THE STUDY OF GENE EXPRESSIONS AND ABIOTIC STRESS RESPONSES DURING GERMINATION AND THE TRANSITION FROM SEED TO SEEDLING IN *ARABIDOPSIS THALIANA*

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ABSTRACT

Spatial-temporal gene expression distribution behaves differently, based on the gene, time and location. A series of GUS reporter lines were used to investigate the gene expression of *Arabidopsis thaliana* during the transition from seed to seedling at several time phases. Embryos were dissected at different times, from seed until prior to germination, then were immersed in X-Gluc for imaging of expression patterns. Expression patterns were present in a 2D whole embryo model. Starch GUS reporters were used to investigate the expression patterns of seed germination imbibed with water and with 20% PEG for five days. Gene expression showed variation in conditions.

Abiotic stressors, such as salinity and drought, are a severe problem in agriculture and the environment. Transgenic seeds gene from *PGMi*, *SEX1i*, *BAM* and *AAM* with an oestrogen inducible system were used to investigate the effect of salinity and drought stress on germination of *A. thaliana*.

Further study of the genes identified in salinity and drought germination was used to find gene orthologues from *A. thaliana* compared to *Capsicum annuum*. RNA samples were collected at 24 HAI (before the gene was induced) and 6 DAI (after the gene was induced). The genes are present, and they are expressed in chilli. The way genes are controlled in *Arabidopsis thaliana* and *Capsicum annuum* are not the same.

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LIST OF ABBREVIATIONS

℃	Degree celcius
%	Percentage
bp	Base pair
g	Gram
L	Litre
Mb	Megabyte
mg	Miligram
ml	Millilitre
mm	Milimetre
mM	Milimolar
ng/µl	Nanogram per microlitre
µg/ml	Microgram per millilitre
µg	Microgram
µL	Microlitre
µM	Micromolar
pH	Potential of hydrogen
v/v	Volume per volume
w/v	Weight per volume

AAM	α-amylase
AAO3	Abscisic aldehyde oxidase 3
ABA	Abscisic acid
BAM	β-amylase

BC	Before Christ
BLAST	Basic local alignment search tool
cDNA	Complimentary deoxyribose nucleic
CO_2	Carbon dioxide
CWRE	Cell wall remodelling enzyme
DAI	Day after imbibition
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
EST	Estrogen
EtBr	Ethidium bromide
EXPA	Expansin
FAO	Food and Agriculture Organization of the United Nations
GA	Gibberellic acid
GUS	β-glucuronidase
HAI	Hour of imbibition
HCl	Hydrochloric acid
HYG	Hygromycin
JG	Just germinate
JTR	Just testa rupture
KOH	Potassium hydroxide
MARDI	Malaysian Agricultural Research and Development Institute
$MgCl_2$	Magnesium chloride
MS	Murashige & Skoog
NaCl	Sodium chloride

NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
PAPI	Periodic acid propodium iodide
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PGM	Phosphogluocomutase
QTL	Quantitative trait loci
RNA	Ribonucleic acid
RPM	Rotation of a machine expressed in revolution per minute
RT PCR	Reverse transcriptase polymerase chain reaction
SEX1	STARCH EXCESS1
SLY	SLEEPY
SOL	Solanaceae
STR	Super testa rupture
T	Generation
TAIR	The Arabidopsis Information Resources
TBE	Tris/Borate/EDTA
UV	Ultra violet
WT	Wild type
X-Gluc	5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid

CHAPTER 1

INTRODUCTION

1.1 Introduction

The global population is currently increasing annually, from the 19th to the 21st century. In 2010, the population was approximately 6 billion, but did not exceed 7 billion. Ten years later (2020), the population hit more than 7.5 billion. The global population is expected to increase to more than 9 million, as shown in Figure 1.1. The population is increasing at the same pace as food production, which will lead to a significant shortage of food due to the increase in global demand.

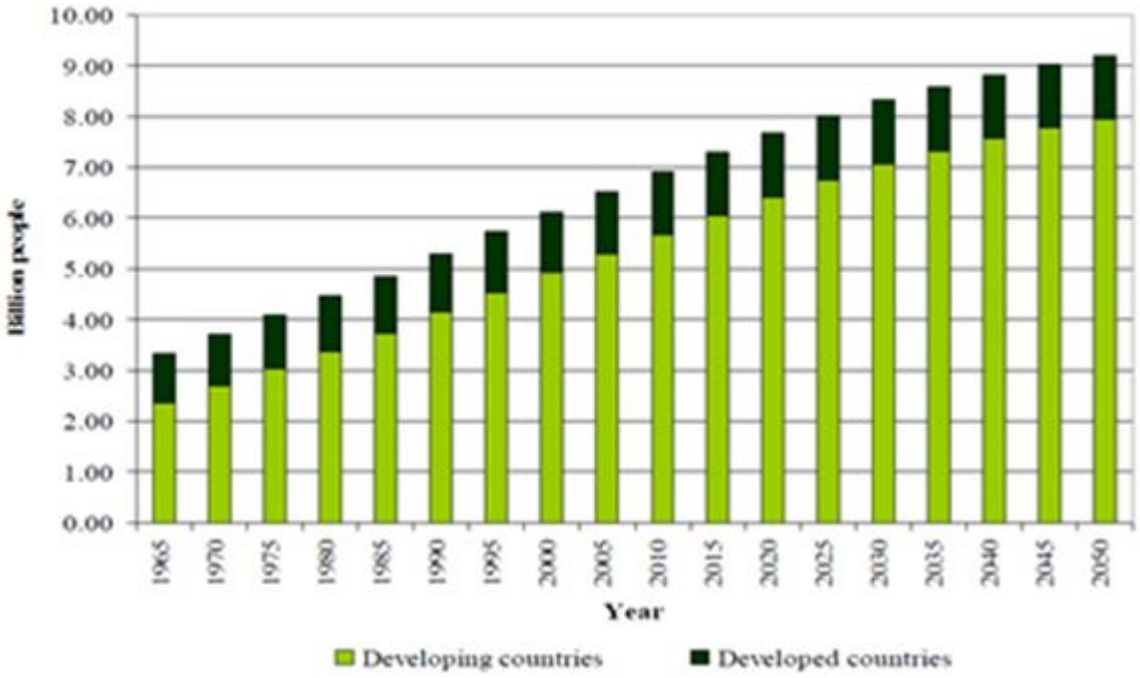


Figure 1.1: The global population for the developing and developed countries from 1965 to 2050. The population has been increasing since 1965 and is expected to increase until 2050. Source: Population Division of the Department of Economic and Social Affair of the United Nations Secretariat (2007).

The objective of food security reflects the population's capacity to access enough nutritious food and must be considered as a requirement of life. If the global population increases at the same pace as the rate of food production, it will lead to a significant shortage of food due to the increase in demand. This implies that global food supply production is correlated with food security. In the same vein, climate change impacts food security because it limits food production output. Climate change has the propensity to affect seed germination; for example, drought causes soil to become too dry, thereby upsetting seed germination due to the absence of water.

Why not grow more crops to produce more food and meet the demand? It is not that simple because environmental factors limit crop yield and food production (Parry *et al.*, 2004). When the production of crops and yield is reduced due to climate change, seed quality will decline and directly impact the next generation of crops (Finch-Savage and Bassel, 2016). Therefore, high-quality crops and originate from high-quality seeds as the starting material. As a result, this research focuses on understanding the seed and its germination process, and finding a solution to low crop yield and production.

1.2 The importance of seeds and germination

A seed is a small part produced by a plant: when sown in the soil, it provides a new plant and establishes a future generation. The seed is the plant's method of ensuring that generations are distributed from time to time, which ensures their success. Seeds are considered as embryonic plants, and are protected by the outer covering layer which enfolds it. As is evident from different seed types, the embryo is bounded by endosperm, perisperm or other tissue that may limit the radicle growth (Georghiou, Psaras and Mitrakos, 1983; Dahal and Bradford, 1990). Contained in each seed is a formed embryo and genetic material

to create a new plant. Seeds form to become seedlings and the mature plant. During this development, the seed expands and continues to nurture.

1.2.1 Economy

Seeds also play an essential role in the agricultural sector of every country. Seed quality is essential for crop productivity and food security (Finch-Savage and Bassel, 2016). Good-quality seeds have a high germination rate, are free from disease, have the same maturity time for harvesting and reduce cost and time. Planting good-quality seeds give a high yield and profit in return. These contribute to the economic growth of the agricultural sector and reduces the poverty of a nation. (Irz *et al.*, 2001) determined that rural poverty is reduced as agricultural job opportunities are created, which enhanced the rural economy.

1.2.2 Food security

The importance of seeds and germination must be considered in terms of food security. Today, the global population is rising daily. Food protection is the individuals' capacity to have adequate access to a healthy food supply to meet their requirements. The increasing population generating the same amount of food would lead to a shortage of food resources due to the rise in global demand. The global food supply output relates to food security. Food protection is intended to ensure the world's food sustainability by providing food at all times. Climate change also affects food security because, with low yields, it could reduce food production. Germination is impaired by climate change; for example, drought causes the soil to become too dry, contributing to water retention failure. The increase in temperatures in tropical regions also appears to reduce the production of crops and yields. Consequently, food production is reduced by climate change. To ensure that performance can meet population growth, high-quality seed production is essential.

1.2.3 Agriculture

Seeds are critical to agricultural activity. Seeds are essential in planting, thus, they play a crucial role in agriculture. The quality of seed will be identified by better yield and high productivity. Low quality seeds may impact the growth and development of the plant. In terms of physical condition, a seed of excellent quality can be classified as being uniform size, weight and free from disease. Delouche (1969) stated that the quality of seed is determined by the ability of the seed to establish a full stand of vigorous uniform seedlings, which can grow into productive mature plants. Rapid uniform germination of the seed will be advantageous in uniform maturity for harvesting. A high-quality seed will possess good root development and efficiency that supports the absorption of nutrients and promotes high yield.

Agricultural operations are based on seeds and germination. Seeds are essential as starting material for the next generation of their species and in sustaining crop production. Seed quality, in exchange for global economic growth, would affect high yields. Seed quality improves germination and contributes to greater yields for harvesting and productivity. In the next growing season, the seed is used by the producer as plant material. Realising the importance of seeds in the agriculture industry, which translates to the global population benefits, the quality of seed is critical.

Seed quality is the first significant characteristic in the development of a new plant. The total genetic complement from the preceding crop will be borne by the seed (Finch-Savage and Bassel, 2016). Therefore, farmers need a seed of excellent quality to ensure that their crop is grown consistently and successfully because the genetics are carried for each generation (Ennos, 1994). Thus, the output's productivity and profitability are ensured by

having excellent seed quality. Farmers can hold the seeds as stock, and develop a seed bank for future use.

1.3 *Arabidopsis thaliana* seeds and the germination process

Arabidopsis thaliana is a tiny plant from the mustard family (Cruciferae or Brassicaceae). It is a flowering plant that forms a simple angiosperm seed. *Arabidopsis* is broadly used as a plant model for biology and molecular genetics. It is widely distributed in North America, Europe and Asia (Meinke, 1998). *A. thaliana*'s has a short life cycle; thus, it is a small plant that can produce a large number of offspring and has a relatively small nuclear genome (The Arabidopsis Genome Initiative, 2000). It is also easy to cross-pollinate *A. thaliana* by hand, as well as artificially inbreed. Furthermore, the number of chromosomes (2n = 10) is low in comparison to other plants.

1.3.1 *Arabidopsis thaliana* seed anatomy

Arabidopsis seeds are produced from the double fertilisation of flowering angiosperm species. The female gamete fuses with the male gamete, providing three separated tissues called embryo, endosperm and testa. The protective coat layer around the embryo is called the testa, which is composed of cells and dies during maturation (Debeaujon *et al*., 2000). The testa is associated with endosperm for additional support and protection of the embryo. They play essential roles in regulating dormancy and seed germination (Debeaujon *et al*., 2000).

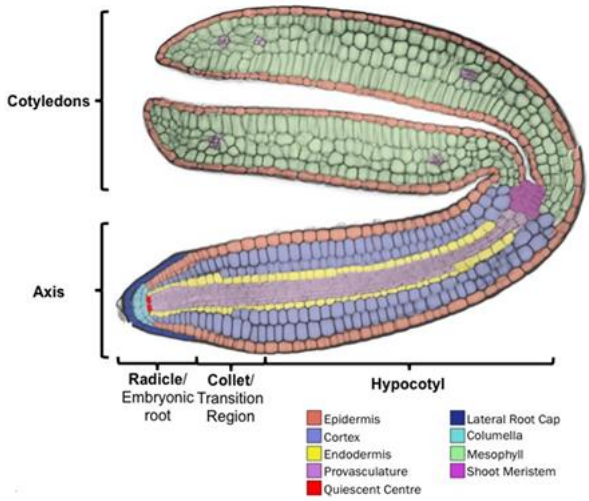


Figure 1.2: 2D model of the seed anatomy of the mature dormant *Arabidopsis thaliana* embryo. Different colours are used to represent separate cells within the embryo of *A. thaliana* seed.

The aleurone layer, also known as the endosperm, is a single-layer cell that connects directly to the embryo. Endosperms play a vital role in regulating seed germination (Bewley, 1997; Kucera, Cohn and Leubner-Metzger, 2005). While an embryo, it is enclosed with endosperm and testa. Once the endosperm ruptures, the seed starts to germinate. The embryo will form roots, stem and cotyledons. The storage component is synthesised and accumulated in the embryo during maturation.

The radicle is embryonic root growth that develops from the seed during germination and grows downward into the soil. After the radicle lengthens, it will expand and rupture the seed coat layer. The stretching of the radicles causes the endosperm to rupture, followed by the breakdown of testa. The rupture of the seed coat results in the protrusion of radicles.

Hypocotyl, the radicle (the root of the embryo), grows out to get the hypocotyl. The short stems are located above the root, pushing the cotyledons above the ground.

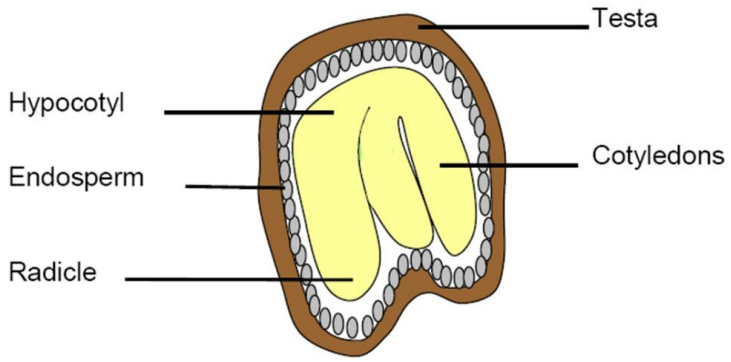


Figure 1.3: *Arabidopsis thaliana* seed components of the separated seed component after the fertilisation. The testa is the outermost layer, and the single layer after the testa is the endosperm. Figure adapted from (Holman *et al.*, 2008).

1.3.2 *Arabidopsis thaliana* germination

Arabidopsis seeds develop from germination. Germination commences with the uptake of water by the seed. The dried seed absorbs the water and interactions of phytohormones start the chemical reaction for seed germination. The seed absorbed with water germinate, and the seeds expand in size. Matured dried seeds undergo a triphasic water uptake (Bewley, 1997; Kucera, Cohn and Leubner-Metzger, 2005; Finch-Savage and Leubner-Metzger, 2006). Based on the germination phase, the triphasic water uptake model by (Bewley, 1997) shows that the germination process comprises various phases (Figure 1.4). The first stage is the imbibition process, which involves a rapid initial uptake of water into the seed. This is followed by the plateau phase, in which the water content changes during water uptake. Next is the post-germination phase, in which there is increased water uptake and water content. During this time, the radicles emerge and embryonic axes elongate.

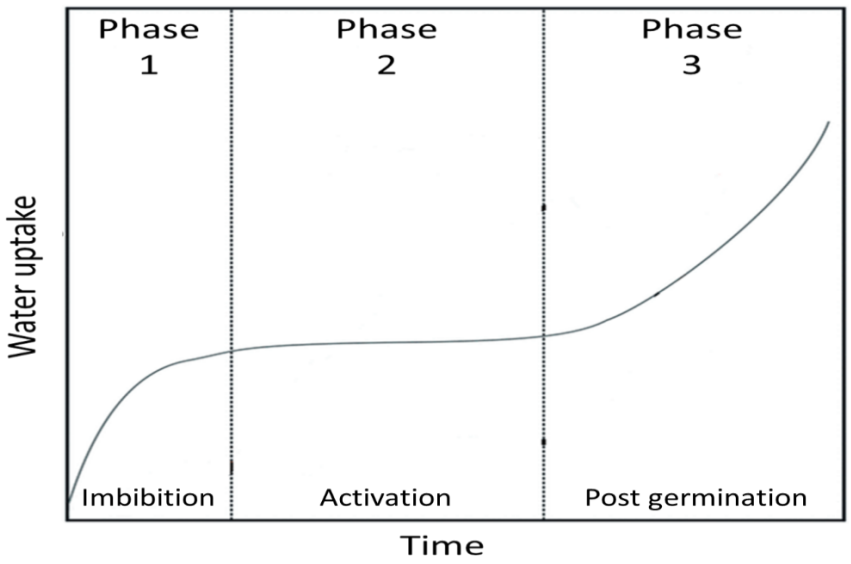


Figure 1.4: The triphasic water uptake and developmental time phase for seed germination, adapted from (Bewley, 1997). The three crucial stages of seed germination are the imbibition phase, activation phase and post-germination. Massive water uptake occurs during the imbibition and activation phases.

