cell region is located at the tip. It contains a set of pluripotent stem cells that are maintained undifferentiated. Daughter cells of stem cells have either stem cell fate or differentiation fate. Peripheral zone is where the stem cell progenies enter into differentiation pathway. The CZ also supplies cells to the rib meristem (RM) located beneath the CZ and the RM cells differentiate and become part of the stem. Cell fate specification in shoot apical meristem is a dynamic process where spatiotemporal gene activation and repression leads to changes in growth pattern. (Reddy, 2009; Satina et al., 1940; Derman., 1953)

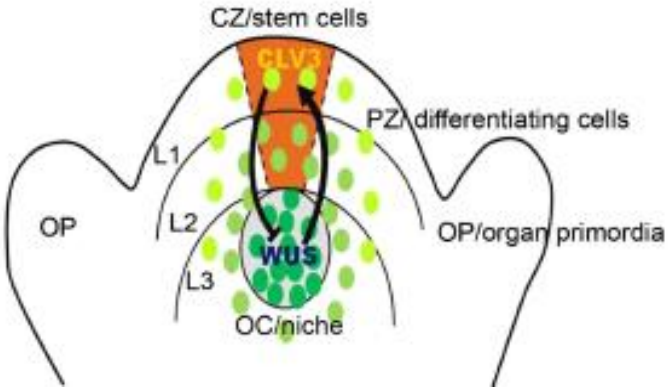


Figure 3. **Schematic representation of Shoot apical meristem**. The SAM is divided into clonally different cell layers L1, L2, L3 and different zones OC- Organizing center, PZ- Peripheral zone, OP- Organ primordia.; Yadav et al (2011)

The organizing center maintains stem cells in undifferentiated state. Organising center has WUSCHEL (WUS) gene expression, which encodes a homeodomain transcription factor required to maintain the stem cells in the shoot apical meristem in an undifferentiated state. WUS protein moves to central zone and activates a small peptide CLV3 that accumulates in the stem cells. Thus WUS specifies stem cells in shoot apical meristem. CLV3, in turn, negatively regulates WUS transcription. The feedback loop between *WUS* and *CLV3* is a self-regulatory system which balances stem cell proliferation and differentiation at the shoot apical meristem. This has been shown by various genetic and molecular analyses. (Laux et al., 2003). Similarly, *WUS* gets activated during the early

stage of flower primordia and it specifies stem cells in floral meristem. Unlike SAMs, floral meristems are determinate and stem cell maintenance is abolished once all floral organs are initiated. AGAMOUS (AG) is required for floral determinacy (Michael et.al 2001). Repression of WUS by AG is essential for terminating the floral meristem and that WUS can induce AG expression in the developing flower. Thus increased AG represses WUS expression in the later stages of flower meristem and the stem cells differentiate to give rise to gynoecium.

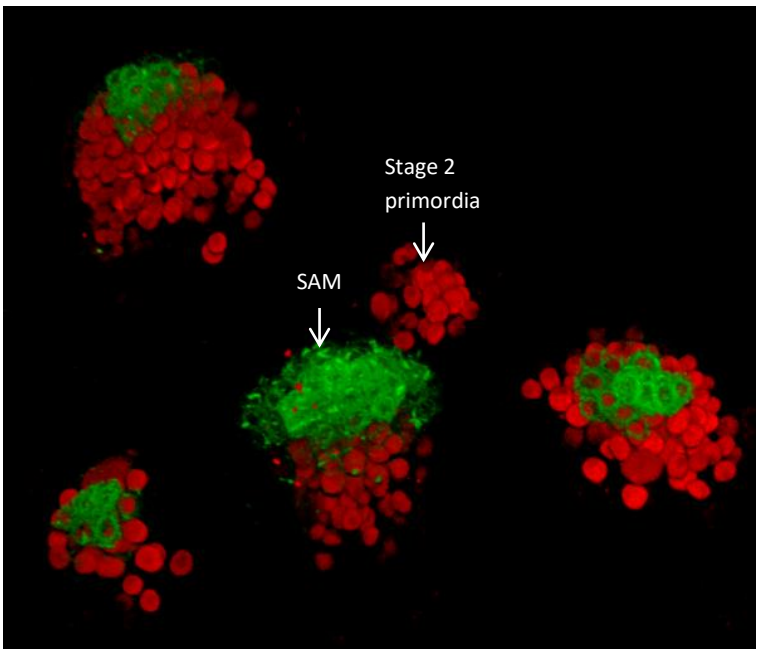


Figure 4. ***WUS* expression in Arabidopsis shoot.** 3d image from confocal sections of shoot apical meristem with different stages of flower development. The stem cells are marked with pCLV3::mGFP5-ER and WUS expressing cells are marked with pWUS::mcherry. The stage 2 floral primordia is attached to shoot and it doesn't have CLAVATA expression.

One of the key challenges in the shoot apex biology is to understand how stem cells differentiate and what kind of division pattern they acquire before they commit to a specific cell type identity. The cells that are away from the central zone of shoot apical meristem moves further to peripheral zone and then to distal zone where they form organ primordia. During the reproductive stage of plant development, flower primordia are developed. The flower primordia establish floral meristem for further development. Plant developmental biologist classified development of flower in to various stages. Stage one is where the flower buttress just arises. Stage two is where flower primordia form. In stage three, flower forms sepal primordia, sepal primordia further grow and overlie the flower meristem in stage four. Stage five begins when the petal and stamen primordia arise. In stage six, the sepals enclose the buds and cover the meristem.

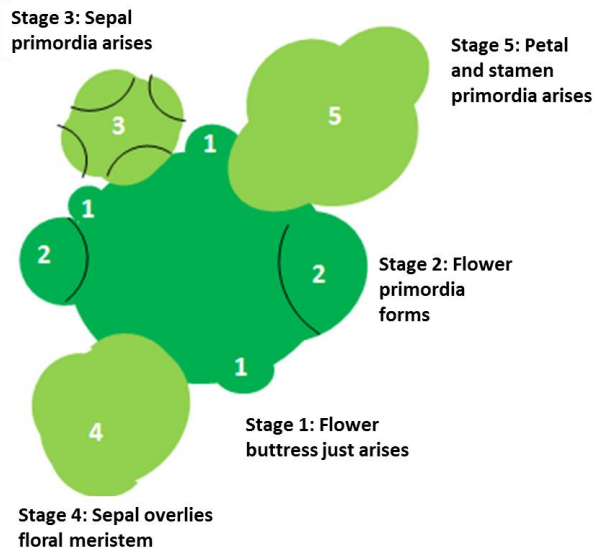


Figure 5. Model of different stages of flower development.

WUS expression comes first during the early stage of flower development. WUS activates CLV3 and stem cells are specified in the floral meristem. WUS expression during early flower development and late stem cell specification suggest that there are other molecular events that follow WUS to specify stem cells in floral meristem.

Auxin has a major role in plant development. Auxin signalling, biosynthesis and transport control auxin maxima at different locations in Arabidopsis shoot. Floral meristem specification is a complex system where different molecular mechanisms and the regulatory networks converge at the time of flowering to give rise to flower buttress as well as later on specification of stem cells so that stem cell can be used later on to form floral organs. Transition to flowering is mediated by various genetic pathways that are responsive for environmental and developmental signalling. (Blazquez et al., 2003; Boss et al., 2004).

Early events that lead specification of stem cell fate in the apical part of the plant embryo are difficult to observe by live imaging in plants. Since stem cell specification occur at later stages of flower development when the floral primordia abut from the shoot apex. This offers the opportunity to explore the de novo specification of stem cells in flowers. This project was aimed at spatiotemporal understanding of stem cell specification at the floral primordia. Some of the unanswered questions include when and where does the stem cell gets specified and how does the stem cell population changes upon time in the floral meristem? We did time series confocal imaging of plants using stem cell specific promoter reporter pCLV3::mGFP5-ER and plasma membrane 35S::YFP29-1.

Chapter 2

Experimental Materials and Methods

2.1 Plant material and growth conditions

The study was carried out on *Arabidopsis thaliana* Col and Ler (ecotype). Plants were grown in small pots with autoclaved soil (solarite, perlite and compost in 2:2:1 ratio). It was grown in controlled environment plant chambers (Percival, USA and Conviron, Canada) with 75% humidity, 130 µmol white light and at 22^{o}C temperature with 16-hour day and 8-hour night. Seeds were directly germinated on soil if they need not have to be selected. In other cases, seeds were allowed to grow on MS plate (with antibiotic for selection) and were kept for 72hr for vernalization in 4^{o}C. They were transferred to growth chambers to grow. Plants were watered thrice in a week and care was taken to give water only when the soil is becoming dry to avoid water stress. The light quality, temperature and day night cycles of the chambers have to be constantly checked for any variations that can affect the plant health.

2.2 Seed Sterilization

Required amount of dry seeds from desiccator were transferred to 1.5ml MCT. Seed sterilization was performed in biosafety cabin. One-minute washes each with Bleach (10% bleach with 0.02% Triton x-100) and 70% ethanol are sufficient for sterilizing seeds. Further 4-5 washes of autoclaved water is required for removing the residual ethanol and bleach from the seeds. With the help of pipet, seeds were spread one by one onto MS agar plates. Care was taken to place seeds a distance apart so that they will not be in stress.

2.3 Preparing soil for growing plants

Mix solarite, perlite and compost in 2:2:1 ratio and autoclave the mixture. Allow the soil to cool down and store it in closed container to prevent contamination. Fill the pots with soil up to a level such that plants can imbibe water and they won't be in water stress. Put seeds as much as needed for germination but plant only 5 to 6 seedlings per pot for optimum growth.

2.4 Pollen germination media and culture apparatus

We used a cellulose-based membrane (Cellophane 325p from AApacking UK) that provides physiochemical environment that mimics the stigma and support pollen germination. Pollen germination media was modified from Downton et.al (2013) and it consisted of:-

- 10% sucrose
- 1 mM CaCl2
- 1mM Ca(NO3)2
- 1mM KCl
- 0.01% boric acid
- 0.03% Muraschine Schoog powder
- 0.01% myo-inositol
- 0.1mM spermidine
- pH 8
- 0.8% agar

Sucrose was dissolved in water first and the media components were added one by one from their stock solution. Further pH was adjusted to 8.0 by adding KOH and 0.8% agarose was added into it. Media was heated in a microwave until it had dissolved completely. Molten media was poured onto slides that were placed on a flat surface to form a flat agarose pad. The slides were left to cool until the media had solidified. Small agarose blocks were cut out from the pad and were transferred to fresh glass slide. Further the glass slide containing blocks were stored in 4^{o}C on in situ glass boxes with water inside to keep it moist. It can be used within a week's time.

A humid chamber was prepared by placing a layer of tissue paper wetted with water at the bottom of the insitu glass box. Slides were placed vertical by equipping slide holders inside the insitu box. Rectangular pieces (2cm x 2cm, smaller than the agar block) of cellulosic Cellophane membrane (325P Cellulose; AA Packaging Limited, Preston, UK; www.aapackaging. co.uk) were cut out. The cellophane membrane was taken up with a forceps and gently placed on top of the block. The membrane folds back but it will revert of its own. If the surface is not flat, add 20µl liquid pollen germination media to wet the cellophane membrane. Pollens were spread onto the media with cellophane membrane without delay.

2.5 In vitro pollen germination

Plants in flowering state that didn't start to exit flower production were chosen for the germination study. These plants would have more unopened flowers than mature flowers and siliques. Flowers were carefully plucked and held on a forceps. Stamen was taken out

using another forceps by plucking from the filament region. This stamen was used as a brush to spread pollens on its tip to the surface of pollen germination media containing cellophane membrane. Slides were immediately placed in a vertical position on humid chamber and were allowed to germinate. Germinated pollens were observed after specific intervals.

2.6 Microscopy of Pollen

Slides were examined using a Zeiss Axio Imager Z.2 microscope and images were captured using Axiocam ICc1. Pollens were spread over the surface of membrane. Different fields were chosen randomly and multiple images were acquired per slide. Care was taken to avoid duplication. Pollen germination was scored from the images and tube length was measured using Image J software. ~ One hundred pollens were scored from one slide and the pollen germination efficiency was calculated from the collated data. Further graphs were plotted in Microsoft excel.

2.7 Preparation of plants for Live-Imaging

Seeds are sterilized (2.2) and dropped one by one onto half MS medium (1% sucrose; 0.22% Murashige and Skoog medium including vitamins, 0.8% agar [pH 5.7] inside biosafety cabin and was vernalized for 72hr and transferred to plant growth chamber at 22^{o}C and 130 µmol white light and allowed to grow for 8 days. Healthy plants were transferred to a fresh half MS media in petridish (110mm x 40mm – Himedia) with the help of sterile tweezers. Plants are carefully observed to identify if it is time for performing imaging. Upon bolting, the shoot apex emerges out of the rosette and the plants are ready for live imaging (approximately 18-20 days). The shoot apex will be visible after removing the floral buds with the help of tweezers. Care was taken to avoid contamination in all steps. Before imaging, dissected apices were left to recover for 12 to 24hr. The plant ready for imaging would have the shoot exposed with some emerging primordia. The petridish was filled with autoclaved water to submerge the plant prior to each imaging session. Each imaging session would last 10 minutes; therefore, per petridish only 4 plants were grown. After imaging, water is discarded and the plants are allowed to grow in the plant chamber. Since we are studying flower development here, the stage 1 and 2 primordia from 0 hr imaging were focused on, thus any big flower that comes into the field were dissected out to clear the light path. The sepals of emerging

flower buds at later stages (stage 6/7) were dissected to see the CLAVATA3 expression at the center.

2.8 Confocal Imaging of Live plants

We have used plasma membrane localized YFP (35S::YFP29-1), which is EYFP fused to a protein tag which targets the protein chimera to the plasma membrane as plasma membrane marker. Alternatively, other dyes such as FM4-64 (100ug/ml) and Propidium iodide (100ug/ml) can be used for plasma membrane marker. pTAR2::mCherry was used as TAR2 reporter. pCLV3::mGFP5-ER reporter was used as stem cell marker. pWUS::mCherry reported was used for *WUS* imaging. pTAR2::mCherry and pWUS::mCherry reporters were made by shalini in the lab. C3ERPM29 (pCLV3::mGFP5-ER and 35S::YFP29-1) seeds were received from Reddy's lab (UC-Riverside)

Dissected apices were imbibed in water and imaged with a Leica TCS SP8 upright confocal microscope with excitation at 488 nm (argon laser), emission filters set to 496-529 nm for EGFP and 529-575 for EYFP, since it overlaps, we scan in sequential mode with EGFP in one sequence and EYFP in another for imaging with both the EGFP and EYFP marker together. A 63x water dipping objective was used to visualize the shoot. Z - stacks were generated consisting of approximately 60 images of 1 µm optical slice thickness and used for 3d visualization and segmentation. Other additional settings are acquisition Mode- XYZ and Sequential, Format- 512 x 512, Image Size- 184.82 µm x 184.82µm, Speed: 400, Bidirectional- On and Line Average- 6

2.9 Image analysis

Confocal images were captured without fixing any particular angle. The 3d reconstructed image was rotated to bring the axis of images in alignment. Image processing was done in Leica LASX software. Filters like Gaussian filter and median filters were used for image optimisation. Time series images were brought into a collage using Picasa. The orthoslice view of primordia was generated in LASX software only. Image J and Amira were also used for other image processing and analysis.

2.10 RNA isolation

Tissue (leaf) from 5-week-old plant was collected in an RNase free MCT and frozen in liquid nitrogen. The tissue can be stored at this stage in -80^{o}C, if required. Pestles were washed with clean water and autoclaved. Label the MCTs and the plant from which tissue was taken so that we can keep track record of plant lines used for later verification.