The rupture of the protective coat (testa rupture), or the rupture of zygotic tissue and the endosperm, is the first external sign during germination. The protrusion of radicles results from germination (Leubner-Metzger *et al.*, 1995; Liu *et al.*, 2005) in plant species, such as Arabidopsis and tobacco. At the time of the absorption of water in phase 1 (Figure 1.4), the seed rapidly swells and changes in size and shape (Robert *et al.*, 2008; Preston *et al.*, 2009), and the changes in seed membrane leads to the rupture, causing the leakage of solute and low molecular weight metabolites into the imbibed solution.

(Woodstock, 1988) verified that dry seeds have very low water potential, which leads to a quick uptake of water during phase I. Phase II, the activation stage, represents the seed's metabolic germination and how water potential increases. The rate of water uptake and changes in seed size and shape begins to stagnant at this stage and, therefore, water remains

stable at this stage. Phase II comprises the testa rupture of the seed. In this phase, the seed's water potential becomes lower than the solute, allowing the water uptake to the seed. The mitochondria are repaired, resulting in the initiation of synthesis. DNA repair is also triggered. During phase III (post-germination), the radicle cell elongates using the mobilised reserved store, then seedling growth starts. The cells divide, and DNA is synthesised to develop into a new seedling (Bewley, 1997).

1.4 Factors that influence the germination process

Environmental factors are the main issue in agriculture that reduce crop productivity (Wang *et al*., 2003) and promote seed dormancy. Seed dormancy is where the matured seeds cannot germinate until optimal conditions of temperature, water and light are met. Seed dormancy is vital for the existence of seeds. During the seed dormancy process, a period of time is allowed for the dispersal of matured seeds. Delays in seed germination (dormancy) are due to the period needed for embryo growth and radicle emergence after the matured seed has been dispersed (Baskin and Baskin, 2004).

1.4.1 Salinisation

Salinisation is the state in which salt deposition accumulates in the soil; because of the change in the possible gradient of osmosis, this will damage the plant. Osmotic pressure can be changed by the water-soluble salt and can prevent cellular activity (De Azevedo Neto *et al.*, 2004; Zhu, 2016). During salinisation, the high salt concentration in the cytoplasm of the plant cell disturbs the ratio of ions, such as Na+/K+. With the increase of Na+, the potential for water absorption is reduced. It can also alter the function of transporter enzymes (Conde *et al*., 2011).

1.4.2 Temperature

Seeds have an optimal temperature range for germination. The minimum temperature is the lowest temperature at which the seed can germinate, while the maximum temperature is the highest temperature at which the seed can germinate. The optimum temperature is the ideal and the best temperature for the seed to grow. If the temperature is either too low or too high, it will damage the seed, leading to dormancy.

(Robert, 1988) reported that the physiological processes of seeds are often affected by temperature. There are three physiological processes. The first process is the impact of temperature on the moisture content, which determines the deterioration rate in seeds. The second process explains the role of temperature in dormancy loss in dry seeds, and the dormancy change pattern in moist seeds. Finally, in dormant seeds, temperature determines the rate of germination. All those processes are interrelated in contributing to seed germination.

When the temperature becomes very high, seeds develop complications. In soybean crops, the quality is affected when subjected to extremely high temperatures during seed development. High temperatures also decrease the rate of seed germination and vigour in growth chamber and phytotron experiments (Dornbos and Mullen, 1991; Zanakis *et al.*, 1994). In seed dormancy, specific high and low temperatures are essential to break seed dormancy to initiate germination. Seed stratification is defined as dormancy is induced as the preparation for germinations through low temperature. When the seed is exposed to low temperatures, it may influence the dormancy or stratification.

1.4.3 Water

Moisture is vital for seed germination. Seeds might not absorb water if the soil is too dry, often leading to death. Seeds absorb water to trigger metabolic functions; a biochemical mechanism required for germination and growth. When the seed absorbs adequate water, the cell goes through turgor pressure and expands in size. The radicles elongate and the embryo ruptures the seed coat. Water plays an important role in softening the hard seed coat of the seed: the seed coat becomes soft or moist when soaked in water to initiate germination. Therefore, water can be responsible for poor germination. Drought restricts the cell's water uptake and is one of the environmental factors that reduces agriculture output. Barley (Hordeum vulgare L.) is an essential cereal widely grown in dry and semi-arid regions in developing countries. It is, therefore, facing severe drought conditions. To resolve this situation, drought tolerance in the plant is necessary to breed the best seeds for drought adaptation (Amini, 2013).

Overwatering can cause the soil to become too moist, which leads to waterlogging. Waterlogged soil has the propensity to reduce the oxygen level in the ground, which can affect germination. When the soil is too moist, it becomes waterlogged. The seed fills with water and drowns, causing the seed to die because it is unable to get enough oxygen from the soil. If the seed is planted too deeply, it uses its stored energy before reaching the soil surface.

1.4.4 Oxygen

Another requirement for germination is oxygen. Soil aeration is essential for oxygen and carbon dioxide exchange and germination. Proper air movement is also beneficial for disease prevention: waterlogged and soggy soil can lead to root disease. It is crucial to have

oxygen as a requirement for seed germination, given its importance for the growth of seed plants. (Bradford *et al.*,2007) conducted a study on oxygen sensibility in determining seed germination by using a population model. The percentage of oxygen available can be scaled to germination rate, demonstrating that the flow of oxygen has an impact on seed germination. A review by (Al-Ani *et al.*,1985) demonstrates that low-pressure oxygen has a different effect on various seeds, depending on their sensitivity. Seeds with a high lipid content tend to be more sensitive to low oxygen pressure than seeds that contain high starch.

1.4.5 Light

Another condition for germination is the availability of light. The absence of light means that seeds remain dormant until light becomes available. Not only the presence of light affects germination, but so does the degree of light and absorption. Some seeds, like Arabidopsis and lettuce, require light to germinate well (Yamaguchi and Kamiya, 2002), some germinate better in full sunlight, while others require darkness. Some seeds stay dormant until they receive sufficient light to initiate growth. Germination is enhanced by light, as red light induces germination. In contrast, far-red light irreversible inhibits germination (Borthwick *et al.*, 1952). As the seed germinates, it will continue to grow as a seedling, requiring light for further establishment. The seedling needs light as energy for photosynthesis and a source of food.

1.4.6 Phytohormones

Plants only can adapt to everyday stress with the help of phytohormones. Phytohormones play an essential role in adjusting to environmental stressors, such as drought and salinity. The level of phytohormones fluctuates based on the changes of genes involved in their biosynthesis, and the responses they regulate (Fahad *et al.*, 2014). The plant hormones

abscisic acid (ABA) and gibberellic acid (GA) promote dormancy and initiate germination, respectively. According to the review by (Donohue *et al.*, 2010), the seed which has a high level of dormancy might delay germination and can then reduce the growing season. Conversely, germination can be observed in seeds which have a low level of dormancy. Phytohormones play essential roles in cell growth development and plant cell maturity. They regulate the speed of development and play crucial roles in the processes of reproduction and death. Phytohormones, also called hormones, are the growth regulators produced naturally by the plant. Plant hormones are very different from animal hormones. The plant coordinates its behaviour against environmental changes using hormones.

There are four essential plant hormones: auxin, GA, cytokinin and ABA, which are involved in plant control and coordination. This research focuses only on GA and ABA because the germination and growth of seeds are interrelated. The signalling pathway of phytohormones is the cell signal, the hormone and plant cell's communication process that coordinates multiple cellular actions in responding to the cellular microenvironment.

1.4.6.1 Gibberellins

GAs are a group of tetracyclic diterpenes that play an essential role in the pathways and mechanisms of growth and development. GAs regulate plant growth and development, including controlling seed germination, stem elongation, leaf expansion and flower development (Yamaguchi, 2008). More than 100 GAs have been identified but the major bioactive GAs included GA_1, GA_3, GA_4 and GA_7. GA_1 has been identified as a command bioactive GA because it is widely present in various plants (MacMillan, 2002).

In higher plants, GAs are generally synthesised from the methylerythritol phosphate (MEP) pathway (Hedden and Thomas, 2012). Bioactive GA is produced from trans-geranylgeranyl diphosphate (GGDP). There are three separate enzymes involved in yielding GA from GGDP: terpene syntheses (TPSs), cytochrome P450 monooxygenases (P450s) and 2-oxoglutarate- dependent dioxygenases (2ODDs). These enzymes must generate bioactive GAs from a precursor, geranylgeranyl diphosphate (*GGDP*) (Yamaguchi, 2008; Hedden and Thomas, 2012). The MEP pathway involves several steps, and takes place within the plastid. In summary, the TPSs enzyme ent-copalyl diphosphate synthease (ent-CPD) and ent-kaurene synthese (KS) convert GGDP into the ent-kaurene compound. The oxidation process happens in the plastid and endoplasmic reticulum when kaurene oxidase (KAO) is associated with enzyme P450s (Hedden and Thomas, 2012). The final step occurs in the cytosol, where oxidation occurs via the two soluble 2ODDs enzymes *GA20-OXIDASE* (*GA20ox*) and *GA 3-OXIDASE* (*GA3ox*). Thus, GA12 is converted into bioactive GA_1 and GA_4 (Yamaguchi, 2008).

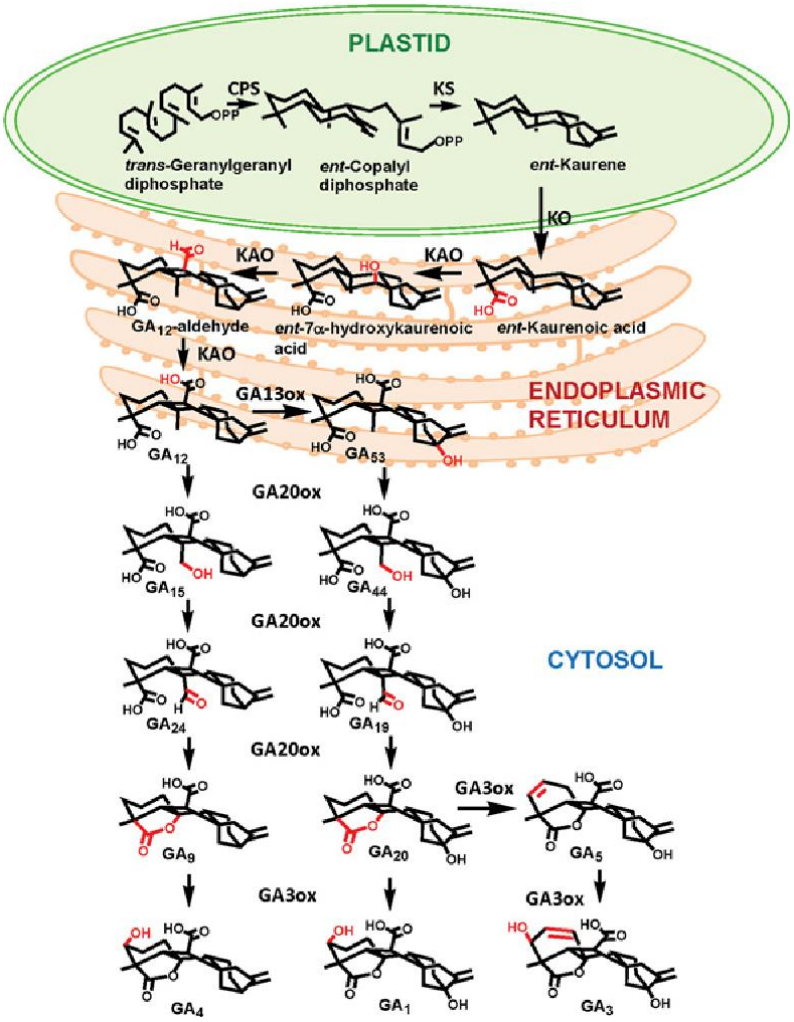


Figure 1.5: Gibberellic acid (GA) biosynthesis pathway from trans-geranylgeranyl diphosphate (GGDP) to GA1 and GA4. The reactions are indicated with three-dimensional structures, and the functionality introduced by each oxidative step is shown in red. The subcellular compartmentalisation of the pathway and the enzymes catalysing each stage are also indicated. GA13ox and GA 13-oxidase. Figure and legend adapted from Hedden and Thomas (2012).

Following the MEP pathway, the bioactive GA is produced. At this stage, the level of endogenous GA increases and starts to be perceived in the embryo. The primary function of GA is in stimulating the germination and degradation of the DELLA protein. DELLA acts as a negative growth regulator in seed germination. A previous study proposed that GA was perceived by an unidentified plasma membrane receptor (Lovegrove *et al.*, 1998); however, the GA Intensive Dwarf Mutant Allele is found in the GID1 (GID1) rice gibberellin receptor (Ueguchi-Tanaka *et al.*, 2007). There are three orthologs of *GID1*, *AtGID1a*, *AtGID1b* and *AtGID1c*, in Arabidopsis (Hedden and Sponsel, 2015). GID1 has a high affinity for bioactive GA (Ueguchi-Tanaka *et al.*, 2007). The GA-GID1 complex results from bioactive GA binding to the GID1 receptor binding pocket. The binding changes the conformation structure before binding the GA-GID1 complex with DELLA protein (Ueguchi-Tanaka *et al.*, 2007). The DELLA protein also changes its structure in the binding of the F-box protein (SLY1 in Arabidopsis, GID2 in rice) (Davière and Achard, 2013). The F-box protein is a vital component of the SCF E3 ubiquitin ligase complex and promotes the degradation of DELLA protein by 26S-proteasome (Davière and Achard, 2013).

Gibberellin reactions occur when gibberellin stimulates the breaking of the DELLA protein. In the process, Gibberellin binds to its receptor Gibberellin insensitive dwarf1 (GID1), which reacts with the DELLA protein. The GID1A-GA-DELLA complex will interact with SLEEPY (SLY). When SLY binds to DELLA, it removes the DELLA, and the DELLA protein is denatured. DELLA blocks germination; since DELLA is removed, GA activates the gibberellin, which responds by allowing germination. Dormancy increases as the accumulation of the DELLA repressor protein increased.

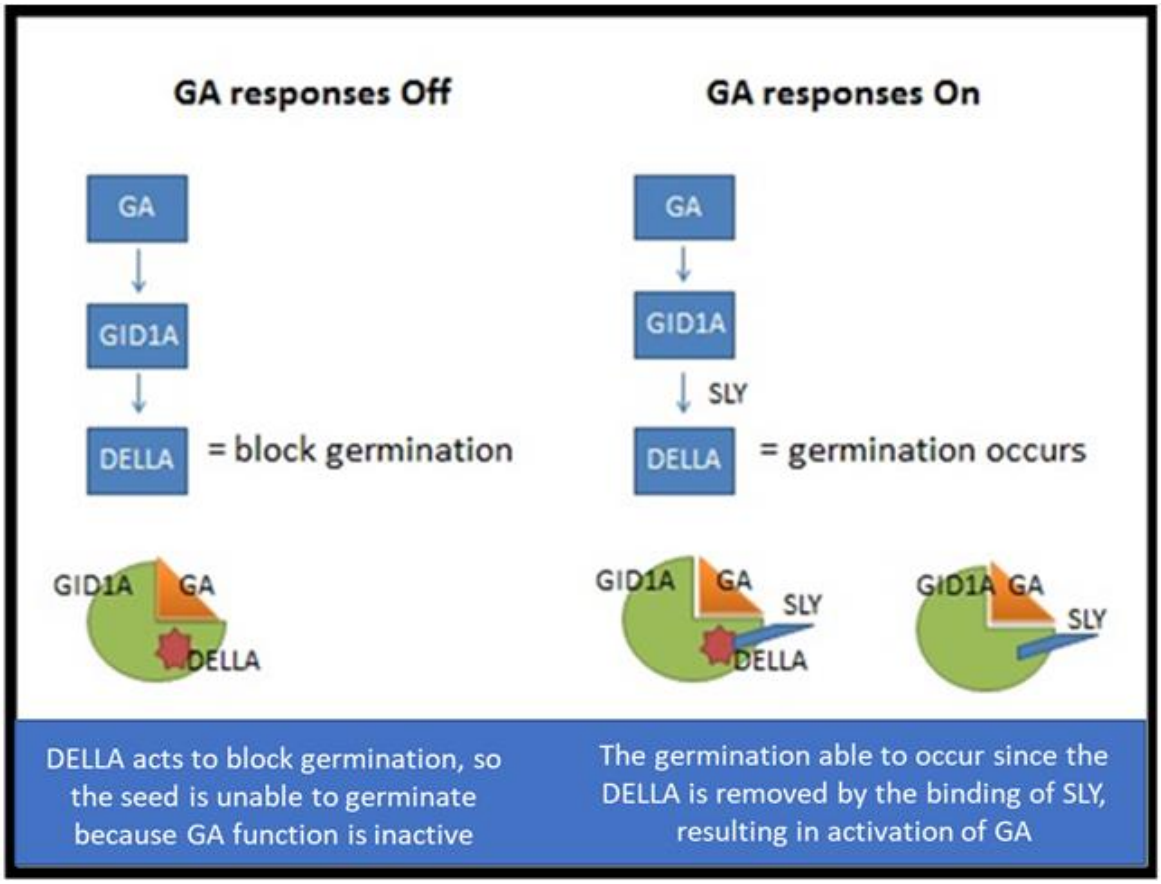


Figure 1.6: Simple illustration of the gibberellic acid (GA) pathway. The DELLA protein is removed when SLY binds to DELLA, which causes germination.