Isolation of total RNA using Qiagen RNaeasy kit.

- Make sure that there is 50-100 mg tissue in the MCT to get good amount of RNA
- Prepare fresh β-Mercaptoethanol (βME) (10µl) + RLC (1000µl) buffer in an MCT
- Set the centrifuge to 4^{o}C
- Crush the tissue with pestles after freezing in liquid nitrogen
- Add 450µl of βME + RLC buffer to the powdered tissue. Mix the tissue with buffer using the pestle to get reasonably clear green solution.
- Transfer the lysate to Qia-shredder spin column in a 2ml collection tube.
- Centrifuge for 2min at 14000rpm in 4^{o}C.
- Transfer the supernatant of flow through to a new RNase free MCT without disturbing the cell debris.
- Add 0.5 volume (~200µl) chilled ethanol to the clear lysate and mix by inversion.
- Transfer the sample in RNAeasy mini spin column.
- Centrifuge for 30sec at 10,000 rpm and discard the flow through.
- Add 700µl buffer RW1 to RNAeasy spin column and centrifuge it for 30 sec at 10,000rpm
- Add 500µl buffer RPE to RNAeasy spin column and centrifuge for 30sec at 10,000rpm
- Repeat 500µl RPE buffer wash again with 2min spin at 10,000 rpm.
- Dry spin for 30 sec.
- Place RNAeasy spin column in a new 1.5ml RNA free collection tube and add 50µl RNase free water directly into the spin column membrane.
- Centrifuge for 1 min at 10,000rpm to elute RNA
- Keep the RNA on ice and check the concentration.
- Store at -80^{o}C if needed.

- According to the concentration, dilute RNA for 1µg / 5µg as required for cDNA synthesis.

2.11 cDNA synthesis

To convert the mRNA in to cDNA, the following reaction components were added in to a PCR tube; the reaction was kept at 65^{o}C for 5 minutes and then placed on ice for 5 minutes.

• RNA	-	1 µg
• Oligo dt	-	1 µl
• dNTP	-	1 µl
• dH_20	-	Makeup volume
		--------
		10 µl

On the top of it, prepare the master mix of the following components and add 10µl.

Master Mix		**1x**
• 10x RT buffer	-	2 µl
• 25 mM $MgCL_2$	-	4 µl
• 0.1 M DTT	-	2 µl
• RNase Out (40unit/µl)	-	1 µl
• Superscript HIRT 200unit/µl	-	1 µl
		--------
		10 µl

Incubate the mixture at 50^{o}C for 90 minutes and terminate reaction at 85^{o}C for 5 minutes.

2.12 RT- qPCR

Semi-quantitative reverse transcription PCR was performed according to the following protocol using SOD and APX-specific PCR primer sequences mentioned below and cDNA. RT- qPCR with several dilutions of cDNA (1:2, 1:3, 1:5) should be done with control (tubulin) to identify the specific dilutions required for sample. Once we identify the dilutions, PCR with our gene specific primers (Table 1) were setup at the particular dilution.

At Cu/Zn-SOD	F	TGCCATGGCGAAAGGAGTTGCAG
	R	ATAGATCTGCGCCCTGGAGACCAATGATG
At APX	F	ATAGATCTGATGGCTGCACCGATTGTT
	R	TAAGTAGTCTTCATCCTCTTCCGGATCTC

Table 1: Primers for RT- qPCR

Prepare master mix according to the table 2 and aliquot into PCR tube. PCR reaction conditions were setup as mentioned in table 3. After completing the PCR, load the samples in electrophoresis gel (2% agarose gel) along with the ladder and run it

Components	**Volume**
Template (cDNA with required dilution)	1 µl
10X Standard Taq Reaction Buffer	1.5 µl
dNTPs (10mM)	1.2 µl
$MgCl_2$ (50mM)	0.45µl
Water	10 µl
Taq DNA polymerase	0.25 µl
10µM forward primer	0.75 µl
10µM reverse primer	0.75 µl
Total	16 µl

Table 2: PCR reaction components for RT- qPCR

Steps	**Temperature**	**Time**	
Initial denaturation	94^{o}C	4 Minutes	
Denaturation	94^{o}C	35x	1 Minutes
Annealing	56^{o}C		1 Minutes
Extension	72^{o}C		2 Minutes
Final extension	72^{o}C	7 Minutes	
Hold	4^{o}C	∞	

Table 3: Conditions for RT- qPCR

2.13 Genomic DNA isolation

- Leaves were harvested and were frozen in liquid nitrogen

- Crush the leaves in a 1.5 ml tube using the pestle
- Add 600µl CTAB buffer and mix well
- Incubate at 65^{o}C for 20 minutes
- Centrifuge at 13,500 rpm for 5 minutes
- Add 600µl Phenol + Chloroform mixture and mix well
- Centrifuge at 13,000 rpm for 5 minutes
- Collect the upper layer of the supernatant and transfer to a fresh tube
- Add 600µl chloroform and shake the mixture for 2 minutes
- Centrifuge at 13,000 rpm for 5 minutes and transfer supernatant into a fresh tube
- Add 0.7 volumes chilled isopropanol and mix by inversion
- Spin down at 16,000 rcf for 5 minutes
- Remove supernatant and wash the pellet with 1 ml 70% ethanol
- Centrifuge at 16,000 rcf for 1 minute and remove the supernatant
- Dry completely by keeping at 90^{o}C for 1 minute
- Resuspend the pellet in 50µl distilled water and Store in -20oC
- Check the concentration and setup a PCR with this genomic DNA and capture the gel image to quantify genomic DNA.

2.14 PCR

- Prepare the master mix and aliquot into the PCR tubes
- Samples were mixed and kept in PCR machine (ThermoCycler, Bio-Rad)
- Setup the PCR conditions mentioned below
- After completing PCR, run the samples along with ladder in an electrophoresis gel.

Components	**Volume**
Genomic DNA	1µl
5x PCR Buffer	5µl
dNTPs (10mM)	2µl
Water	13.4µl
DMSO	0.5µl
Phusion polymerase	0.5µl
10µM forward primer	1.2µl
10µM reverse primer	1.2µl
Total	15µl

Table 4: Master mix for PCR

Steps	Temperature	Time	
Initial denaturation	94°C	3 Minutes	
Denaturation	92°C	35x	30 seconds
Annealing	58°C		30 Seconds
Extension	72°C		1 Minutes
Final extension	72°C	5 Minutes	
Hold	4°C	∞	

Table 5: Conditions for PCR

2.15 Agarose gel electrophoresis

- 0.8g of agarose (0.8% w/v calculations) was added to 100mL 1X TAE buffer.
- Dissolve the agarose in 1X TAE buffer completely by heating.
- Let it cool (around 50℃).
- Add 0.2µL of Ethidium bromide (0.5µg/mL) and mix it well
- Pour into gel casting tray with required combs and allow it to solidify
- The solidified gel was placed in the electrophoresis gel tank (Biorad) filled with 1X TAE buffer. The wells were placed near the negative terminal of the electrophoresis unit.
- The PCR product was then mixed with 6x loading dye (Bromophenol blue) and loaded in the wells of agarose gel.
- Ladder was added in one of the well in a row.
- Voltage (90- 120V) was applied until the sample runs 2/3rd of the gel.
- Image of the gel were taken using gel documentation system (Biorad)
- In case of gel band purification, required band was cut out from the gel under UV trans illuminator.

2.16 REAGENTS AND CHEMICALS USED

High fidelity polymerase (Phusion) and standard molecular weight markers (1 Kb DNA ladder) were obtained from Thermo scientific. Taq DNA polymerase was made in the lab. All other chemicals and reagents used in the study were of molecular biology/analytical grade and were obtained from Sigma-Aldrich (USA) or Hi-media.