1.4.6.2 Abscisic acid

ABA is a natural plant hormone involved in most plant developmental processes. ABA promotes dormancy in seed and bud opening, organ development and encourages stomata closing. It is crucial to respond to environmental changes, such as salinity, drought, flooding, heat and heavy metals (Finkelstein, 2013). ABA-mediated signalling also plays an essential role in the plant's response to environmental stress and plant pathogens (Zhu, 2016). ABA is produced in the root and at the top of the plant bud to decrease soil water potential when under stress. The alteration of osmotic water potential causes the shrinking and closing of the stomata due to ABA's translocation to the leaves. ABA is produced in the

terminal bud of the plant in preparation for winter. During the cold season, plant development is slowed to protect the dormant buds (Wang *et al.*, 2016).

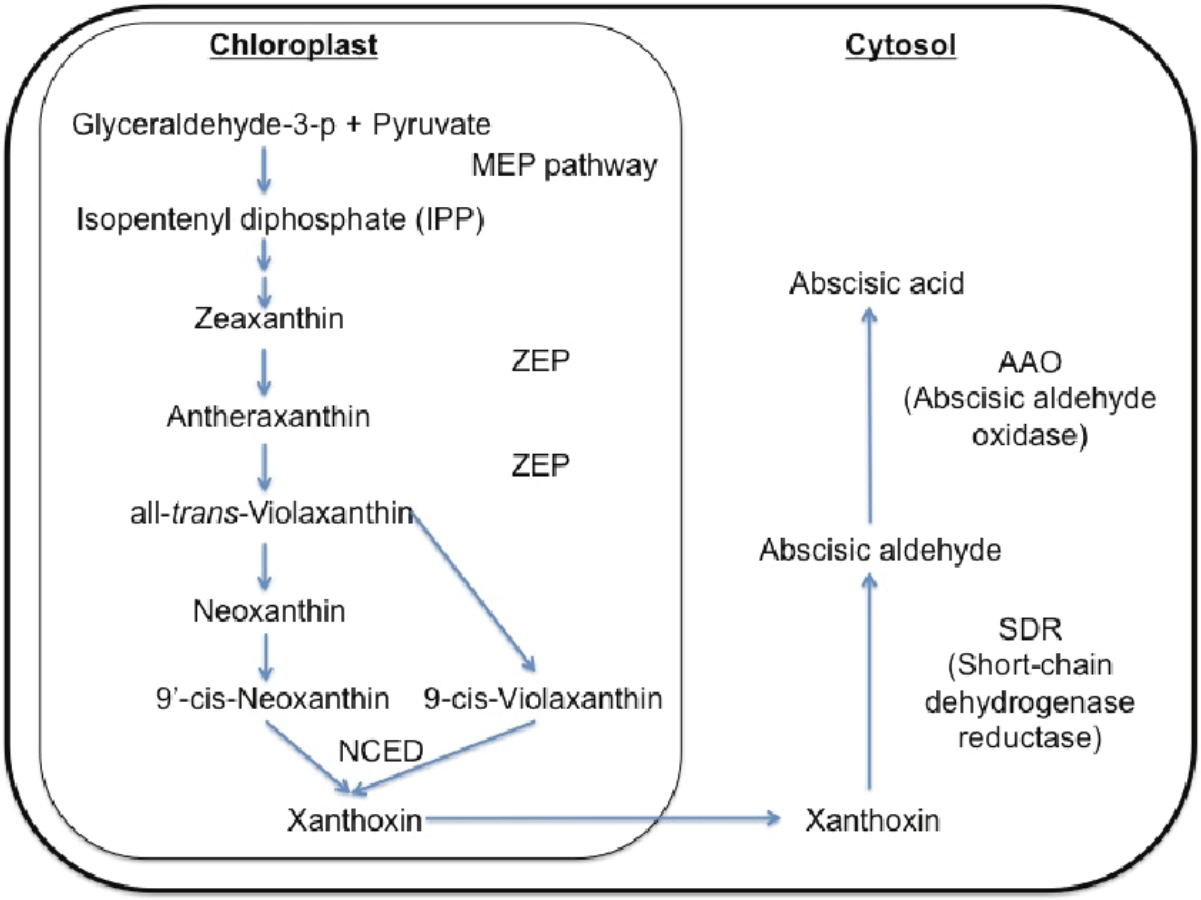


Figure 1.7 Abscisic acid (ABA) biosynthesis in the plant, figure adapted from (Kao, 2014). MEP occurs in the chloroplast. The IPP is converted to zeaxanthin by the ZEP enzyme, then is deoxidised by NCED into xanthoxin. The ABA 2 turns the xanthoxin into AAO3.

Following the MEP/IPP pathway, an embryo of the seed perceives an increase in endogenous ABA. ABA receptor pyrabactin resistance (PYR), type 2C protein phosphate (PP2C) enzyme and subfamily 2 SNFL-related kinase (SnRK2) serine/threonine kinase enzymes control the responsive of ABA. When ABA is absent, protein kinase (subfamily 2) (SnRK2s) activity is inhibited by type 2C protein phosphate (PP2C) enzyme through the

removal of activating phosphate. Intracellular PYR/PYL then binds to the ABA to form the ABA receptor-PP2C complex (Park *et al.*, 2010). The formation of the ABA receptor-PP2C complex inhibits the activity of PP2C in ABA-dependence and allows the activation of SnRK2s. When ABA is present, the phosphate activity of the receptor is blocked, protein kinase is released and phosphorylate regulates ABA signalling's critical target.

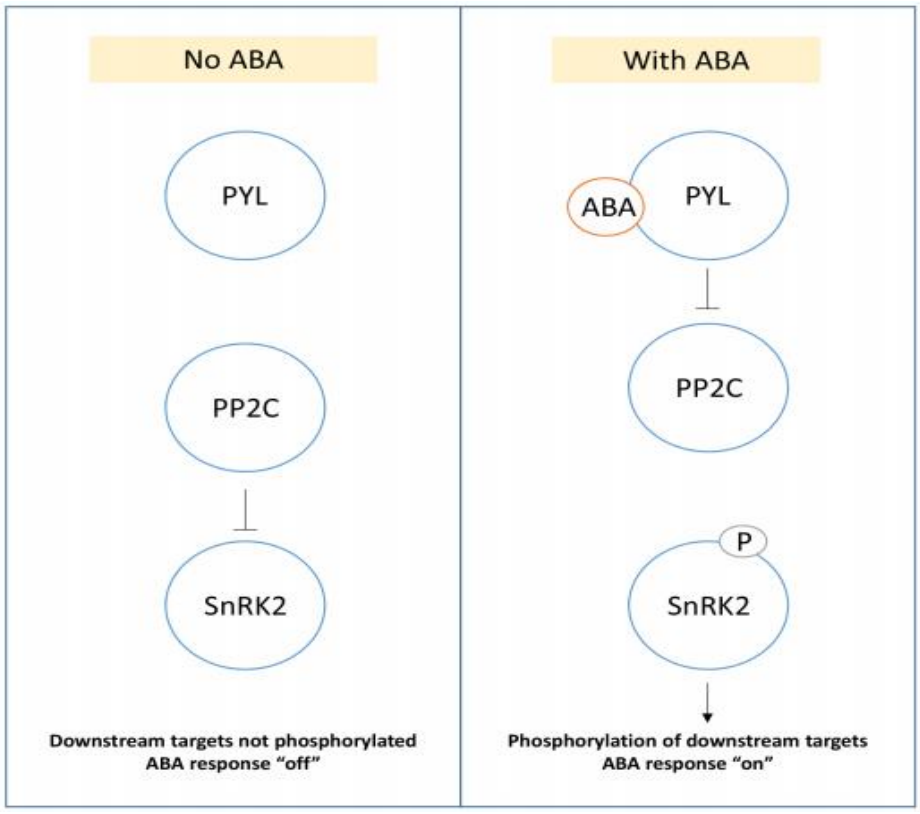


Figure 1.8: Schematic representing the abscisic acid (ABA) signalling pathway. In the absence of ABA, PP2C phosphatases inhibit the activity of SnRK2 kinases, preventing the downstream phosphorylation of downstream substrate targets. In ABA's presence, the PYR receptor binds ABA forming the PYR-ABA complex, which binds PP2C, alleviating inhibition SnRK2. Activated SnRK2 then phosphorylates downstream substrate targets, enabling ABA responses. Figure and legend adapted from (Ng, 2016).

1.5 Seed dormancy

Seed dormancy is interconnected with germination. Seed dormancy refers to the situation in which matured dry seeds cannot germinate until conditions become conducive for germination. Dormancy is essential to avoid the seed from growing; it guarantees that the seed only germinates in optimal temperature, water and light. Seed dormancy is vital for the existence of seeds. During the seed dormancy process, a period of time is allowed for the dispersal of matured seeds. Delays in seed germination (dormancy) are due to the period needed for embryo growth and radicle emergence after the matured seed has been dispersed (Baskin and Baskin, 2004). In contrast, a non-dormant seed is characterised by its ability to germinate under favourable conditions of steady temperature, water and sufficient light.

A non-dormant seed can germinate after the dormant stage, under physical environment factors. During seed dormancy and germination, natural plant hormones ABA and GA promote dormancy and initiate germination, respectively. According to the review by (Donohue *et al.*, 2010), seeds with a high level of dormancy might delay seed germination and can then reduce the growing season. Conversely, germination can be observed in seeds which have a low level of dormancy. In cereal, seed quality is reduced in seeds with low dormancy, because the quality has been reduced during sowing and the period before pre-harvesting sprouting (Gubler, Millar and Jacobsen, 2005). Seed dormancy can be terminated in a variety of ways to induce germination, including seed coat disturbance (scarification), cold treatment (stratification), dry storage (after-ripening) and light exposure (Finkelstein *et al.*, 2008).

1.5.1 Primary seed dormancy and secondary seed dormancy

Dormancy is vital in ensuring that seeds germinate under favourable conditions. Dormancy is classified into primary dormancy and secondary dormancy. Primary dormancy is when the seed is released from the mother plant already in a dormant state, and dormancy has been induced since seed maturation (Hilhorst, 1995). At this time, the high concentration of ABA prolongs and prevents the seed from germination. Primary dormancy is essential because it is related to seed development and maturation. It is present at dispersal. Secondary seed dormancy is the seed's situation after being released due to environmental factors. It occurs after seed dispersal and is subject to an annual dormancy cycle. Secondary dormancy is the continuation of primary dormancy. If too many seeds are in the soil, dormancy is induced when non-dormant seeds do not have favourable conditions for germination, including water, temperature, oxygen, light and nitrates (Hilhorst, 1998). During secondary dormancy, the seed becomes sensitive to GA, breaking the dormancy and initiating germination.

1.6 Starch

Starch is composed of two glucose polymers, amylopectin and amylose. To form linear chains, the glucose units are joined by alpha-1,4-glucosidic bonds, then connected to the amylopectin branches of alpha-1,6-glucosidic bonds (Streb and Zeeman, 2012). Amylose is a more tiny molecule because it has a much lower branching point, while amylopectin is more solid because it takes 70–90% of the granules' weight (Denyer *et al.*, 2001). Starch is usually accumulated in leaves, but in non-photosynthetic plant tissue, starch often accumulates in seeds, roots and tuber (Streb and Zeeman, 2012). This is referred to as "storage starch", stored in the heterotrophic cell. Amyloplasts produce energy for future use.

Starch is the main energy source of plants. It is essential to convert starch into the simplest molecule as an energy fuel for germinating seeds and developing plants. The endosperm is contained in seeds as food storage. In rice, after four days of germination, the breakdown of reserve polysaccharides (starch) in the endosperm starts (Murata *et al*., 1968). For Arabidopsis and most vascular plants, starch plays a crucial role in the daily metabolism of carbohydrates in the leaf (Streb and Zeeman, 2012). Starch accumulates in many Arabidopsis tissues in the daytime, but mainly in leaves. At night, starch is degraded to provide a constant source of carbohydrates in the absence of photosynthesis (Streb and Zeeman, 2012).

Cotyledons store food in seeds, and it provide nutrients and energy for plant growth. Starch is turned into the simplest soluble molecules (sugar) by amylase enzymes. The stimulated gibberellin encourages the production of amylase with the presence of water. Then the starch is broken down to maltose, this allows ATP to produce energy in the embryo to facilitate germination. Arabidopsis is an oilseed; little is known about the role of starch accumulation in the developing oilseed embryo. During seed development, the starch is believed to act as a carbon reserve to synthesise lipid and sugar (Leprince *et al*., 1990).

1.7 Plant stress responses

During development, plants are exposed to abiotic or environmental stress. Drought and salinity are common in agriculture, and causes a problem during seed germination. A reduction of more than 50% in average crop yields has been attributed to abiotic stress (Wang *et al*., 2001). Plants only can adapt to everyday stress with the help of phytohormones. Phytohormones play an essential role in adjusting to environmental stress, such as drought and salinity. The level of the several phytohormones fluctuates based on the

changes in genes that are involved in their biosynthesis, and the response they regulate (Fahad *et al.*, 2014).

The environmental influences that restrict crop productivity must be considered. Abiotic stressors include drought, salinity, chemical poisoning and high temperatures. Abiotic stress in the natural environment and agriculture can result in degradation (Wang et al., 2003). The most common agricultural, causing up to 50% reduction in crop production) is salinity and drought (Boyer, 1982). Without environmental stress, plants will grow healthier.

The plant does not move like an animal, therefore, abiotic stress must be endured. The root is the most significant plant component for mitigating abiotic stress (Brussaard et al., 2007). The root ensures that the plant has a high chance of survival and that the soil can keep the plant healthy. Most plants can withstand stress, but tissues tolerate stress differently, e.g. the tissue involved in stress during transcription varies from the stress-based tissue involved in stress (Cramer *et al.*, 2011). The main problems in the agricultural sector are drought and salinity, resulting in a decrease in output. Osmotic pressure on the plant is caused by both drought and salinity. Drought is the restriction of the cell's water uptake.

Salinisation is the state in which salt deposition then accumulates in the soil, the change in the possible gradient of osmosis will damage the plant. Osmotic pressure can be changed by water-soluble salt and can prevent cellular activity (De Azevedo Neto et al., 2004; Zhu, 2016). During salinisation, the high salt concentration in the cytoplasm in the plant cell disturbs the ratio of ions such as Na+/K+. With increased Na+, the potential for water absorption is reduced. Salinisation can also alter the function of transporter enzymes (Conde, Chaves and Gerós, 2011).

1.8 Aims and objectives of this study

Arabidopsis thaliana is widely used in plant model in biology and molecular genetics. It has a short lifecycle, it is a small plant that can produce large numbers of offspring volume, it has a relatively small nuclear genome (The Arabidopsis Genome Initiative, 2000) and is easy to cross-pollinate. This research focuses on plant cell expansion during seed germination, which is a crucial process in germination. This study examines the gene expression involved in seed vigour, development and germination. Seed development and germination involve the relationship between phytohormones GA and ABA; both of these hormones have specific functions in regulating the development of germination and dormancy. Furthermore, the effect of stress responses influences germination. Further studies about seed germination on stress responses are discussed in a different chapter.

1.8.1 Objectives represented as individual chapters

The objectives are represented as individual chapters. For separate chapters, as below, the critical goals for the thesis are addressed.

1.8.1.1 Chapter 3

To analyse gene expression patterns in *Arabidopsis thaliana* during the seed to seedling transition, using "Spatial-Temporal Distributions" on 2D embryo models and the starch GUS reporter system.

1.8.1.2 Chapter 4

To determine the genetic effect of estrogen and the phenotypi effects of salinity and drought on *Arabidopsis thaliana* seeds.

1.8.1.3 Chapter 5

To identify the genetic similarities of starch modulation in *Arabidopsis thaliana* and *Capsicum annum.*

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and apparatus

Most of the chemicals used in this study were purchased from the University chemistry store. The apparatus and instruments used during the study were from the Bassel lab.

2.1.2 GUS reporter line

Arabidopsis thaliana seeds were from the Bassel lab group. All GUS reporter lines of *A. thaliana* used in the experiment were also from Bassel's Lab group. A list of all GUS reporters used in this study is shown in Table 2.1.

Table 2.1: The GUS reporter line for protein fusion and translation fusion for the GUS reporter study

Genotypes	
Protein fusion	**Translation fusion**
GA3ox1::GUS	*AAO3::AAO3-GUS*
GA3ox2::GUS	*ABA2::ABA2-GUS*
SCL3::GUS	*CYP707A2::CYP707A2-GUS*
RAB18::GUS	*GAI::GAI-GUS (10)*
EXPA1::GUS	*GID1A::GID1A-GUS*
EXPA8::GUS	*GID1C::GID1C-GUS*
EXPA9::GUS	*RGA::RGA-GUS (3)*
EXPA10::GUS	*RGL2::RGL2-GUS (2)*
EXPA15::GUS	*RGL2::RGL2-GUS (8)*
XTH9::GUS	*SLY::SLY-GUS (7)*
XTH18::GUS	*SLY::SLY-GUS (12)*
XTH19::GUS	-

2.1.3 Starch GUS reporter line

All starch GUS reporter lines were from the Bassel lab group. A list of all starch GUS reporters used in this study is shown in Table 2.2.

Table 2.2: The starch GUS reporter lines used for the starch GUS experiment.

Locus	Starch reporter lines	Full name
AT1G27680	*APL::GUS*	ADP-glucose phosphorylase large subunit
AT1G05610	*APS::GUS*	ADP-glucose phosphorylase small subunit
AT5G51820	*PGM::GUS*	PHOSPHOGLUCOMUTASE
AT3G21720	*ICL::GUS*	Pump induced coiled-coil (PICC) PICC-LIKE (PICL) ISOCITRATE LYASE
AT1G04710	*pKAT::GUS*	3-Ketoacyl-CoA Thiolase
AT5G24300	*SS1::GUS*	STARCH SYNTHASE 1
AT3G05970	*Lacs6-1/lacs7-1*	Long-chain acyl-CoA synthase (6/7)

2.1.4 Transgenic seeds of the oestrogen-inducible system

All the transgenic seeds of the oestrogen-inducible system were created by our previous lab member, Dr Petra Stamm. The list of constructs to make transgenic *A. thaliana* is shown in Table 2.3

Table 2.3: Transgenic *Arabidopsis thaliana* created by our previous lab member, Dr Petra Stamm and used for starch and germination studyas below:

Locus	The transgenic plant
AT1G10760	*SEX1i*
AT5G51820	*PGMi*
AT3G23920	*β-amylase*
AT1G69830	*α-amylase*

2.1.5 *Capsicum annuum* seed

Chilli seeds (*Capsicum annuum*) were obtained by the Malaysia Agriculture Research and Development Institute (MARDI).

2.2 Histochemical staining

The staining process involves several steps, each with its own function to stain the GUS.

2.2.1 Seed imbibition

2.2.1.1 Imbibition with water for GUS reporter lines

Approximately 50 mature seeds were soaked with water in petri dishes on filter paper. Plates were then placed in a regulated light growth space at 22℃. Water absorption to soften the seed coat will affect the testa and endosperm rupture. Using the other plate of the petri dish, the seeds were also imbibed to be dissected over a time series: I HAI, 3 HAI, 6 HAI, 18 HAI, 24 HAI, JTR, STR, JG, HOOK SHAPED, COTYLEDONS EXPENDED, SEEDLING I and SEEDLING II.

2.2.1.2 Imbibition with water for wild type Columbia (Wt Col) for starch GUS reporter line

The 50 seeds were impregnated with water in a petri dish on filter paper. Then plates were placed in a regulated light growth room at 22°C. For a time series (I HAI, 3 HAI, 6 HAI, 18 HAI, 24 HAI, JTR, STR and JG), the seeds were soaked, and approximately 30 seeds were dissected. The dissected seed was GUS stained in an incubator at 37°C. The samples were fixed and cleared, then imaged.

2.2.1.3 Imbibition with 20% Polyethylene glycol (PEG)

The seed were soaked with 20% PEG. This concentration was chosen because it blocks the germination of the seed. The seeds were imbibed and dissected after five days. The dissected embryo was GUS stained, then fixed and cleared. After being placed in 30% glycerol, the cleared samples were imaged.

2.2.2 Seed dissection

The seeds were dissected under a Leica Light Microscope, using forceps and a scalpel to remove the seed coat from the embryo for 30 seeds of *A. thaliana* for a specific time: 1 HAI, 3 HAI, 6 HAI, 9 HAI, 18 HAI, JTR, STR, JG, HOOKSHAPED, COTYLEDONN EXPANDED, SEEDLING 1 and SEEDLING 11. Before beginning the dissection, the tools were cleaned with 70% ethanol. The forceps were pressed softly on the seed to keep it from shifting aside, then it was gently nicked using the scalpel, and the seed coat was squeezed to extract the embryo.

2.2.3 GUS staining

Two separate 100X concentrated cyanides were used: 100 mM potassium ferricyanide and 100 mM potassium ferrocyanide. Each cyanide (1/100) was freshly applied to 98/100 GUS staining (X-Gluc) to form a GUS solution. X-Gluc contains 100 mm sodium phosphate buffer, 0.1% (v/v) Triton X-100, and 2 mm X-Gluc. The dissected embryos were placed and immersed in the GUS solution in 0.5 ml tubes, and the sample was incubated at 37°C.

A phosphate buffer (pH 7) was made for 10 ml of stain. The two stock solutions, A (1 M sodium monophosphate) and B (1 M sodium diphosphate), were combined at a ratio of 39:61 (A: B). Therefore, solution A, 390 µl and solution B, 610 µl. To dilute the 1M phosphate buffer, 9 ml of water was applied to the solution to make 100 mM (pH 7). The sum of the X-Gluc substrate was measured and applied to the buffer, and the solution was vortexed until fully dissolved. For 10 ml of 2 mM, 10.4 mg was sufficient for X-Gluc. Triton was added to 0.1% (v/v) and then pipetted into the buffer containing X-Gluc. Prior to use, the two cyanides were added into the GUS solution. The tissues samples of the embryo were immersed and covered in GUS solution.

2.2.4 Fixation

The 30 dissected seed samples were put in 300 µl fixative solution (3:1 ethanol: acetic acid + 1% Tween v/v). The termination of the ongoing biochemical reaction is the fixative. This process aims to keep the cell close to its natural state. Fixatives also modify and strengthen the tissue structures for stability and rigidity. To ensure that β-glucuronidase activity is not lost, it is vital to utilise short fixation, for not more than three days. Following fixation, the tissue was washed three times with water.

2.2.5 Clearing

Following the fixation step, 30 embryos per 0.6 ml tube were treated with 300 µl clearing solution (0.2 mol NaOH, 1% SDS) and the sample was left on a 3D mini rotator for at least overnight. Viewed under the microscope, the cleared embryos become transparent like glass: this is the most reliable indicator of a successful clearing process. The fixation and clearing steps were repeated until embryos were fully translucent in the clearing solution.

2.2.6 Slide mounting of samples

Cleared samples were pipetted onto the microscope slide by cutting the end of the tip to create a wide opening to avoid destroying the samples. Excess water on the slide was removed, as much as possible. Glycerol (30%) (replaced by Hoyer's solution) was pipetted around the sample in a ring. A coverslip was then carefully put on top of the sample and the slides were ready to be imaged.

2.2.7 Microscope imaging of samples

The prepared slides were imaged using a Leica microscope. To view embryos and seedlings, 20X and 10X magnification lenses were used, respectively. The captured sample should contain at least 50–70% similarity, or 15–20 seeds of the samples which have a similar staining pattern, to be considered acceptable for making the figures.

2.2.8 Making embryo and seedling figures

Captured images with at least 50–70% similar patterns were stitched and combined using Hugin software. Total mixed embryos and seedlings were arranged using Adobe Photoshop. Based on the overall staining, the good figure seeds were chosen for the final results.

2.2.9 Confocal imaging

As a result of the mobilisation of oil to starch, starch was present in the germinated crop. To test this hypothesis, WT (Col) was used. The embryo was dissected in a time series (1 HAI to the point of germination). Embryos were incubated and cleared with 70% ethanol until they were translucent embryos. The embryos were stained with Periodic acid propodoium iodide (PAPI) and imaged on a confocal microscope. The starch granules can be seen with confocal imaging. The abundance of starch present in each construct shows whether or not the construct affects the abundance of starch. The stained embryos were imaged with confocal LSM 710 microscopes.

2.2.9.1 Sample preparation

The seeds were saturated with estradiol and water. The plates were placed and dissected in the monitored light growth space at I HAI, 3 HAI, 6 HAI, 18 HAI, 24 HAI, JTR, STR and JG. The dissected embryos were put in a microcentrifuge tube, with 70% ethanol and left on a rocker (3D Sunflower Mini Shaker) overnight.

2.2.9.2 Fixation

The same process with the fixation step was applied (Fixation).

2.2.9.3 Clearing

The same process with the clearing step was applied (Clearing).

2.2.9.4 Periodic acid

The tissue was rinsed with water twice. Periodic acid (250 µl, 1%) was added for 40 min at room temperature.

2.2.9.5 Schiff and propodium iodide

The tissues were then rinsed three times with water. The staining solution was prepared and 100 µl of staining solution was added to each 0.6 ml tube. Schiff reagents (100 µl of 100 mM sodium metabisulphite and 0.15 N HCl) were added for staining. A final concentration of 100 µg/ml of propidium iodide was freshly applied before staining. Stain (100 µl) was applied and gently mixed into each tube. Samples were incubated for a minimum of 3 h until the stains were fully penetrated into the tissue.

2.2.9.6 Chloral hydrate clearing solution

After PAPI staining, a 200 µl pipette was used to drain as much water as possible and 400 µl of solution for chloral hydrate clearing was added. The mixed chloral hydrate contained 4 g chloral hydrate, 1 ml glycerol and 2 ml water. The solution was thoroughly inverted gently with the remaining water left in the specimen. Samples were then put on the rocker and left to mix for a week before the embryo's pink stain was removed. The embryos fell to the bottom in the tube, showing they were properly stained and ready for imaging.

2.3 Initial seed screening for transgene seeds, (oestrogen-inducible lines system)

The available seeds were T2 generation, and heterozygous. The transgene seeds used for this experiment were:

- (*PGM*) - The first step of starch synthesis
- *STARCH EXCESS1* (*SEX1*) - A protein required for starch breakdown
- *α-amylase* (*AAM*) - An enzyme that degrades starch
- *β-amylase* (*BAM*) - An enzyme that degrades starch

The constructs that were used to make transgenic *A. thaliana* were created by our previous lab member, Dr Petra Stamm, as below:

- XVE::*Beta-amylase* (this will induce a starch breakdown enzyme)
- XVE::*Alpha-amylase* (this will generate a starch breakdown enzyme)
- XVE::*PGMi* (this will influence an amiRNA which will degrade the *PGM* mRNA)
- XVE::*SEX1i* (this will induce an amiRNA which will degrade the *SEX1* mRNA)

These transgenic plants' seeds must be screened to determine the homozygous line for phenotyping and possible experiments.

2.3.1 Seed phenotyping with PEG and β-estradiol

Screening of transgene seeds was performed by pre-testing germination on 19% PEG-6000, with and without 30 μM β-estradiol on filter paper. Ethanol was applied to the PEG solution for a control, as well as β-estradiol added. The solution was pipetted onto a petri tray. One by one, three filter papers were put in petri dishes to ensure that filter papers were wetted with PEG. Filter papers were flattened to ensure there were no air bubbles. Excess PEG was pipetted out, which guarantees that the seed does not fall into the solution. Seeds were put on filter paper (seeds were treated with 10% bleach to prevent contamination). To lose their dormancy, the plates were put in a controlled cold room at 4°C for three days for seed stratification, then the plates were moved to a controlled light growth room at 22°C. If required, further PEG was further added to the plates. The germination percentages were scored in a controlled growth room after a week. Table 2.4 used for seed screening by phenotyping with 19% PEG (+/-EST).

Table 2.4: Seed screening by phenotyping with 19% PEG (+/- EST)

Locus	The transgenic plant (line)	Imbibed seed
AT1G10760	*SEX1i* (1,2,3,4,6,7,8,11)	80 seeds
AT5G51820	*PGMi* (1,2,3,4)	80 seeds
AT3G23920	*β-amylase* (1,2,3,4,5,6,7,9,10,13,14,15, 16,17,18)	80 seeds
AT1G69830	*α-amylase* (1,2,3,4,5)	80 seeds
-	Wild type Columbia	80 seeds

2.3.2 Homozygous lines

Subsequent to the preliminary 19% PEG (+/- EST) screening, the selected lines showing strong germination were selected to establish homozygous plants, such *SEX1i*- lines 3, 7, 8, 11; *PGMi*- lines 1, 2, 3, 4; *β-amylase*- lines 1, 2, 3, 6, 15 and *α-amylase*- lines 2, 3, 4, 5. The seeds available for the development of homozygous seeds were generations of T2. The phenotype ratio for this step was 3:1, as followed by the Mendel Law of Segregation. It should undergo further plant generation (T3) to make plants homozygous.

2.3.3 Seed plating on hygromycin media

To pick the resistance plants that can live on it, the hygromycin (HYG) agar selection process was carried out. HYG is an essential agent for good seedling selection. HYG media is exceptionally toxic, so it is difficult for seedlings to thrive: only resistant plants can live on HYG. For the resistant seedlings, suitable formations of cotyledons that have real leaves and form a good root penetrate to the media. At the same time, seedlings susceptible to HYG are unable to survive. The HYG-sensitive seedlings (non-resistant) can be seen as pale green, do not develop real leaves and do not have any roots.

The agar medium (1 L) contains 0.5 w/v Murashige & Skoog (MS) medium, 0.8% w/v Agar and HYG (30 µM). Germination media contained 0.8% w/v agar with 0.5 w/v MS salt. MS (2.3 g) was added to 1 L of distilled water. Before autoclaving, the pH of the solution was adjusted to pH 6.2 with 0.1 M KOH (pH decreased to 5.7 after autoclaving). Agar (8 g) to a volume of 1 L was added to the media. The papers were autoclaved for 2 h, then the autoclaved media was left to cool on the shaker. Once the medium was cold, antibiotics like HYG were applied. The media was poured onto 90 mm Petri plates.

2.3.3.1 Seeds plating on HYG agar

Seed sterilisation was performed in a laminar airflow chamber. The seeds were sterilised on a surface with 10% bleach for 5 min, then washed three times with sterile distilled water. The treated seeds were then plated with sterile multi pipette tips on HYG agar media. To stop the dormancy, the planted seeds were placed in a 4°C cold room for seed stratification. The plates were then covered with parafilm and after three days, were moved to the 22°C regulated growth lightroom and left for 5 to 7 days to allow germination.

2.3.4 Transplanting the resistant seedlings to soil

Germination was achieved when the radicles protruded through the endosperm. Resistant seedlings (T3) were picked and transplanted into small pots when the first real leaves. The transplanted seedlings were covered for a few days with cling film to reduce the loss of water by transpiration.

2.3.5 Growing and harvesting of plants

The cling film was removed from the environmentally adapted plants. When the plants began to bloom, the plant sleeves (bagging) were mounted. For seed collection, plants were left to grow. Plants began to dry after 10 to 12 weeks and were ready for harvest. After a

week, the harvested plants (T3) were ready to be cleaned. They were then left to dry and placed in a small labelled bag.

2.3.6 Hygromycin selection to identify the homozygous line

To test the homozygous line, the T3 seeds were sown on 30 μM HYG plates. To test the resistant plant line, the seedlings were grown and the plant's survival was measured over two weeks. The heterozygous plant was light green, with no development of true leaves and no root penetration to the media, as a responsive non-transgenic irresistant. By having green leaves, the development of true leaves and root penetration into the media, the homozygous plant showed resistance to HYG. As homozygous plants, 100% immunity can be considered.

2.3.7 Planting the homozygous line

For massive seed selection, the selected homozygous lines (T4) were grown in large pots. When the pods turned orange, the plants were harvested. The T4 transgene seeds were sued for the starch and germination experiments.

2.4 Nucleic acid protocol

To detect the expression of RNA abundance, reverse transcription polymerase chain reaction (RT PCR) was used. Complementary DNA was used in the detection of gene expression. RT PCR was used to validate gene expression by adding β-estradiol to the gel electrophoresis and without adding β-estradiol.

2.4.1 Sample preparation to collect seedlings

Seed were soaked with 10% bleach, then rinsed with sterile water. Sterile seeds were plated on 0.5 MS treated agar, a control agar with 0.8% agar with 30 mM β-estradiol, and absolute

control ethanol, respectively. For stratification, the seeds were placed in a dark, cold room at 4°C. For seedling settlement, the plates were moved to a light growth room at 22°C. It took approximately 3 weeks from sowing until the seeds became seedlings.

2.4.2 Preserving the seedling samples

The seedling sample was collected and positioned within a small hole in an Eppendorf (to avoid explosions within the sealed tube due to liquid nitrogen when the lid is closed). Liquid nitrogen was immediately inserted to reduce the expression of new mRNAs due to tissue wounding or plant detachment. The sample was stored in liquid nitrogen after the seedling was collected, then stored at -80℃.

2.4.3 RNA extraction from seedlings

RNA was extracted from *A. thaliana* plant tissue using the RNeasy® Plant Mini Kit, from QIAQEN, following the manufacturer's protocol. All equipment was autoclaved prior to use. The mortar and pestle were cooled with liquid nitrogen, then the seedling tissue samples were disturbed and mashed into a fine powder with liquid nitrogen, ensuring that the sample did not thaw during the grinding process. The powdered samples were added to Eppendorf's 450 µl Buffer RLC (with β-mercaptoethanol added) and energetically vortexed. The lysate was moved to the QIAshredder spin column (lilac) and was then centrifuged at maximum speed (14 000 rpm) for 2 min. The supernatant was then transferred into a 2 ml tube and 0.5% absolute ethanol (225 ml) was added to the cleared lysate and mixed by pipetting to make precipitated nucleic acid.

The samples (650 µl) were transferred to the RNeasy Mini spin column and centrifuged at ~8000 x g for 15 s and the flow is discarded. The spin-column membrane was then washed

for 15 s with 700 µl of Buffer RW1 by centrifugation at 800 x g (10 000 rpm) and the flow was discarded. RPE buffer (500 µl) was added to the column and centrifuged for 15 s at 8000 x g (10,000 rpm). The flow was then discarded and this step was repeated by adding 500 µl Buffer RPE to the column, then centrifuging it at 8000 x g (10 000) for 2 min. The flow was discarded again, then the columns were centrifuged for 1 min at maximum speed (14,000 rpm) to dry the membrane. RNase free water (50 µl) was added to the spin column membrane and centrifuged for 1 min at full speed to elute the RNA. The collected RNA samples were processed for immediate use at -20°C, or kept for long-term storage at -80°C.