2.16.1 Preparing MS agar plates (1000ml)

Prepare the media (pH 5.7) and autoclave. Appropriate concentration of antibiotic is added if required when the media becomes luke warm. Mix and pour the media on plates and store them on $4^{o}C$.

- MS - 4.32 g (0.1%)
- Sucrose - 10.00 g (1%)
- dH_2O - 1000ml (makeup volume)

Set pH to 5.7 before adding agar

- Agar - 8.00 g (0.8%)

2.16.2 MS media for plants

Sometimes liquid MS media is applied on the plant tray instead of water so that the plants would be healthy with all the nutrients. Dissolve 2 gm. of Murashine schoog media in 1000ml distilled water and set the pH to 5.7 or manually add the micronutrients and macronutrients as mentioned below

- Macro elements - 100 ml
- Micro elements - 50 ml
- NPK - 100mg/ml

1000ml dH_20

Composition of Macroelements

- NH4NO3 - 1650mg
- KNO3 - 1900mg
- CaCl2.2H2O - 440mg
- MgSO4.7H2O - 370mg
- KH2PO4 - 170 mg

1000ml d H_20

Composition of Microelements

- H3BO3 - 6.2 mg
- MnSO4.4H2O - 22.3 mg
- ZnSO4.7H2O - 8.6 mg
- KI - 0.83 mg
- NaMoO4.2H2O - 0.25 mg

- CuSO4.5H2O - 0.025 mg
- CaCl2.6H2O - 0.025 mg
- FeSO4.7H2O - 27.8 mg
- Na2EDTA.2H2O - 37.3 mg

1000ml dH_20

2.16.3 10X TAE buffer

The following components were mixed together in a 1L flask.

- Tris base - 108g
- Glacial acetic acid - 57ml
- 0.5 M EDTA - 40ml
- dH2O - 795ml

2.16.4 6X Loading dye components (10mL)

- 1ml Glycerol, 0.025g Xylene cyano and 0.025g bromophenol blue were mixes in a falcon tube and the volume was brought to 10ml with distill water. This was then aliquoted in several 1.5ml MCT.

2.16.5 Preparing dNTP stocks

- Add 50µl of 100mM GTP, ATP, CTP and TTP each into a 2.0 ml tube
- Add 1.8ml distilled sterile water and mix well
- Aliquot 50µl into 1.5µl tubes

2.16.6 Preparing antibiotic stocks (50mg/ml)

- Add 0.5g of antibiotic on a 15ml oak ridge tube
- Fill sterile water up to 10ml and mix properly for dissolving completely
- Aliquot 1ml each in 1.5ml MCT and store in -20^{o}C

2.16.7 Ethidium Bromide

- Dissolve 1g Ethidium Bromide powdered form in 100 ml sterile water
- Stir for several hours to ensure that the dye has completely dissolved
- Store in a dark bottle

Chapter 3

Results and Discussion

3.1 Investigating to role of Antioxidant enzymes SOD and APX in pollen oxidative stress defence

3.1.1 Expression analysis of SOD and APX by RT-PCR

Seeds of stable transgenic lines over expressing APX and SOD were obtained from IHBT Palampur. To verify the integrity of these lines for ectopic expression of APX and SOD, semi-qRT PCR was carried out. cDNA was synthesised from mRNA and RT-qPCR was performed with primers specific to *Cu/Zn*-SOD in different lines of interest to verify the ectopic expression. RT-qPCR analysis of two single copy insert of *Cu/Zn*-SOD was performed with WT as control and found that S26 line was having higher expression than the S15 transgenic line (Fig. 6). Further S26 was used for pollen germination analysis. Different stable transgenic lines of APX were previously tested for its expression (Tejpal and Ahuja, 2010). All lines showed high expression of the *APX* transcript. Lines #2, 18 and 20 were used for pollen germination assay. SOD and APX transgene were combined together by crossing the individual transgenic line for generating double transgenic plant (CA18O or SOD'APX).

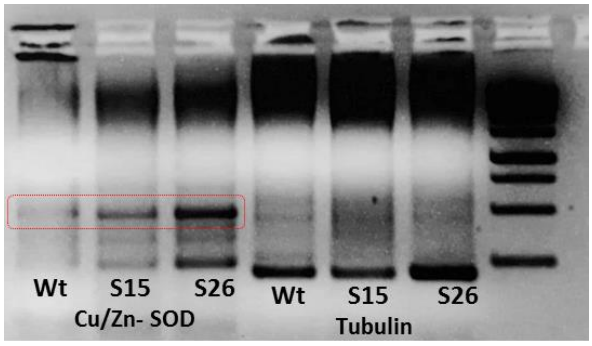


Figure 6. PCR amplification of cDNA with Cu/Zn-SOD specific primers of single copy inserts lines and control PCR with Tubulin specific primers Lane 1,2 and 3 are with Cu/Zn-SOD specific primers (Wt, S15, S26) and lane 4,5and 6 are with tubulin specific primers (Wt, S15, S26). Lane 7 is 1kb ladder.

3.1.2 Pollen germination at different temperature

In-vitro pollen germination assay was conducted using the pollen from WT *Col-0*, SOD (S26), APX2, APX18, APX20, and SOD'APX (CA18O). Solid pollen germination media was used to test efficiency of various lines first at ambient temperature. Pollen germination media was modified from Downton et.al (2013) and it consisted of 10% sucrose, 1 mM CaCl2, 1mM Ca(NO3)2, 1mM KCl, 0.01% boric acid, 0.03% Muraschine Schoog powder, 0.01% myo-inositol, 0.1mM spermidine, pH 8 and 0.8% agar (mentioned in 2.4). Pollen germination at 22^{o}C, 25^{o}C**,** 30^{o}C and 34^{o}C was performed, respectively, to see the effect of temperature on pollen germination and tube length. WT pollens maintained high germination efficiency and tube length from 22^{o}C to 30^{o}C. However at

34°C the WT pollens display drastic reduction in the germination efficiency and pollen tube length. Similar observation was made for respective SOD and APX lines except the APX #20. Surprisingly, APX #20 showed high germination efficiency and pollen tube length at 34°C. SOD'APX double transgene lines showed poor germination at all temperature, suggesting that perhaps excessive antioxidant quenching negatively influence the pollen germination and tube length. (Fig 7).

A

	22°C	25°C	30°C	34°C
Wt	87.61	81.35	83.21	44.02
S26	85.44	76.85	83.75	35.5
APX2	69.94	57.4	47.32	42.3
APX18	67.95	72.1	77.47	21.11
APX20	76.17	74.5	73.42	76.53
SOD'APX	31.87	23.52	32.22	5.2