2.4.4 RNA quantity measurements using a Nanodrop

The NanoDrop® Spectrophotometer measures the absorbance of the RNA sample from the seedling extraction to determine the concentration. The NanoDrop is a DNA spectrophotometer that uses small-volume samples to measure the absorbance of 1–2 µl of RNA. The spectrophotometer's receptacle laser cell (the sample loading area) was cleaned with RNase free water. The initialisation of the Nanodrop program was then accompanied by the selection of the nucleic acid measurement tab. RNA 40 was chosen as the sample. As the RNA sample was eluted with nuclease-free water, 1 µl of nuclease-free water was used as a blank on the receptacle. Before proceeding to the cDNA and PCR process, the RNA concentration should be determined. The blank (water-free of nucleases) was loaded and weighed. The reading shows 0.0 ng/µl for null, then the RNA sample was loaded, and the Nanogram reading registered. RNA (ng/µl) 260/280 and 260/230 ratio concentrations were observed. The 260/230 ratio represents protein contamination.

2.4.5 Determining RNA integrity by agarose gel

The RNA integrity was determined by agarose gel electrophoresis on 1% agarose. Using a w/v percentage solution in an Erlenmeyer flask, 1% agarose gel and salt buffer (10X Tris-

borate, TBE) was prepared. The solution was heated and left to cool in the microwave; before setting, 0.5 μg/ml ethidium bromide (EtBr) was applied to the molten agarose gel solution to visualise the RNA. The cooled gel was then poured to create wells in containers with combs. Before loading onto the gel, DNA binding dye was applied to the RNA. An electric current was applied t to pull the RNA fragments through the gel. To see the RNA band integrity, the gel was imaged under UV light. The strength of the bands depends on the number of nucleotides that are present in each molecule: 28S (closer to the wells) and 18S (further from the wells) were two light bands on the gel. A high 28S/18S ratio shows that the purified RNA is intact and has not degraded. If a smeared or poor band is shown, then the RNA is likely degraded.

2.5 Primer design

Primers are the DNA short strands used in PCR to start the chain reaction and to determine which DNA fragment will be amplified. The BLAST method and NCBI Tool Primer-BLAST scan were used to design primers. The gene and locus name of *Arabidopsis thaliana* were identified from the website.. The designed primers and sequences are presented in Table 2.5. The website used as below:
(https://www.arabidopsis.org/servlets/Search?type=general&search_action=detail&method=1&show_obsolete=F&name=At1g69830+&sub_type=gene&SEARCH_EXACT=4&SEARCH_CONTAINS=1).

Table 2.5: The designed primers and sequences from the BLAST tool.

Primer	Locus	Size	Sequences
PGMi	At5g51820	333 bp	Forward: TCATCGCAACAAGGACACGA Reverse: GCTGAGCCAGTTCCGGATAA
SEX1i	At1g10760	317 bp	Forward: CCTTGATTCGCCTCTGGTGT Reverse: GGACGACATAGAGCTTCCCG
β-amylase	At3g23920	302 bp	Forward: CTGTCACTATTCCTCTGCCTCA Reverse: GGGACCACTTTGTACAAGAAAGC*
α-amylase	At1g69830	297 bp	Forward: ACTGGCTCTACACAGGGTCA Reverse: GGGACCACTTTGTACAAGAAAGC*

BAM and *AAM*, for a reverse primer with (*) were from the Bassel lab group. All the designed primers were ordered in a lyophilised state from Eurofins Genomics, at a stock solution of 1:10. Working solutions were then stored at -20°C.

2.6 Reverse transcriptase PCR (RT PCR)

2.6.1 DNase 1

This process is to get the pure RNA into oligodeoxy-ribonucleotides containing 5'-phosphate, by digesting single and double-stranded DNA. DNase 1 Amplification Grade Kit (Thermo Scientific, Invitrogen) was used for real-time analysis during cDNA synthesis. The primary aim of the DNase 1 kit is to obtain pure RNA and remove any traces of DNA. The components are shown in Table 2.6.

Table 2.6: DNase 1 reaction components.

Reagent	Volume
RNA	1 µg
10X DNase 1Reaction Buffer	1 µL
10X Amplification Grade (DNase 1)	1 µL
DEPC-treated water/nuclease-free water	Up to 10 µL

RNA sample (1µg), 10X DNase 1 Reaction Buffer (1 µl), 10X Amplification Grade DNase 1 (1 µl), and DEPC-treated water/nuclease-free water (up to 10 µl per tube) was added to PCR tubes. The solution was mixed by pipetting, and the tubes were incubated at room temperature for 15 min. To inactivate DNase 1, 1 µl of 25 mM EDTA was applied to the channel. The mixture was incubated for 10 min at 65℃ on a PCR machine.

2.6.2 cDNA

Following the DNase 1 treatment reaction, the cDNA (complementary DNA) process began. The synthesis of cDNA was carried out with GoScript TM Reverse Transcription System Kits (Promega). RNA from treated DNAse 1 and Oligo (dT)15 Primers was combined in the PCR tube (Table 2.7) and then incubated for 5 min in the PCR machine at 70°C. The samples were immediately chilled for 5 min on ice.

Table 2.7: cDNA synthesis reaction components.

Component	Volume
RNA	4 µL
Oligo $(dT)_{15}$ Primer	1µL

The reverse PCR mix was added into the tubes following the cDNA reaction. According to Table 2.8, the reverse RT PCR was mixed up to 15 µL, then 5 µL cDNA synthesis was added. Reaction components were mixed according to the manufacturer's protocol.

Table 2.8: RT PCR reaction components.

Component	Volume
5X Reaction Buffer	4 µL
$MgCl_2$	1.5 µL
PCR Nucleotides Mix	1 µL
Recombinant RNasin® Ribonuclease Inhibitor	0.5 µL
Reverse transcriptase	1 µL
Nuclease-Free Water	7 µL

Following the mixture for Table 2.8, the next step was the final reverse transcription reaction. The PCR machines were set for 1x cycle, as shown in Table 2.9.

Table 2.9: Reaction conditions for the final reverse RT PCR reaction.

Temperature	Time	Step
25ºC	5 min	Anneal
42ºC	1 h	Extended
70ºC	15 min	Inactivation
4ºC	-	Hold

2.7 Polymerase Chain Reaction (PCR)

2.7.1 Taq PCR reaction

The RT PCR for the samples used Taq DNA Polymerase with Standard Taq Buffer from (New England BioLabs), as shown in Table 2.10:

Table 2.10: Components of Taq for the PCR reactions.

Component	For 25 µl reaction
10X Standard *Taq* Reaction Buffer	2.5 µl
10 mM dNTPs	0.5 µl
10 µM Forward Primer	0.5 µl
10 µM Reverse Primer	0.5 µl
Template cDNA 1	1 µl
Taq DNA Polymerase	0.125 µl
Nuclease-free water	up to 25 µl

All the reactions were combined by pipetting. Relevant to the gene, the forward and reverse primers were used. Actin forward/reverse was used to monitor the targeted genes as a guide. A fast spin was done before the PCR was performed to collect all the solution at the bottom. The PCR machines were set up as in Table 2.11.

Table 2.11: Reaction conditions of the Taq PCR reaction.

Temperature	Time	Step
95℃	30 s	Initial Pre Denaturation
95℃ 55℃ 68℃	15 s 20 s 45 s	Amplifying
68℃	5 min	Final Extension

2.7.2 PCR cycles

The PCR run consists of three phases: denaturation, annealing and extension. The high temperature of 95℃ is part of the denaturation process that allows the double strands of DNA to be divided into two single strands. The hydrogen bonds are broken at this stage. The annealing is a primer annealing operation, at a lower temperature of 55℃, it connects to the DNA template. This allows the DNA primer to bind to the template DNA when the temperature is reduced to 55℃. To determine the different stages of the amplifying of the PCR, three different cycles (25, 30 or 35 cycles) were carried out to see the saturated stages of the PCR; 35 cycles were the ideal.

2.8 Gel electrophoresis

The effect of PCR can be seen by gel electrophoresis. This isolates the DNA fragment according to its size. For the development of the gel, agarose gel and a specific buffer were used. The higher the agarose concentration, the smaller the pores formed in the gel, and the harder it is for the larger DNA fragment to pass through the gel. As a result of the DNA binding dye used, the bands will appear.

2.8.1 Agarose gel preparation

Using the w/v percentage solution in the Erlenmeyer flask, 1% agarose gel and salt buffer (10X TBE) were prepared. The solution was swirled to mix and heated to melt in the microwave until the agarose dissolved completely. They were allowed to cool the dissolved agar and EtBr (0.5 µg/ml) was added to the solution. The comb was positioned to create wells in the container, then the molten gel into the bottle and left until it was solid. Before loading into the wells, gel loading DNA binding dye was added to the DNA fragment to track how far the DNA fragment travelled, which can be seen as bands. A negative electrode (cathode) was attached to one end of the box, and the other end was connected to the positive electrode (anode). To cover the surface of the gel, 1X TBE buffer was added. To pull the DNA fragments through the gel, electric current was applied to the bottle.

2.8.2 DNA fragment movement through the gel

The current starts to flow through the gel once the strength of the gel box has been turned on. As the phosphate groups are in their sugar-phosphate backbone, the DNA molecules are negatively charged. The DNA fragments will shift towards the positive end. A standard reference of known DNA length was reserved for a DNA hyper ladder for one well. The correct voltage was applied on average to 80-120 v depending on the size of the containers. A high voltage made the gen run quickly, while a low voltage made the gel run slowly. On the gel's positive end, the small DNA fragments were closed, and the longest DNA fragment remained near the wells.

2.8.3 Gel electrophoresis analysis

The power source was turned off after completing the electrophoresis. The lid of the container was removed. The gel jar was removed, and the excess buffer was drained out of

the gel surface. The gel was imaged under fluorescent light using a gel documentation system, ChemiDocTM MP Device with Image LabTM Software for the gel imaging process. The DNA hyper ladder (1 kb) from the Bioline marker was used to estimate the estimated fragment of the DNA size as a reference.

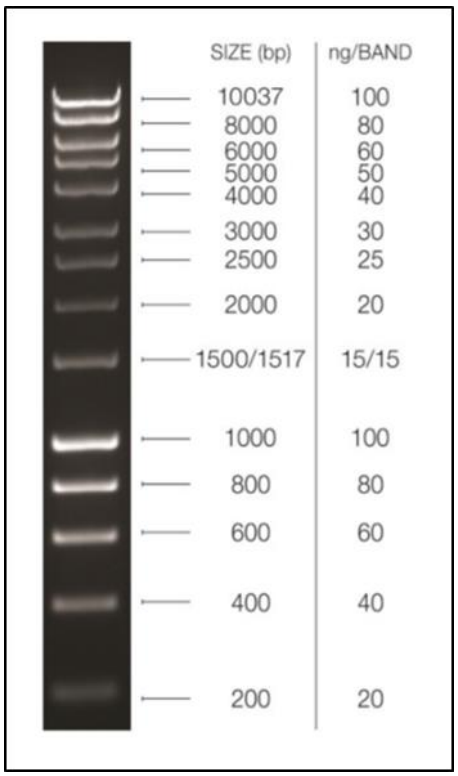


Figure 2.1: HyperladderTM 1KB used as a guide to the size of the target gene in DNA imaging (Source: https://www.bioline.com/uk/hyperladder-1kb.html#RL:).

2.9 Phenotyping seed germination on the stress condition

To see the ability of seed germination against water potential on polyethene glycol (PEG) and sodium chloride (NaCl) salt.

2.9.1 Phenotyping on PEG

For WT Columbia, several different concentrations of PEG were used. In pre-testing, 20% PEG demonstrated better germination. A 20% PEG solution was used for all transgene seeds based on this. Seeds were treated with 10% bleach and washed to rinse and clean the seeds of excess silica with water. The water was then withdrawn, and replaced with PEG solution. Three filter papers were put on the petri dish without air bubbles. The filter papers were wetted with PEG and the seeds pipetted on.

The plated seeds were placed for seed stratification for three days in a 4°C room. Then the plates were moved to light growth for the seed germination process. The germination was measured in the regulated light growth room after seven days. PEG concentrations of 20.5%, with and without estradiol, would be subject to lines that have shown a positive hypothesis. The following showed a positive hypothesis: *PGMi 1-7*; *PGMi 3-3*; *SEX1i 3-6*; *SEX1i 7-5*; *β-amylase 6-8*; *β-amylase 3-2; α-amylase 4-1 and α-amylase 5-3*.

2.9.2 Phenotyping on NaCl

The following concentrations of NaCl were used for pre-testing on seed germination: 0, 100, 150, 200, 250, 300 mM). β-estradiol (30 µM) was added to the treatments because the seed sample was from an oestrogen-inducible system. Ethanol without β-estradiol was added for a control, for testing on WT Columbia. NaCl (200 mM) showed the highest germination according to the pre-testing. All transgenic seeds were plated on 200 mM NaCl and then put in a 4°C cold room after three days in the lightroom. Selected lines that showed positive hypothesis were: *PGMi 1-7*; *PGMi 3-3*; *SEX1i 3-6*; *SEX1i 7-5*; *β-amylase 6-8*; *β-amylase 2-3*; *α-amylase 4-1* and *α-amylase 5-3*.

2.9.3 Data analysis for phenotyping

An independent t-test (two tail) was carried out using EXCEL to compare the mean from two groups regarding the germination. The analysis was conducted on the phenotyping data germination of PEG and NaCl to see the differences of the group before germination.

2.10 *Capsicum annuum* study

2.10.1 Seed germination on *Capsicum annum*

On three filter sheets, the chilli seeds were soaked with water in the petri dish. For each plate, 30 chilli seeds were impregnated, this was repeated with three plates. Then the plates were mounted in the regulated light space. If possible, water was applied to the petri dish, and the paper was not allowed to dry. The plates were tracked daily, and the germinated seeds were counted day after day. The seeds absorbed water at an early stage and enabled the chemical composition to begin the process of germination. The protrusion of the radicle suggests that there was germination. *C. annum* is a dicotyledon plant, meaning it has two cotyledons and two leaves grow from the germinating seeds.

2.10.2 Gene search similarity using SOL Genomics Network

The protein sequences from *A. thaliana* encoded for genes *Phosphoglucomutase (PGM)*;*Starch excess 1 (SEX1), Starch synthase (SS1)* and *Phosphoglucoisomerase (PGI.* To classify the homologous genes in *C. annuum* seeds, unique protein sequences from the specific genes were used in BLAST, then copied into the Solanaceae Genomic s Network, as in Figure 2.2. The Genomics Network was used with the protein sequences as follows: > find the sequences by similarities > by selecting PEPPER > then fill in the information as below and paste the protein sequence in the given space. The screenshot shows descriptions

of the genes used to assess the resemblance of the gene to *A. thaliana*. (sources from the website (https://solgenomics.net/tools/blast?db_id=217)).

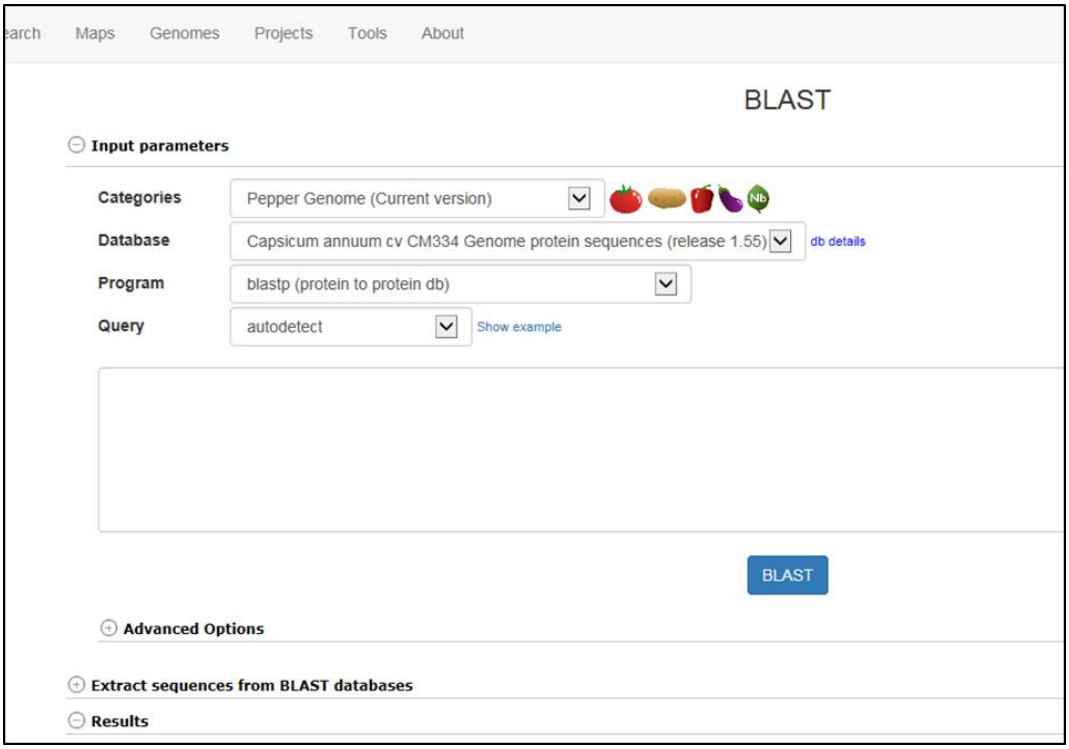


Figure 2.2: The screenshot of the input parameter

to run the BLAST to find other information for primer creation.

Several similar genes were identified on the SOL Genomic Network website with the ID names, after the input parameter was completed. The completeness of each similarity was found by the name of the ID. The subject ID was then used to obtain the cDNA to build the primer to run the PCR. The ID of the similarities are presented in the following screenshots (Figures 2.3-2.6).

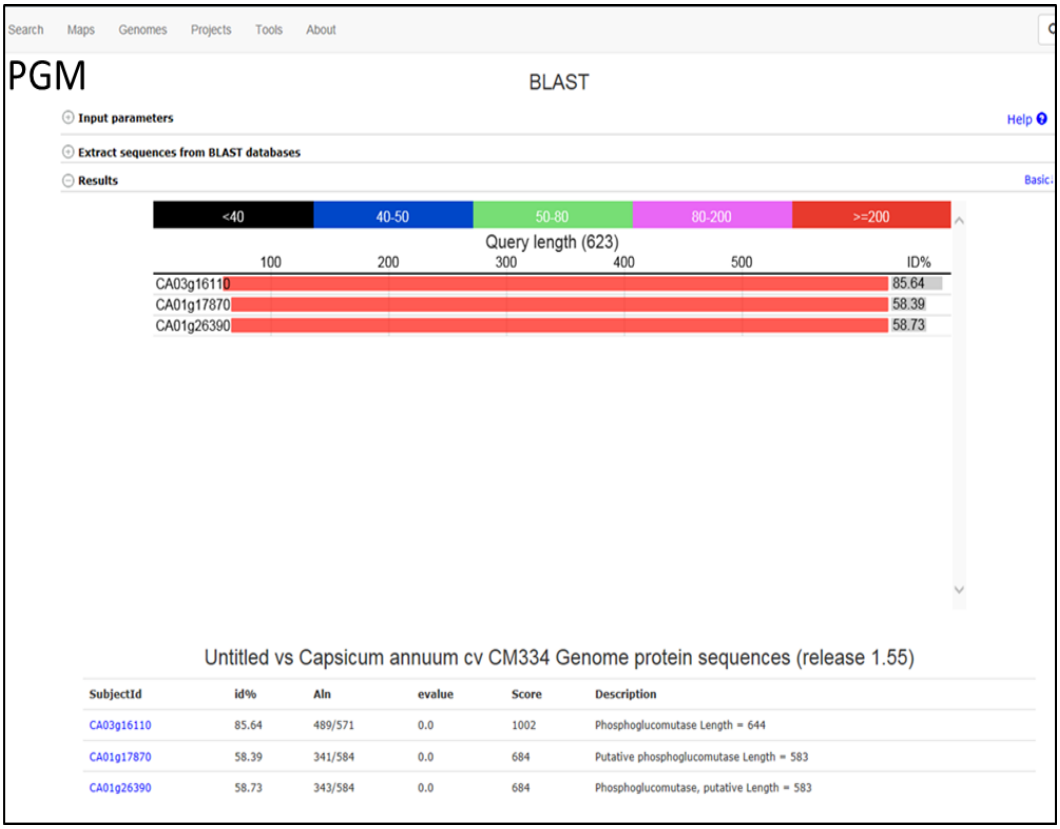


Figure 2.3: The screenshot of the similarity matches found for gene *PGM*. It showed three matches on the SOL Genomics Network website and can be used to create a specific primer.

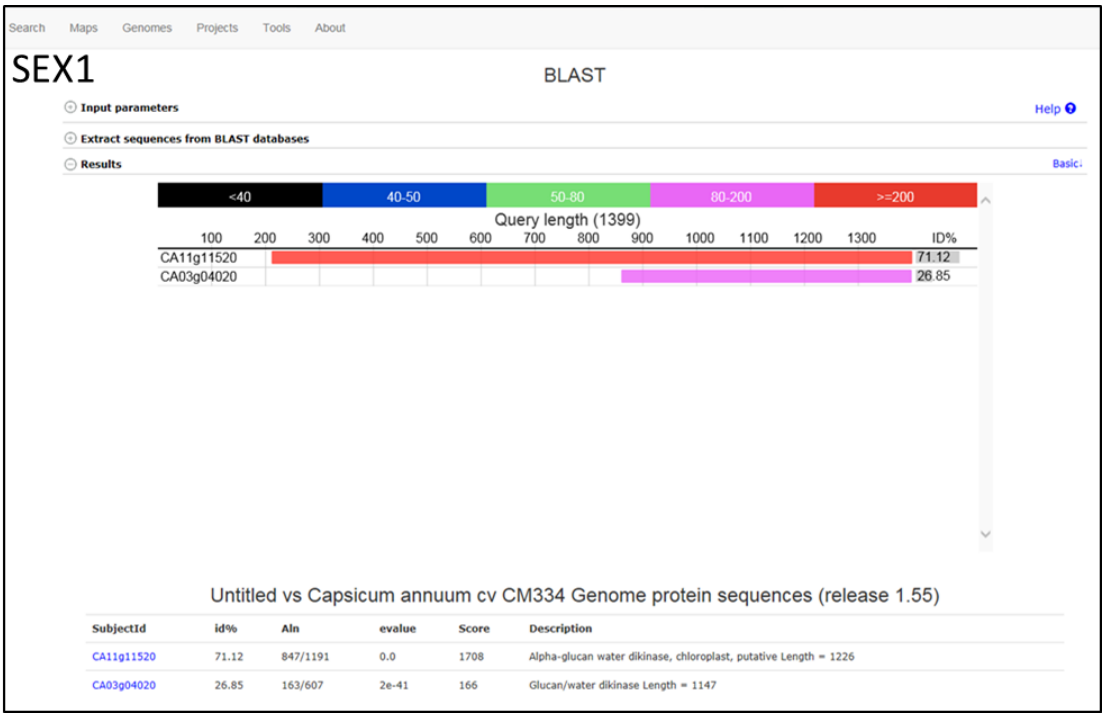


Figure 2.4: The screenshot of the similarity matches found for gene *SEX1.*There are two matches found on the SOL Genomics Network website and it will be used for further primer creation.

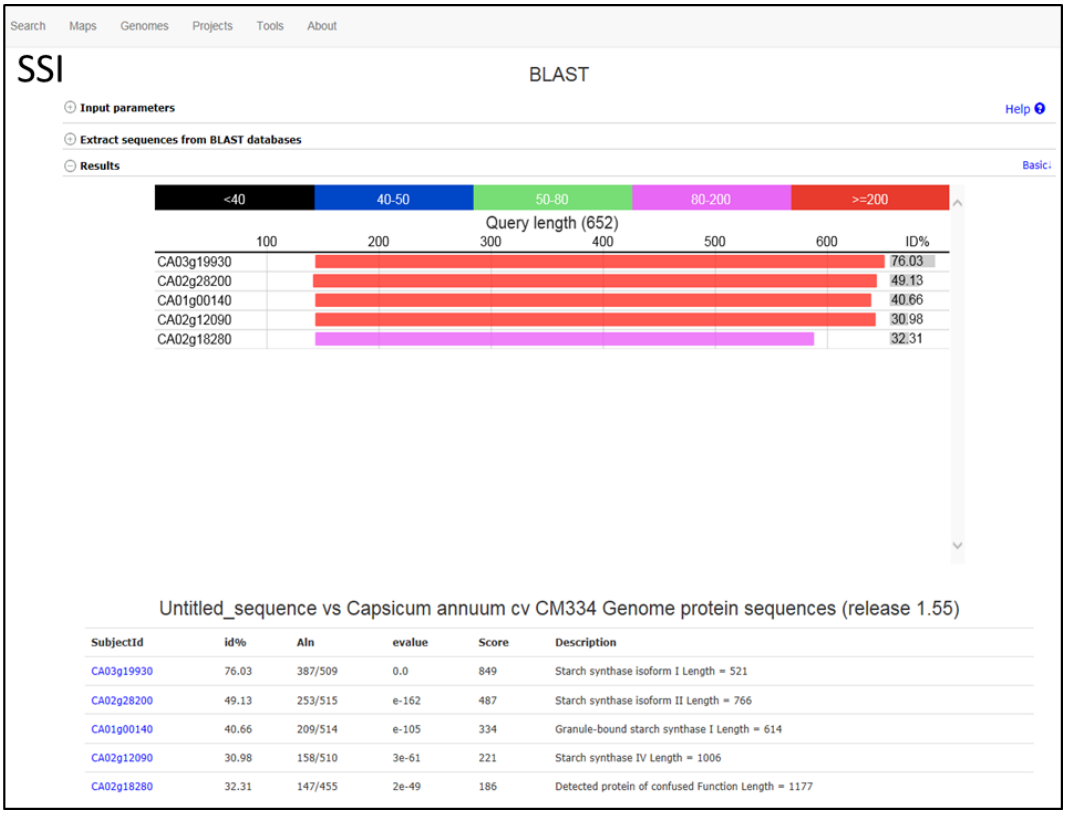


Figure 2.5: The screenshot of the similarities matches found for gene *SSI.* There were five similarities matches found on the SOL Genomics Network website for SSI, and it will be used to create the primer.

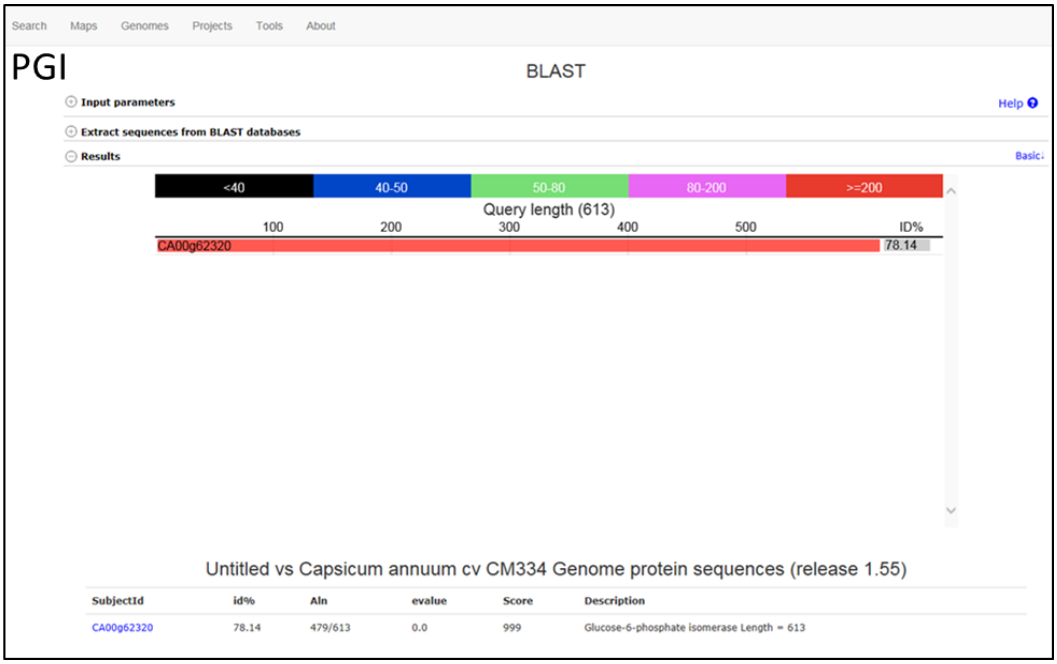


Figure 2.6:The screenshot of the similarity matches founded for gene *PGI*. Only one similarity match was found for gene PGI in *Arabidopsis thaliana* on the SOL Genomics Network website, to be used to compare with *Capsicum annuum* for primer creation.

2.11 Primer creation

The primers from the SOL genomic website were designed from the cDNA, using the BLAST search method to build a primer. The same process was used for the design of primers for *A. thaliana*, which was also used to design the *C. annuum* primer (Primer). The unique primers were then used to amplify the target gene during the PCR reaction in the Taq PCR reaction.

Table 2.12: The designed primers and the sequences resulting from the BLAST tool to be used for PCR.

Primers	ID Name	Size	Sequences
PGM 1	CA03g16110	200	Forward: TGACTACGAGGCAAGCACATT Reverse: AAACAAACCGAACCCCCTGT
PGM 2	CA01g17870	223	Forward: ATCATTCGCCAACACTGGGT Reverse: AGACCGAACCATCAATGGGG
PGM 3	CA01g26390	98	Forward: TGGTTCAGTCTCGCAGCATC Reverse: TAGTAGCACCCTCTGAGCCA
SEX1	CA11g11520	312	Forward: TGGGAAGGTAGTTTGCTTTGC Reverse: GGAATTCCCACCCAGGAAGG
SSI 1	CA03g19930	421	Forward: GGGCTATGGTCCACTGCTTT Reverse: CTCCATACCATTCCGGAGGC
SSI 2	CA02g28200	293	Forward: TTTTGGTGGCTGCAGAATGC Reverse: CACACGGTTCCCTCCGTAAA
SSI 3	CA01g00140	726	Forward: TAAACGTGGGGTTGATCGCA Reverse: CTGTCAACAGGCAAGCCAAC
SSI 4	CA02g12090	624	Forward: TGCCATGATTGGCAAACAGC Reverse: ATGTGGCACTGGGCTAGAAC
PGI	CA02g00010	212	Forward: CAATCAGCAGACTCGGGGA Reverse: AAATCTCAAGGCAGGGGGC
ACTIN7 1	CA03g11540	235	Forward: AACGGAGGCACCCCTTAATC Reverse: ACGACCAGCAAGGTCCAAAC

All the designed primers were ordered in a lyophilised state from Eurofins Genomics, and the 1:10 working solutions were stored at -20℃.

2.12 RT PCR confirmation for *C. annuum*

2.12.1 Sample preparation for *C. annuum* seeds

For 24 HAI and DAI, 50 chilli seeds were impregnated with water. The magnification was calibrated for suitability using the Leica DM500 microscope prior to viewing for dissection.

The chilli seeds were dissected after 24 HAI in the growth room by cutting using forceps and a blade in a cross-section. The embryos were separated from the seed. The dissected embryos were isolated from the endosperm. The embryos were collected for RNA extraction.

2.12.2 RNA extraction for *C. annuum* seeds

C. annuum RNA was extracted using the Qiagen RNeasy Package. In this extraction, however, the embryos were used. A cooled pestle and mortar were used to ground the 50 dissected embryos in liquid nitrogen. They were then put in an Eppendorf tube and 450 µl β-mercaptoethanol-containing RLC buffer was added to it. This was accompanied by the same method as seedling extraction (**2.4.3 RNA extraction from seedling**) . The extracted RNA concentration readings were read using the NanoDrop® Spectrophotometer.

2.12.3 DNase 1 treatment

The RNA samples were treated with DNase1 from Thermo Fisher, following the extraction of RNA to remove genomic DNA contamination. The DNase1 treatment followed the previous steps (DNase 1).

2.12.4 cDNA

The same techniques were used in the previous process (cDNA) for reverse transcriptase.

2.13 PCR for *C. annuum*

2.13.1 Taq PCR reaction

The same technique for *C. annuum* was used as for *A. thaliana* (**Taq PCR reaction**). However, during the PCR reaction, variations in annealing temperatures were used for each primer. The annealing temperature applied to the primers are shown in Tables 2.13 and 2.14.

Table 2.13: Reaction conditions for Taq PCR reactions. The temperature for different primers.

Temperature	Time	Step
95ºC	30 s	Initial Pre-Denaturation
95ºC 55°C 68ºC	15 s 20 s 45 s	Amplifying
68ºC	5 min	Final Extension
4ºC	-	Hold

Table 2.14: The different annealing temperatures used during the PCR reaction based on the different primers.

Primers	Annealing temperature (ºC)
PGM 1	45
PGM 2	45
PGM 3	50
SEX1	45
SSI 1	45
SSI 2	45
SSI 3	45
SSI 4	45
PGI	50

ACTIN7 1	45

CHAPTER 3

SPATIAL TEMPORAL DISTRIBUTION OF GENE EXPRESSION DURING TRANSITION FROM SEED TO GERMINATION AND SEEDLING IN *ARABIDOPSIS THALIANA*

3.1 Introduction

Plants experience a sequence of seed-to-seedling expansion during phase transition. Environmental signals, such as light, temperature and water help germinate and produce seeds. The normal hormonal abscisic acid (ABA) acts, and gibberellic acid (GA) promotes germination to encourage dormancy. In the seed development stage, the ABA level is high, and is low level during germination and seedling establishment, which differs from GA. Unlike ABA, GA is at a low level during seed production, while during seed germination it is at a high level. Seeds are a vital element in the production of agricultural products. They are the most significant factor that initiates a further step in developing a plant in the germination and seedling process.

Germination occurs when all the favourable conditions are given to the seeds to start germination and terminate dormancy. Seed problems will lead to failure to germinate and become a seedling and, later, a plant. High-quality seeds have a high return in the future and ensure food protection. Germination is a crucial and complicated process that acts as a starting point to shift from the quiescent stage to germination and seedling establishment. During the seed development stage to seed germination, the plant cell expands and enlarges in size. The plant cell's expansion is essential in seed development and seed germination. Cells are growing within the cells. Thus, it drives the embryo's enlargement and leads to germination. The cell activity is related to gene expression, where the process of information from the genes is used to synthesise a functional gene product. During seed

development involved cell expansion and hormone synthesising, we do not know the specific gene that drives seed germination.