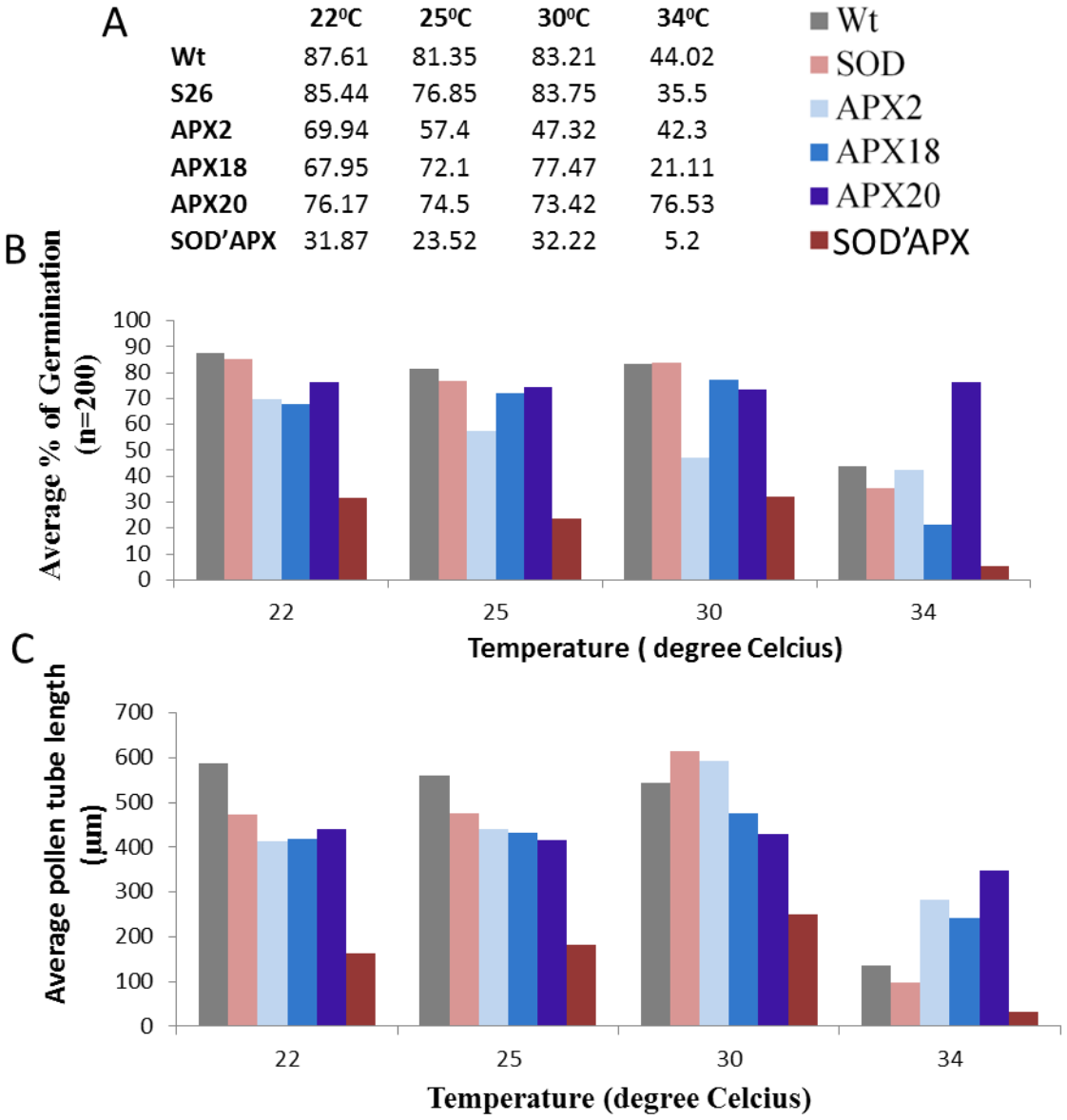


Figure 7. **Pollen germination and tube length** A: Germination percentage of different lines used for the study; B: Average percentage of pollen germination at different temperature counted over 200 pollens; C: Average pollen tube length of the pollens od different lines of interest. Germination and pollen tube length was counted after 11 hours of incubation at different temperatures. The different bars represent pollens taken from Wt, Cu/Zn-SOD, APX2, APX18, APX20 and SOD'APX double overexpression.

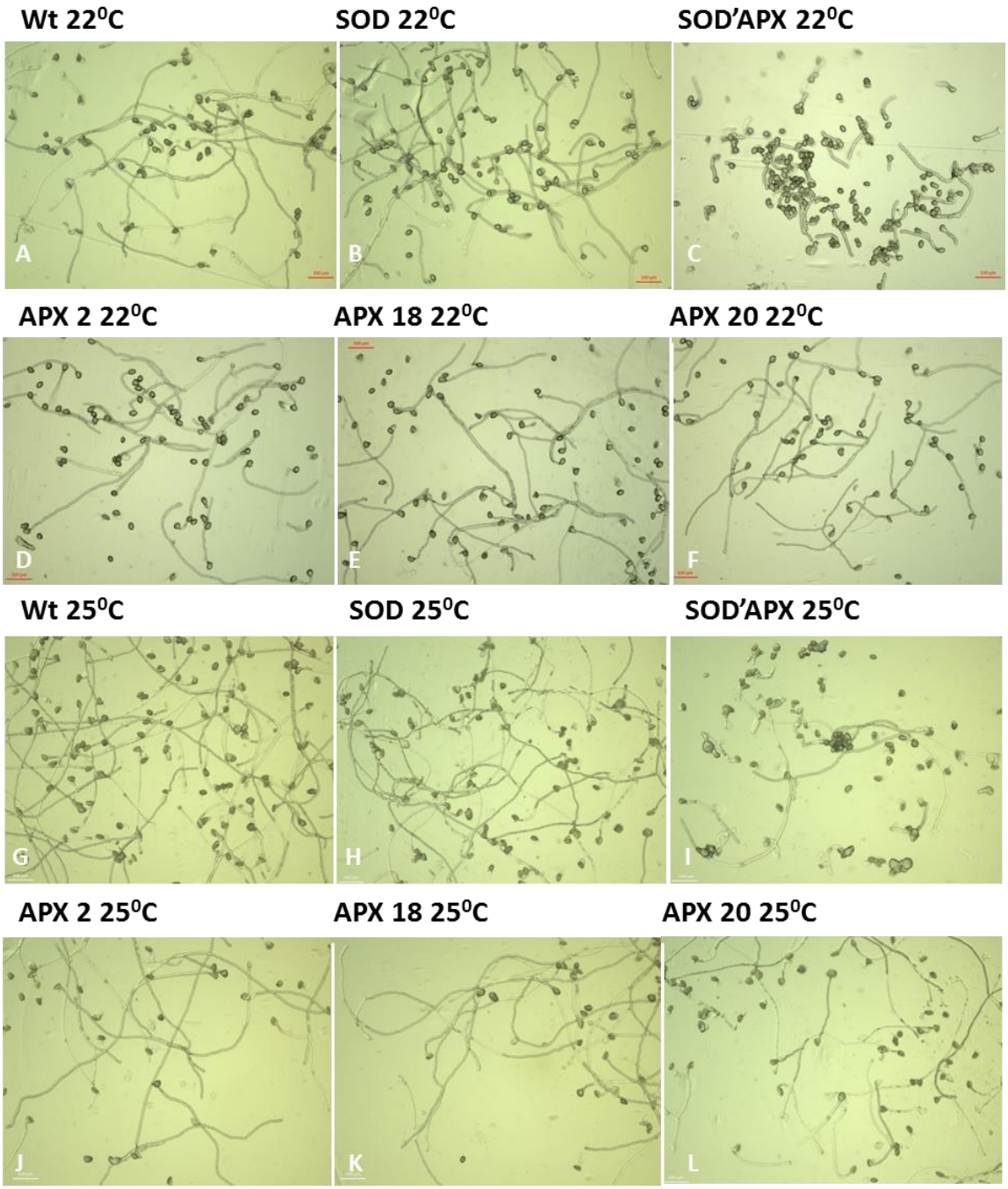


Figure 8. **Pollen germination at different temperatures – 22°C and 25°C**. Bright field images of in-vitro pollen tube growth of Arabidopsis thaliana Wt and antioxidants overexpressing lines of SOD, APX and SOD'APX double overexpression captured after 11hours of incubation at specific temperatures 22°C and 25°C. Scale bar is 100µm

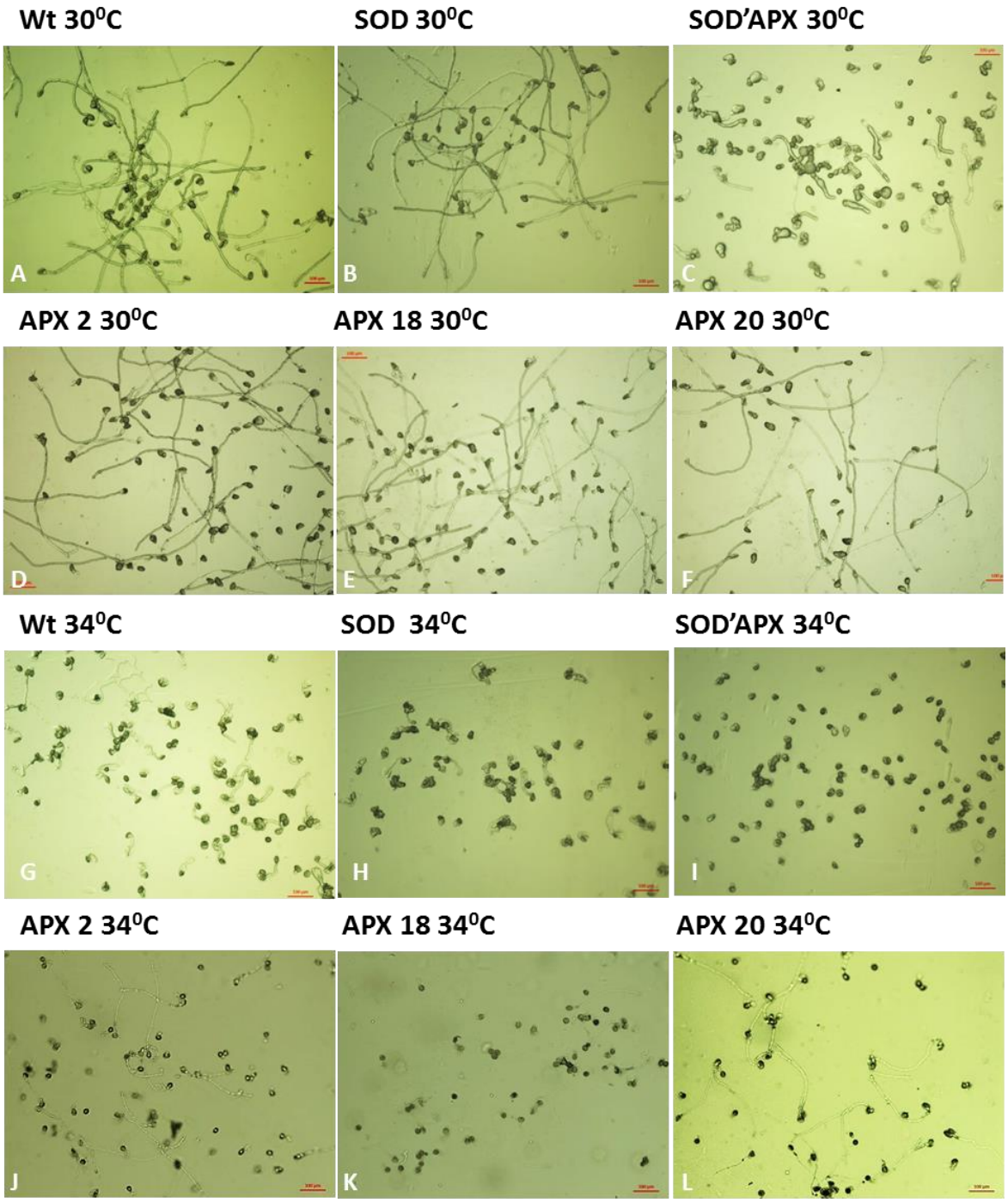


Figure 9. **Pollen germination at different temperature – 30°C and 34°C**. Bright field images of in-vitro pollen tube growth of Arabidopsis thaliana Wt and antioxidants overexpressing lines of SOD, APX and SOD'APX double overexpression captured after 11hours of incubation at specific temperatures 30°C and 34°C. Scale bar is 100µm

3.1.3 Developmental consequences of Cu/Zn- SOD, APX double overexpression

Pollen germination and tube length study from Figure 7, 8 and 9 demonstrate that SOD'APX double overexpression had defect in pollen germination. Double overexpression had approximately 70% reduced germination with respect to Wt at respective conditions. The poor germination percentage along with lower pollen tube length (Fig.7b,c) leads to developmental consequence. The productivity of SOD'APX double overexpression is very low when compared to Wt (Fig.10a). The double overexpression had an average of 40% reduced seeds per silique (averaged over 15 silique) when compared to Wt of the same age and grown at same conditions. It can be said that the developmental consequence is due to the combined pollen tube length and germination defect, because the ovules in the lower part of most of double overexpression silique are unfertilized and they are retarded (Fig.10c).

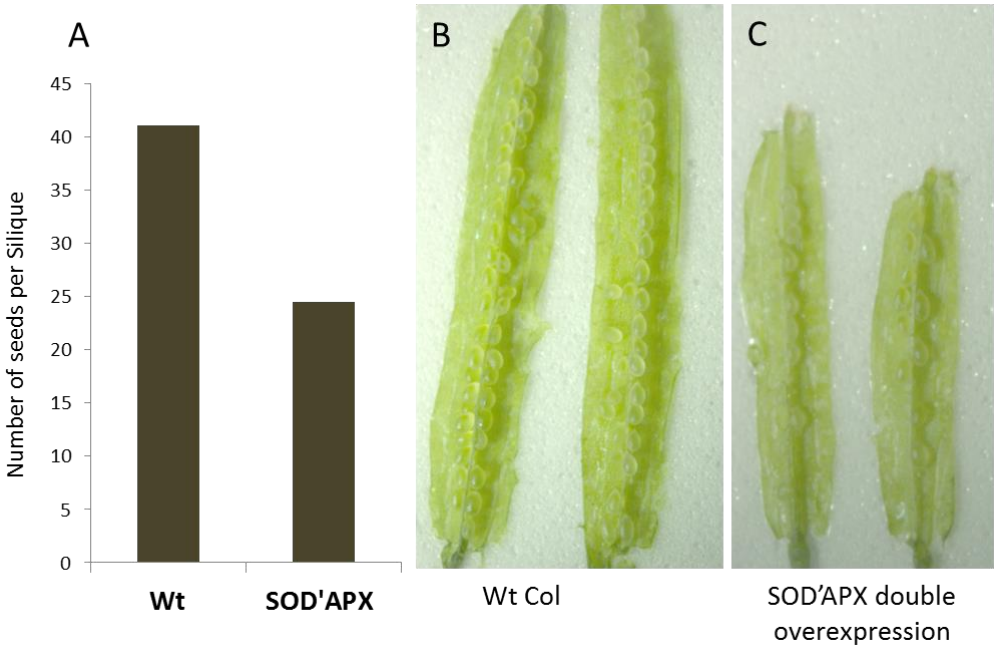


Figure 10. Developmental consequence of SOD'APX double overexpression.

3.1.4 APX overexpression induces pollen germination at elevated temperature

The Ascorbate peroxidase overexpressing transgenics had moderately high enzyme activity (1.9-2.2 folds) in comparison with the enzyme activity of the WT plants and also showed higher root length and rosette area in comparison with the WT plants (Tejpal and Ahuja, 2010). The pollen germination analysis from figure 7 demonstrates that the pollen germination percentage of APX overexpression line APX #20 is comparatively higher

with respect to others at higher temperature. APX #20 had 44% higher germination than Wt pollens at 34^{o}C. The pollen tube growth of Wt, SOD and SOD'APX at 34^{o}C was declined in most cases (Fig 7). It may be also because of low moisture content and drying of media at higher temperature that resulted in lower germination and tube length. APX #2 and APX #18 had lower germination percentage, but their tube length was comparable to APX #20. This demonstrates that Ascorbate peroxidase overexpression gives a positive phenotype at elevated temperatures also. APX #20 line can be further forwarded for beating up the environmental stress associated defect in pollen germination.