To overcome issues related to cell wall expansion and hormone synthesising induced by seed germination in *Arabidopsis thaliana*, I used the promoter's GUS gene reporter and translational fusion to quantify and evaluate the behaviour and pattern of the gene being expressed during the transition of seed to seedling transition. This chapter will cover the preparation of seed material and application in a lab-based experiment. Spatiotemporal distribution is the ideal term used in this study because the observation of the gene expression involved a separate time, starting from seed to germination and seedling. The spatiotemporal gene expression can be accessed through the GUS reporter, and it helps to examine and observed the pattern of the molecular event during the germination.

3.1.1 GUS reporter system

The GUS reporter system method is widely used in biology (Jefferson *et al*., 1987). The purpose of the GUS reporter system technique is to analyse the promoter activity. *Escherichia coli* was applied in the GUS reporter system. It encodes β-glucuronidase as a gene fusion marker analysis of gene expression in a transformed plant. It then transforms X-gluc into the coloured product (normally blue staining). The GUS system becomes a command method. It is crucial to analyse the promoter's transcriptional activity (in terms of expression of the gene under that promoter), quantify the GUS staining and microscopy of the images. The histochemical technique is the most common GUS analysis method (Jefferson, Kavanagh and Bevan, 1987). It allows researchers to visualise tissue structures in the cell. The reporter genes are used to make it easier for the researcher to identify and track

where, when and how the promoter's activity is actively translocating the promoter's event transcript.

Table 3.1: The GUS reporter line for protein fusion and translation fusion for the GUS reporter study.

Reporter line	Locus	Fusion
GA3ox1:: GUS	At1G15550	Protein
GA3ox2:: GUS	At1G80340	Protein
GID1A:: GID1A-GUS	At3G05120	Translation
GID1C:: GID1C-GUS	At5G27320	Translation
SCL3:: GUS	At1G50420	Protein
SLY:: SLY-GUS	At4G24210	Translation
EXPA1:: GUS	At1G69530	Protein
EXPA9:: GUS	At5G02260	Protein
EXPA10:: GUS	At1G26770	Protein
EXPA15:: GUS	At2G03090	Protein
XTH9:: GUS	At4G03210	Protein
XTH18:: GUS	At4G30290	Protein
XTH19:: GUS	At4G30290	Protein
GAI:: GAI-GUS	At1G14920	Translation
RGA:: RGA-GUS	At2G01570	Translation
RGL2:: RGL2-GUS	At3G03450	Translation
ABA2:: ABA2-GUS	At1G52340	Translation
AAO3:: AAO3-GUS	At2G27150	Translation

3.1.1.1 Gibberellin biosynthesis genes

GA biosynthesis is involved with the GA3-oxidase enzyme. The multigene family of *GA3ox* encodes four: *AtGA3ox1* to *ATGA3ox4*. *AtGA3ox3* and *AtGA3ox4* are believed to have an essential role in the limitation of germinating seeds. Thus, they are entirely expressed in

flower development and siliques (Sun, 2008). In this study, I focused on *AtGA3ox1* and *AtGAxox2* genes, in line with previous research. This gene was shown to be expressed during plant development. *AtGA3ox2* is expressed in the development of embryos and the young seedling (Yamaguchi *et al.*, 1998). Both genes are also expressed in the cortex and endosperm in developing embryo (Yamaguchi *et al.*, 1998; Mitchum *et al.*, 2006). The endosperm is crucial because it plays a vital role in supporting embryonic growth and supplying nutrition (Yan *et al.*, 2014).

3.1.1.2 Gibberellin signalling genes

After GA biosynthesis, the GA-GID1 complex results from the GA receptor GID1 binding to GA. This sophisticated binding plays an essential part in GA perception. As mentioned earlier in the previous chapter, the *A. thaliana* GID1 receptor is encoded by *GID1A*, *GID1B*, and *GID1C* genes.

3.1.1.3 Gibberellin responsive genes

Following the perception of GA, the GA responsive genes involve the family of Expansin, EXPA (*EXPA1*, *EXPA9*, *EXPA10*, *EXPA15*) and xyloglucan endotransglucosylase/hydrolase, *XTH* (*XTH9*, *XTH18*, *XTH19*). Both *EXPA* and *XTH* are involved in enabling turgor pressure, which drives cell expansion. However, they act in different ways. The Expansin protein is one of the groups of the pH-dependent cell wall loosening protein, driven by cell expansion during the development event (McQueen-Mason, Durachko and Cosgrove, 1992; Cosgrove, 2000). The Expansin superfamily comprises four groups: α-expansin, β-expansin, expansion-like A and expansin-like B (Sampedro and Cosgrove, 2005). Both α-expansin and β-expansin are known to have cell

wall loosening activity and are also involved in cell wall enlargement (Sampedro and Cosgrove, 2005).

About 33 *XTH* members have been examined (Becnel *et al.*, 2006), in a study of the variable spatiotemporal expression pattern through GUS analysis. For this study, the three *XTH* gene, *XTH9*, *XTH18*, *XTH19* were considered they have previously been correlated with cell expression (Bassel *et al.*, 2014). The *XTH* gene also encrypts enzymes involved in the non-hydrolytic cleavage of xyloglucan chains, by splitting the β-1,4 linked poly glucose backbone of xyloglucan molecules (Hyodo *et al.*, 2003). The cell wall's primary element is the hemicellulose xyloglucan, which is vital in giving structure to the plant cell. The *XTH* enzymes are essential in restricting cell walls (Becnel *et al.*, 2006; Eklöf and Brumer, 2010).

3.1.1.4 Abscisic acid biosynthesis associated genes

The ABA biosynthesis gene plays a role in promoting seed dormancy and inhibiting germination events. For the immature seed embryo, *ABA2* and *AAO3* were localised at the provascular cell during histochemical GUS analysis (Seo *et al.*, 2006). Nevertheless, the GUS expression activity starts to decrease following the imbibition process, then localised at the radicle tip cells (Seo *et al.*, 2006).

3.1.1.5 Abscisic acid response-associated genes

To assess ABA's presence within plant tissues, the ABA sensitive *RAB 18* is often used. In the plant, the accumulation of *RAB18* mRNA was identified by an experiment using the aba-1 mutant ABA synthesis and abi-1 mutant ABA response when exposed to cold temperature, water stress and exogenous ABA (Lang *et al.*, 1994) *RAB18's* GUS activity in WT seedlings also depends on ABA's exogenous application (Ghassemian et al., 2000).

According to image analysis, *RAB 18* is localised within the outer layer of radicles at the epidermis and root cap (Topham *et al.*, 2017).

3.1.2 Starch GUS reporter system

The available seed obtained from the Bassel lab was the starch GUS reporter seed. In this analysis, the starch GUS reporter used the staining pattern to see which locations represent a particular moment. The expression of GUS staining can vary, based on time and place. The GUS staining distribution pattern of the expression activity is used to map the 2D embryo model. Metabolic switches are also correlated with different gene expression patterns during seed maturation and germination (Fait *et al.*, 2006).

To equate the gene expression imbibed with water and 20% PEG for this experiment, particular time and phases for the seed to germinate for imbibition with water, such as 1HAI, 3HAI, 6HAI, 18HAI, 24HAI, JTR, STR and JG were used. In contrast, it was imbibed for five days with 20% PEG to block germination.

In this analysis, the starch GUS reporters used gene expression during seedling to observe the germination are shown in Table 3.2.

Table 3.2: The starch GUS reporter and the Arabidopsis gene coding for the protein.

Starch reporter line	Protein abbreviation (alternative name)	Locus	Description
APL:: GUS	ADP-glucose phosphorylase large subunit	AT1G27680	Responsible for ADP-glucose phosphorylase activity-regulatory role in rate-limiting step in starch biosynthesis
APS:: GUS	ADP-glucose phosphorylase small subunit	AT1G05610	Responsible for ADP-glucose phosphorylase activity-small subunit is the catalytic isoform
PGM:: GUS	PHOSPHOGLUCOMUTASE	AT5G51820	Response to drought stress resulting in starch degradation
ICL:: GUS	Isocitrate lysate	AT3G21720	Encodes a glyoxylate cycle enzyme isocitrate lyase (ICL)
pKAT:: GUS	3-Ketoacyl-CoA Thiolase	AT1G04710	Involved in fatty acid degradation β oxidation
SS1:: GUS	Starch Synthase 1	AT5G24300	Required for the synthesis of amylose in the endosperm
Lacs6-1/lacs7-1	Long-chain acyl-CoA synthase (6/7)	AT2G47240	Activate free fatty acid and play critical roles in fatty acid metabolism

3.1.3 Aims and hypothesis of the study

It is not known where and when the natural hormones ABA and GA are placed in the plant during the seed-to-seedling transition. Where do genes respond to locations during the change, and when do they respond (the transition stage)? This project will define where and when GA signalling and downstream cell wall modifications occur in seedling growth to drive seedling establishment. The basic time frames were used, beginning with 1 h of imbibition, until the seedling stage. The "spatial-temporal distribution" was used to represent this study, which refers to time and location. This chapter's main focus was to

observe the gene expression pattern of the seed-to-seedling transition in *A. thaliana* seeds using the GUS reporter system's technique applied to the transgenic seed in *A. thaliana*. ABA and GA were assessed by protein localisation and expression of the selected gene. The chosen gene was associated with synthesis, perception and response. The localisation of the gene expression enables us to identify the specific molecular marker related to the seed germination starting from seed to seedling. The same idea applies to the starch GUS reporter to observe the gene expression pattern during the transition of seed to germination.

3.1.3.1 Objectives of the study

During this research, there were many targets to be accomplished, such as:

i. To characterise the trends in GA and ABA signalling during the transition from the seed to the seedling.

ii. To describe the pattern of cell wall changes that alter gene expression during the transition from seed to seedling.

iii. To characterise the pattern of gene expression changes of starch GUS reporters during the transition of seed to germination.

3.2 The hormonal interaction during seed germination

During every phase of plant development, natural plant hormones interact with each other. A large range of hormones is found in the plant itself. GA, ABA, cytokinin, auxin, ethylene, jasmonate, brassinosteroid and peptide hormones are plant hormones (Bari and Jones, 2009). These plant hormones are chemicals, much the same as animal hormones that assist in developing, growth and plants. During seed dormancy and seed growth, continuous interaction is crucial. Plant hormones function differently at various stages of plant growth. As an essential endogenous regulator, phytohormones, such as GA and ABA are widely

recognised. These hormones may help in the phases of plant growth and environmental responses. Both GA and ABA can directly or indirectly impact their direction of signalling.

3.2.1 Dormancy

A dormant seed is a seed that, under any combination of standard physical environmental factors and favourable germination conditions, cannot germinate during a defined time. The situation where the seeds are unable to germinate, even under the desirable condition of germination, is a clear definition of seed dormancy. So for dormant seeds, it blocks germination. Unlike non-dormant seeds, it may germinate in the normal situation. A completely non-dormant seed will grow over the broadest possible range of standard genotypical physical environmental factors (Baskin and Baskin, 2004; Finch-Savage and Leubner-Metzger, 2006).

3.2.2 The importance of dormancy

Depending on the seed species, the period of seed dormancy varies. It could be a few months or a few years of dormancy. Dormancy blocks germination and assists in the process of survival at some stage. Dormancy in nature protects the seed from an environment that is temporarily ideal for germination, which soon returns to a condition that is too harsh for the young seedling to survive. Therefore, during the dry season, a seed coat that is relatively impermeable to moisture will avoid germination, while allowing germination during the long rainy season (Karanth, 2013).

After dormancy is released, an unfavourable condition for germination, storage, and imbibition of the non-dormant seed will promote secondary dormancy. This will help ensure that the seed is postponed until the condition is met, and causes seasonal dormancy cycling

(Cadman *et al.*, 2006; Footitt *et al.*, 2011). This will help ensure that the seed is postponed from germination until the condition is met. A high degree of dormancy is slowly released for freshly harvested seeds during dry seed storage after ripening. Slow germination can occur for seeds that are stored in the soil under appropriate conditions. Dormancy is slowly released when seeds are exposed to low temperatures (stratification). The imbibed seed also contributes to the released dormancy (Finch-Savage and Leubner-Metzger, 2006).

3.2.3 GA and ABA interactions

For seed germination, GA and ABA communicate with each other. The dormant seed is incapable of germinating under the favourable conditions of the seed. In dormant seeds, the ABA level is increased to protect the seed before increasing until it reaches a suitable germination requirement. Meanwhile, during the germination of seeds, the GAs are associated.

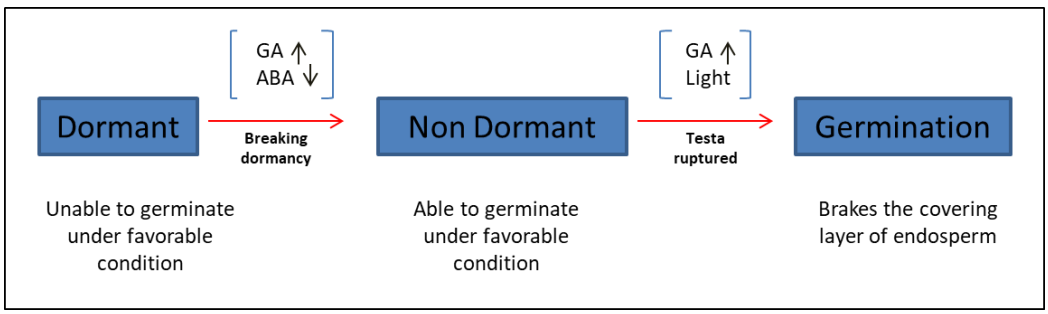


Figure 3.1: Pathway of dormant seed to germination. The GA level is high, whereas the ABA level is at a low level as GA promotes germination, and ABA reduces the dormancy during the breaking of dormancy toward germination.

Figure 3.1 shows an overview of the pathway from dormant seed to germination. For seed germination, GA is required. ABA during seed germination does not inhibit the ruptured

testa, however, it does prevent the development of radicles and endosperm rupture (Table 3.3).

Table 3.3: Relationship of abscisic acid (ABA) and gibberellin acid (GA) in dormancy and germination.

	GA (Gibberellin acid)	ABA (Abscisic acid)
Biosynthesis	*GA3Ox1* *GA3Ox2*	*AAO3* *ABA2*
Signal	*GID1A* *GID1C* *DELLA* *(GAI 10/RGA 3/RGL2(2)/RGL2(8)* *SCL3* *SLY (7/12)*	
Responses	*EXPA* *XTH*	*RAB18*

Seed lines are reporter constructs to visualise the localisation of GA and ABA signalling components.

Table 3.4: Summary of the gene descriptions.

Gene	Other names	Description
GID1A/GID1C	GIBBERELLINE INSENSITIVE DWARF1	GA receptor
SLY7/12	SLEEPY	Promote GA
RGL2(2) *RGL2(8)*	RGA-like 1	RGL2 regulates seed germination in response to GA RGL2 is a negative regulator of GA responses that act specifically to control seed germination rather than stem elongation. control seed germination
RGA 3	Repressor of gal-3	Negative plant regulator for hormonal gibberellin acid (GA) signalling in Arabidopsis
XTH9/XTH18/ XTH19/	XYLOGLUCAN ENDOTRANSGLUCOSYLASES/HYDROLASES	responsible for cell wall construction XTH loosens the cell wall by hydrolysing cell wall components XTH splitting and reconnecting molecules and tissues elongation & differentiate region of the root
EXPA 1/ /9/10/15	EXPANSIN	cause wall stress relaxation and irreversible wall extension It is linked to the cell enlargement and cell wall changes induced by plant hormones

		by disrupting hydrogen bonding. (allow the cell wall to become weak, and will enable the cell wall to become bigger)
ABA2/AA03	ABSCISIC ALDEHYDE	Enzyme producing ABA
RAB 18	RESPONSIVE TO ABSCISIC ACID	

3.3 Plant hormone interactions during seed development

During germination, plant hormones in the plant are essential. GA and ABA are also interrelated. Based on (Jacobsen, Gubler and Chandler, 1995) previous study, the interlinked reaction is the cereal's aleurone layer. Hormones also contribute to the regulation of gene expression and endosperm function following germination. The hydrolytic enzymes are water-activated during the seed's water absorption, and the storage proteins are degraded into amino acids. In stimulating the aleurone cell, the GA was observed to secrete various hydrolytic enzymes in cereals (Ritchie *et al.*, 1999).

In small-seeded plant species, such as tomatoes and Arabidopsis, several researchers have discovered two major roles of GA in stimulating germination (Hooley, 1994; Debeaujon and Koornneef, 2000; Yamaguchi and Kamiya, 2002). The mechanical reaction of the tissue surrounding the embryo, such as aleurone and testa, is resolved by GA. Plus, it serves as a GA that functions as induction for the radicle's protrusion to disrupt the tissue surrounding the embryo (Silverstone *et al.*, 1997; Telfer, Bollman and Poethig, 1997). The other function of GA is to help increase the embryo's growth potential.