3.1.5 Discussion

A common aspect of all abiotic and biotic stresses is the enhanced production of reactive oxygen species (ROS). These ROS, namely $O2^{-}$ and OH^{-} oxidize cellular constituents such as lipids, proteins and nucleic acids and can initiate chain reactions triggering cellular apoptosis. However, at low concentrations, they participate in signalling events. Plants have highly regulated and controlled enzymatic and non-enzymatic mechanisms to modulate their intracellular ROS concentrations to ensure minimum damage and optimal functioning. Enzymatic mechanisms include superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase to counter balance the free radical production

The present study focused on characterising the antioxidant over expressing A. thaliana for high temperature induced pollens oxidative stress. Antioxidant genes (Cu/Zn-SOD and APX) from heterologous plants, single as well double transgenics expressing both Cu/Zn-SOD and APX genes in A. thaliana were used for the study. The over expression of the genes under the constitutive CaMV35S promoter did not result in retardation in growth and development of the transgenic plants, but Cu/Zn-SOD and APX double overexpression leads to less productivity.

The simultaneous scavenging of $O2^{-}$ and H_2O_2, by Cu/Zn-SOD and APX, is important for the maintenance of plant productivity under harsh conditions where single gene does not suffice for providing stress tolerance (Lee et al., 2007a, 2007b). The double overexpression line used for the study was generated by crossing plants with high transcript level of SOD and APX. Earlier finding suggest that transgenics having both Cu/Zn-SOD and APX are more tolerant to cold stress in comparison to the WT as was evident by increased enzyme activity and decreased in situ ROS production in the double

transgenics as compared to the WT plants (Tejpal and Ahuja, 2010). We further hypothesise that the developmental consequences are due to high ROS scavenging and a minimum threshold of ROS is required for normal cell functioning and signalling. We plan to alter the media compositions such that the double overexpressing pollen would germinate. This would let us understand the molecular elements involved in inhibiting pollen germination and tube growth on double overexpressing plants.

APX is an essential enzyme for the conversion of H_2O_2 to water and molecular oxygen in the cells (Mittler, 2002). Over expression of APX provided tolerance against a wide range of environmental cues like chilling (Kornyeyev et al., 2003; Wang et al., 2005c), drought (Yan et al., 2003), paraquat induced oxidative stress (Murgia et al., 2004), salt (Wang et al., 2005c) and high light (Rossel et al., 2006). Our results suggest that APX overexpression helps in pollen oxidative stress defence and pollen tube development at elevated temperature. Thus, APX overexpression has application in agricultural industry hoping to generate transgenic lines capable of defending pollen germination and tube growth at elevated temperature.

3.2 Understanding stem cell specification during flower development

Stem cells in the shoot apical meristem are maintained undifferentiated and all the post embryonically formed above ground organs and tissues are derived from shoot apical meristem. But how does the stem cell differentiate into specific cell types, tissue and organs are still not clear. The shoot apical meristem stem cell specification at embryonic stage can't be studied over time, but the floral meristem development and stem cell specification can be captured live. Since flowers development is homologous to shoot apical meristem development, various mechanisms underlying stem cell specification in the floral meristem would be applicable to shoot apical meristem also. Thus the model of flower development can be studied to better understand stem cell initiation at the shoot apical meristem and propose or validate various models for stem cell maintenance and differentiation.

Phytohormone auxin regulates cellular activities (division, elongation and differentiation), pattern formation, organogenesis, reproduction, sex determination, and responses to abiotic and biotic stress. Auxin biosynthesis, transport, perception, signalling and responses are responsible for several of these regulations. Stem cell homeostasis in the shoot apical meristem is maintained by WUS and stem cells are maintained undifferentiated by STM. From live-imaging studies it was shown that WUS suppresses auxin responses in the stem cell niche cells. However, it was not clear how it mediates this response. When we analysed the gene expression data of plants over expressing *WUS*, we found that the transcripts of the genes responsible for auxin biosynthesis such as ASA1, TAR2 and TSA1 were down regulated. Since auxin is involved in multiple aspects of plant development and growth, we wanted to understand the role of auxin biosynthesis gene *TAR2* in shoot and flower development. We hypothesise based on the live-imaging observation that WUS might be involved in repression of TAR2 in floral meristem.

3.2.1 Spatiotemporal expression of stem cells in developing flower primordia

Stem cells in the shoot apical meristem divide to give rise to stem cells and cells that undergo differentiation. Those cells that are in the differentiation fate move away from the shoot apical meristem to the peripheral zone where incipient primordia will come. Incipient primordia develop into floral primordia. Different stages of floral primordia are categorized based in its morphology and time (Smyth et.al, 1990, Fig 5). The floral primordia establish stem cells at some point of time and it gets terminated once the flower

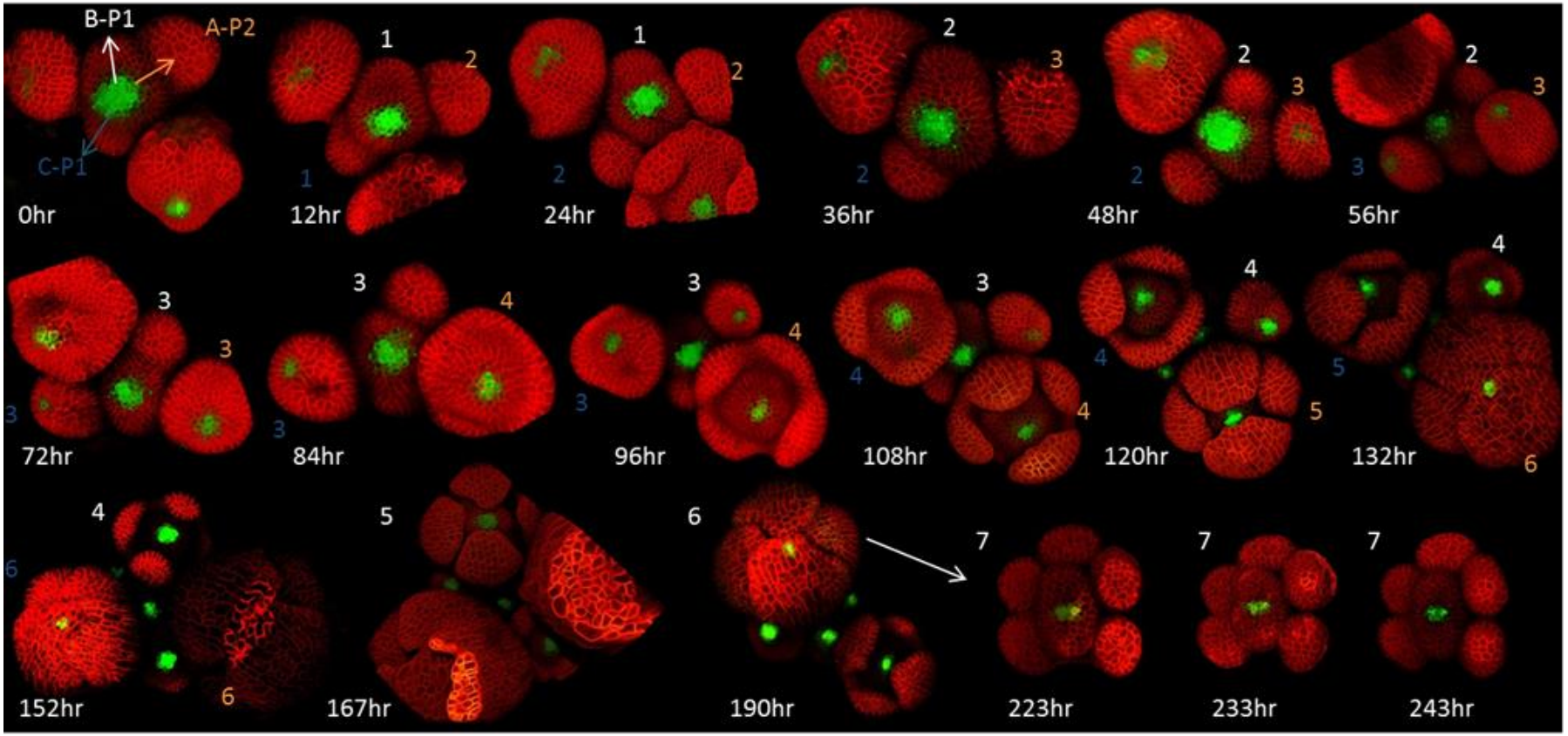


Figure 11. **Spatiotemporal CLV3 expression in Arabidopsis shoot**. The figure represents 3d image of live Arabidopsis shoot with different stages of flowers obtained from confocal z stacks over a period of time. Stem cells are marked green (pCLV3::mGFP5-ER) and plasma membranes in red (35S::YFP29-1). The arrows of different colours represent different primordia of interest (A, B and C). The primordia stages are marked with respective colours and are tracked over time. Sepal and petal primordia were dissected after stage 6 and further imaged for few hours in 223,233 and 243hr data.

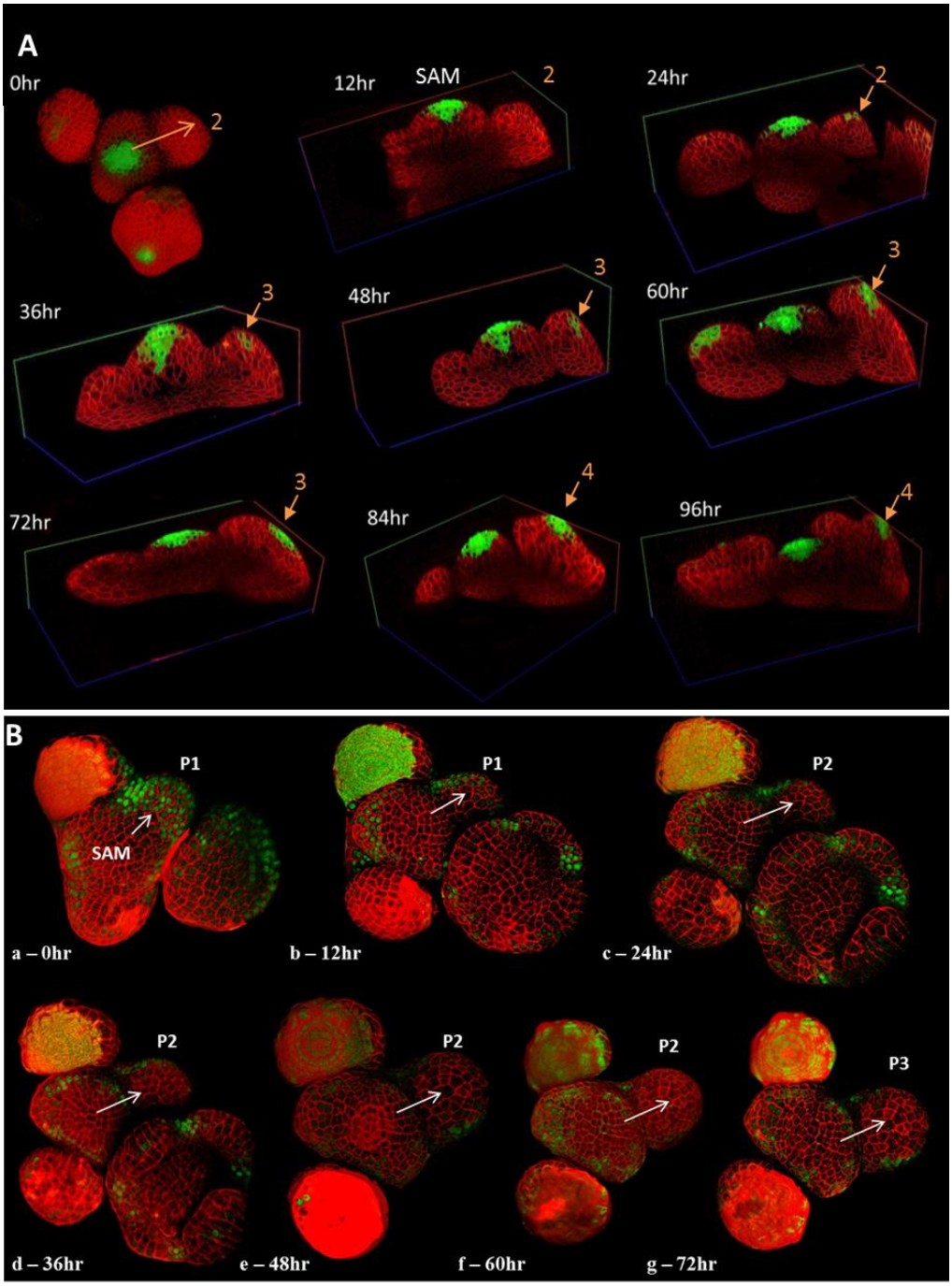


Figure 12. *CLV3* and *TAR2* expression in Arabidopsis shoot A: Stem cell specification in floral primordia. Depth analysis of the 3d view by taking transverse section and vertical section together. Primordia A from Fig 11 was followed here. Arrows indicate the stem cell in the floral meristem. The different stages of flower primordia were marked according to fig 12. 24hr data shows that stem cells are specified in L1 layer. Stem cells are marked green (pCLV3::mGFP5-ER) and plasma membranes in red (35S::YFP29-1). B: Spatiotemporal *TAR2* expression in Arabidopsis shoot. Figure represents the 3d image of live Arabidopsis shoot with different stages of flowers obtained from confocal z stacks over a period of time. pTAR2:: mcherry was marked green and plasma membranes in red (100ug/ml Propidium Iodide).

organs are initiated. Time series live imaging with *CLV3* and *PM29* marker reveals the expression pattern of stem cells in the floral primordia. In confocal imaging emerging flower primordia a, b and c was followed and three-dimensional representation of flowers of various stages were obtained (Fig. 11). The flower stages (till 7 stages) are marked according to the morphology. The stem cell localization in different stages of flowers can be seen in 3D (Fig. 11). But stem cell initiation occurs in few cells of floral primordia and it can't be visualised in 3D.

Stem cell specification can be better looked at the vertical section (Fig. 12a). The zero hour data points at flower primordia (Fig. 12a), which was followed. Stem cells are specified in between stage 2 and stage 3 of floral primordia (Fig. 11 and Fig. 12a) and stem cell specification occurs in epidermal or L1 layer (Fig 12a- 24hr). Stem cell domain expands from L1 layer to L2 layer during the later hours of development (Fig. 12a). This opens more ideas related to L1 specific molecular mechanisms in specifying stem cells on flower primordia. The data also gives a clear view of the morphology of different stages of flower primordia in the same plant.

3.2.2 Spatiotemporal expression of Auxin biosynthesis gene *TAR2* during flower development

Auxin triggers organ initiation at the periphery of shoot apical meristem by activating its biosynthesis. Previous studies have reported that PIN1 auxin transporter generates local auxin maxima and is responsible for organ initiation and cell identity changes. The live imaging data shows that auxin biosynthesis gene *TAR2* gets down regulated from stage 1 onward (Fig 12b). Thus, a local auxin minimum is required for stem cell specification in floral meristem. This further confirms perhaps WUS mediated repression of auxin biosynthesis is relevant to stem cell specification.

3.2.3 Discussion

This project presents more insight into the spatiotemporal specification of stem cell in floral meristem. Spatiotemporal cell fate maintenance, specification of cell identity and position in rapidly dividing cell population of floral meristem requires dynamic cellular communication. Previous findings suggest that stem cell specification is to repress differentiation, but how is stem cell specific gene expression of CLV3 is happening and what other molecular factors that gave them the freedom of pluripotency is still unknown.

Auxin promotes differentiation. Auxin transport integrates with auxin biosynthesis and other hormone signalling in order to have a coordinated cell behaviour and differentiation in the complex dynamic microenvironment. This project has expanded our understanding of auxin biosynthesis and stem cell specification in the floral meristem. Our data reveal that stem cell specification at the floral meristem happens in L1 layer and gets expanded to L2 layer and stem cells are specified in between stage 2 and 3 of flower development.

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