3.3.1 GA signalling

In regulating almost all plant growth phases and development, plant hormones play an essential role as signalling molecules, starting from embryogenesis to senescence. Monitoring, cell division organisation, production and differentiation are the crucial functions of plant hormones. GA is essential in seed germination, while ABA inhibits germination. In the plant model, both hormones have unique rules to control dormancy and germination, such as in *A. thaliana*. According to a study conducted by Peng and Harberd (2002), GA has a particular role in promoting seed germination to encourage hypocotyl and stem elongation and control the production of pollen and flower initiation.Gibberellins can alleviate dormancy from seeds (Kucera, Cohn and Leubner-Metzger, 2005; Gubler *et al.*, 2008). Due to its short life cycle, *A. thaliana* is widely used as a plant model in molecular and plant genetics, and has rapid developmental growth.

The three *GIBBERELLIN INSENSITIVE DWARF1 GID1A, GID1B* and *GID1C* receptors serve as receptors for gibberellins in *A. thaliana*. The product of gibberellin stimulation to synthesise and form hydrolases in seed germination is alpha-amylase. GA reactions occur when the breaking of the DELLA protein is triggered by gibberellin. GA, then, binds *GIBBERELLIN INSENSITIVE DWARF1* (*GID1*) to its receptor. This leads to the DELLA protein being dealt with in turn. *SLEEPY* (*SLY*) can interact with the *GID1A-GA-DELLA COMPLEX*. It eliminates DELLA as SLY binds to DELLA, allowing the DELLA protein to denature. DELLA works to block germination. Since DELLAs have been removed, by enabling germination, it will activate the GA that responds.

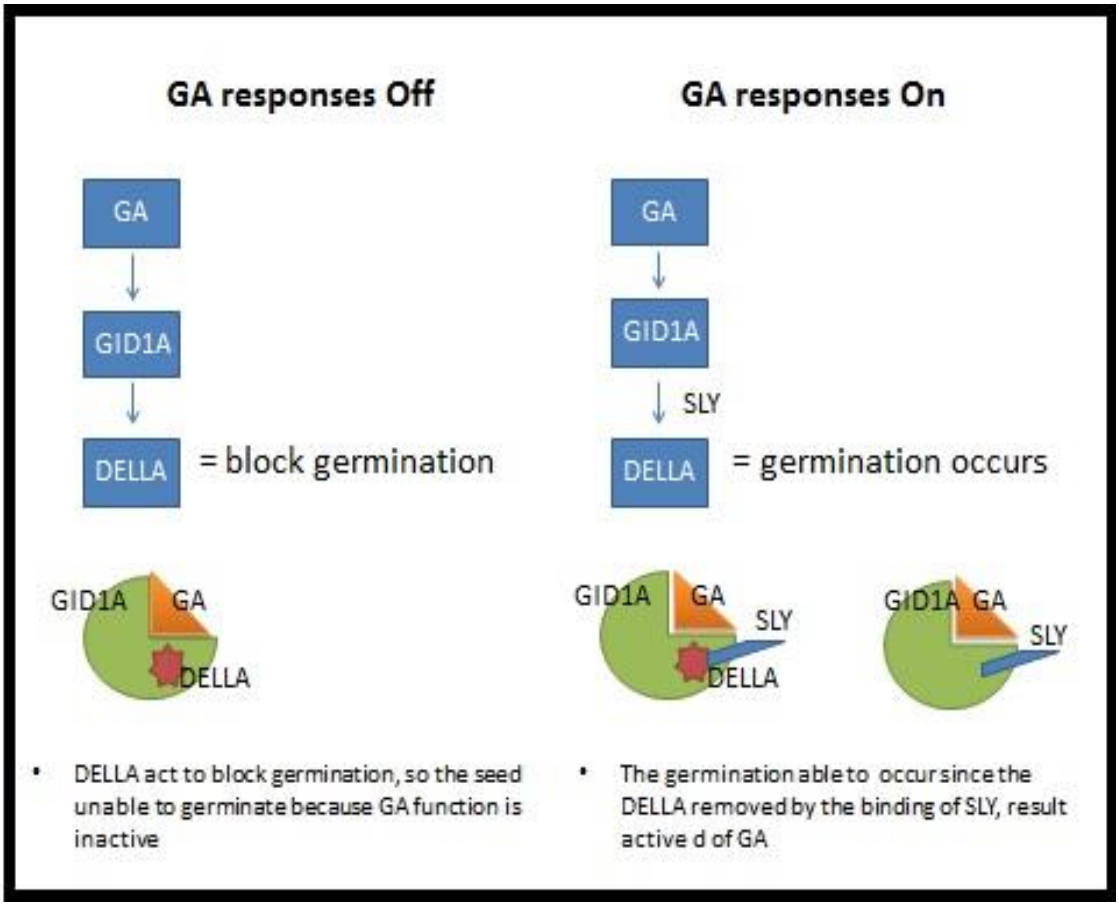


Figure 3.2: Simple illustration of the pathway of gibberellin (GA).

The simple diagram can be seen above for the path of GA. The DELLA was removed when SLY bind to DELLA, which makes way to germination

3.3.2 GA responses

Expansin (EXPA) makes it possible to weaken the cell wall before extending the cell wall, called the 'Cell Wall Remodelling Enzyme' (CWRE). EXPA can be divided into two large alpha-expansin (EXPA) and β-expansin (EXPB) families. In cell wall loosening activity, both alpha-expansion (EXPA) and beta-expansion are documented and experimentally proven to function, and are also involved in cell expansion and other cell growth (McQueen-Mason, Durachko and Cosgrove, 1992; Cosgrove, Bedinger and Durachko, 1997).

There is a solid cellulose structure in the cell wall. Cellulose easily forms hydrogen bonds with itself, which bind and build a cellulose chain with other cellulose. The long cellulose chain will provide microfiber that results in wall strength. A diverse community of carbohydrates is the hemicelluloses. Hemicelluloses do not mix, so there is no creation of microfibers. They are linked by cellulose and hemicellulose. EXPA helps to loosen the wall of the cell by rendering cellulose-unbinding hemicelluloses. The binding becomes weaker and loose, leading to the cell wall being further formed and extended. Both hormones are regulated responses that change the cell wall to make it more expandable or softer, or isolate it more easily from other walls.

3.3.3 Cell wall modifying gene expression

A significant structure in the plant is the cell wall. The mechanical strength is then given to determine the shape of the cell. The cell wall is a rigid structure. As a protection for the cell inside, it gives a barrier layer. Expanded by expansion enzymes, the cell wall of *A. thaliana* can be weakened. EXPA facilitates the cell wall's expansion by the weakening of the cell, which causes the cell wall remodelling enzymes (CWRE) to increase in size. The expansion includes EXPA 1, 3, 8, 9, 10, 15 and 20. According to (Voegele *et al.*, 2011), GA

stimulation results from the deteriorating layer of endosperm by distressing cell wall protein changes. The hormone signal is transferred to the seed coat to release the coat's dormancy, which is later accompanied by germination.

Some characteristics, such as porosity, permeability, elasticity and compressibility to the cell wall were initiated by pectin methyl arrangement (Wolf *et al.* 2009; Peaucelle *et al.*, 2012). Besides, pectin methylesterases (PME) promote the demethylesterification of pectin from the cell wall. Altering the cell walls, the PME (pectin modifying enzymes) facilitates certain plant physiological and biochemical processes (Micheli, 2001; Wolf, Sebastian. Mouille,Gregory. and Pelloux, 2009; Peaucelle, Braybrook and Höfte, 2012). Research by Muller et al. (2013) has discovered that the different stages of seed germination in Arabidopsis can differently control PME activity. The rate of seed germination depends on the concentration level of PME in the cell wall.

3.3.4 ABA signalling

ABA is defined as a repressor of completion of germination (Finch-Savage and Leubner-Metzger, 2006). ABA will prevent the germination of seeds during imbibition. In general, by preventing endosperm rupture, ABA can block seed germination but not testa rupture (Müller, Tintelnot and Leubner-Metzger, 2006). In nature, during seed dormancy, the ABA concentration level will increase and decrease during seed germination so that GA can participate in promoting seed growth. Overexpression of ABA biosynthesis contributes to a rise in seed ABA concentrations, increases seed dormancy and prevents the seed's germination (Frey *et al.*, 1999). Abscisic aldehyde oxidase 3 (AAO3) during ABA biosynthesis is the that produces ABA. During seed dormancy, where ABA blocks germination, the degree of ABA is boosted. When the seed reaches germinating status, the ABA concentration decreases, and the seed begins to increase GA sensitivity. As reviewed

by (Hilhorst and Downie, 1996; Benech-Arnold *et al.*, 1999; Beaudoin *et al.*, 2000; Grappin *et al.*, 2000; Koornneef *et al*., 2002), these are correlated with the transition from dormant to non-dormant seeds mediated after ripening. One family of proteins is ABA. ABA's receptor is the pyrabactin resistance/PYR1-like/regulatory portion of the ABA receptor (PYR/PYL/RCAR). ABA is not used in the germination of seeds. It will inhibit the elongation of the hypocotyl instead.

3.4 Testa rupture and endosperm rupture in germination

In general, the micropylar region of endosperm called "endosperm cap" is triggered to initiate germination before the radicle emerges in seeds. In several animals, activation is predominant, and completion of the germination process is crucial. Then, the rupture of the endosperm occurs, the seed coat splits and the appearance of radicles follows. By providing nutrients, the endosperm layer plays a crucial role in promoting embryonic development. It also serves as a mechanical obstacle to protecting the embryo and regulate the development of the embryo. Among various plant species for mature, dry seeds, the endosperm structure is divergent and specialised (Yan *et al.*, 2014). It is widely understood that the embryo secretes a signal that serves to expand the embryo as a message.

3.5 Morphological changes for seedling development

The complex germination process of *A. thaliana* consists of many stages, including the imbibition phase, the plateau phase, the water absorption phase and the late germination phase. In each step, beginning from imbibition to seedling, the physical and morphological changes were observed differently. Due to the elongation of radicles from the seed coat, the final stages of germination were the radicle's protrusion, followed by the seedling establishment. During the imbibition process, several processes include 1 HAI, 3 HAI, 6

HAI, 18 HAI, 24 HAI, JTR, STR, JG, HOOK SHAPED, COTYLEDON EXTENDED SEEDLING I and SEEDLING II. Every step has numerous physical changes and is named based on its reforms (Figure 3.3).

Figure 3.3: The un-dissected *Arabidopsis thaliana* seed for a specific time during imbibition until germination. The *A. thaliana* seed under the microscopy without dissection involves several phases of changes during seedlings' transition. The stages involve A: 1 h of imbibition; B: 3 h of imbibition; C: 6 h of imbibition; D: 18 h of imbibition; E: 24 h of imbibition; F: just testa ruptured; G: super testa ruptured; H: just germinated; I: hook shaped; J: cotyledons expanded; K: seedling 1 and L seedling 2.

Table 3.5: The morphological changes for each stage for *Arabidopsis thaliana* starting from early imbibition until late germination.

Stage	Morphological changes
A. 1 HAI	Day 1, imbibition, the seed absorbs water, nothing happens
B. 3 HAI	Day 1, imbibition, the seed absorbs water, nothing happens
C. 6 HAI	Day 1, imbibition, the seed absorbs water, nothing happens
D. 18 HAI	Day 1, imbibition, the seed absorbs water, and the seed coat becomes soft
E. 24 HAI	Day 1, imbibition, the seed absorbs water, and the seed coat becomes soft
F. JTR	Day1, a tiny testa rupture of seed coat due to seed expansion in absorbing water
G. STR	Day 1, a super and major testa rupture of seed coat as a result of a cell increase in size
H. JG	Day 1, a further increase of water uptake caused the event of "just germinate" is a significant change in cell expansion which caused the embryo axis elongates the radicle emergence and protrusion from the seed coat and result in germination
I. HOOK SHAPED	Day 1, hypocotyl enlarges and bent like hook-shaped
J. COTYLEDONS EXPANDED	Day 2, cotyledons expand from shoot meristem
K. SEEDLING 1	Day 3, shoot meristem enlarges and becomes a leaf, root hair appeared
L. SEEDLING 2	Day 4, enlargement of leaves from shoot meristem, seedling increases in size

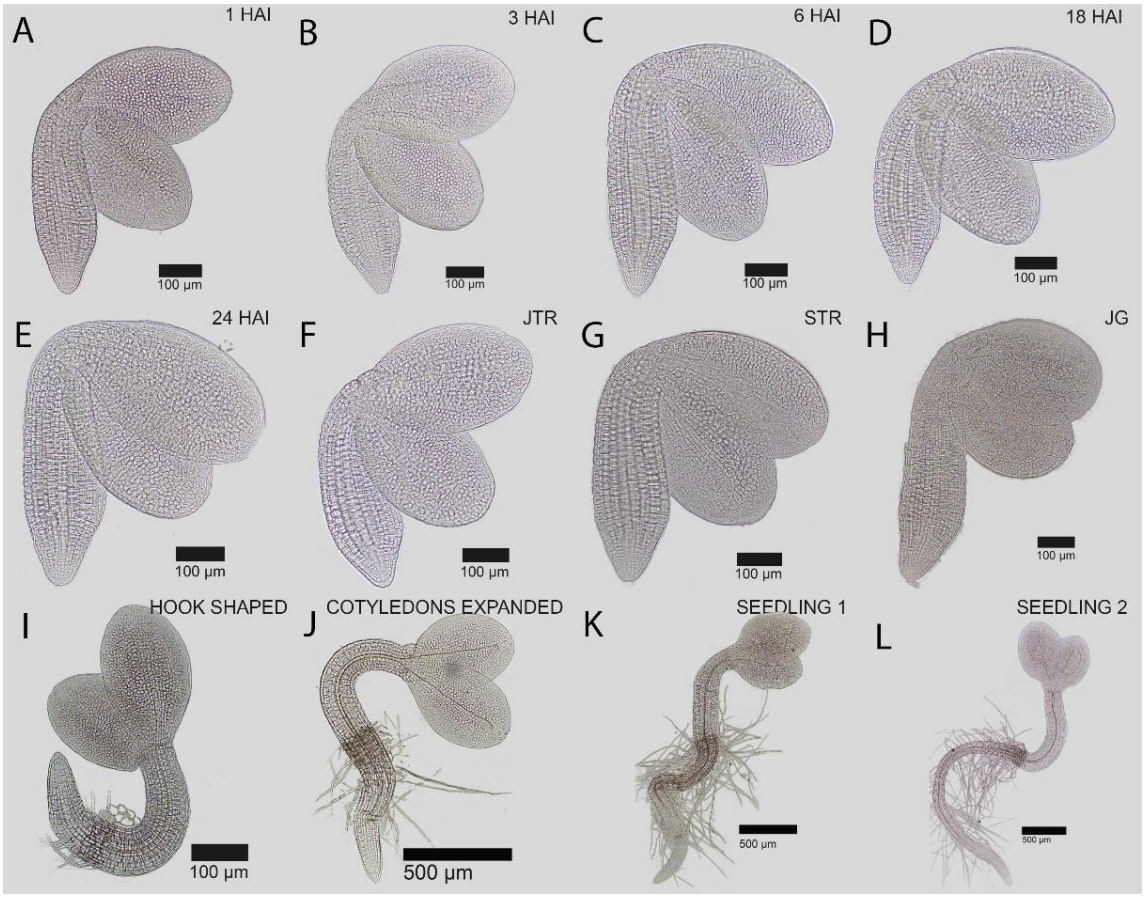


Figure 3.4: Process until seedling under light microscopy, the dissected seed without GUS staining at different times. Captured photos of the dissected embryo and seedling seed of *Arabidopsis thaliana* from seed imbibition to seedling. The different stages of physical changes of seed: A: 1 h of imbibition; B: 3 h of imbibition; C: 6 h of imbibition; D: 18 h of imbibition; E: 24 h of imbibition; F: just testa ruptured; G: super testa ruptured; H: just germinated; I: hook shaped; J: cotyledons expanded; K: seedling 1 and L: seedling 2.

3.6 Results

For each gene, the expression results are presented separately using a time series for each figure. The images of figures shown for each gene reflect the separate gene expression. The others resulted from combining the gene expression sequence using the 2D embryo model and the seedling model for gene expression biosynthesis, signalling and responses. The

same goes for the starch GUS reporter; the results are presented using the 2D embryo model.

3.6.1 GUS reporter system line

The outcome of the GUS reporter lines was present in a sequence of periods. Every figure shows the distinct gene expression behaviour of the dissected seed of *A.thaliana*.

3.6.1.1 GA biosynthesis gene expression on GUS

Figure 3.5 shows the GA biosynthesis gene at the early stage of imbibition. The promoter activity expression was distributed at the radicle cells, except at the columella cells (Figure 3.5 A-C). The *GA3Ox1* promoter then became restricted and focused on the hypocotyl's central area (Figure 3.5 D-L) during the germination progression. There was no expression at the cotyledon for the GA biosynthesis gene *GA3ox1* at the germination and in the rest of the seedling (Figure 3.5 H-L).

The other GA biosynthesis gene is *GA3ox2* (Figure 3.6). *GA3ox2* acts differently compared to the other GA biosynthesis gene, *GA3ox1* (Figure 3.5). At the early stage of seed imbibition (Figure 3.6 A-C), the localisation of the expression of *GA30x2: GUS* reporters are focused within the quiescent centre. Following the germination process progression, the expressions start to distribute along the hypocotyl at 18 HAI up to JG (Figure 3.6 E-H) but did not show any expression at the radicle, except at stage G (Figure 3.6 E-H). The rest of the stages show there is the expression of the *GA3ox2* gene actively at the quiescent area for the germinate stage until seedling 2 (Figure 3.6 H-L).

3.6.1.1.1 *GA3ox1::GUS*

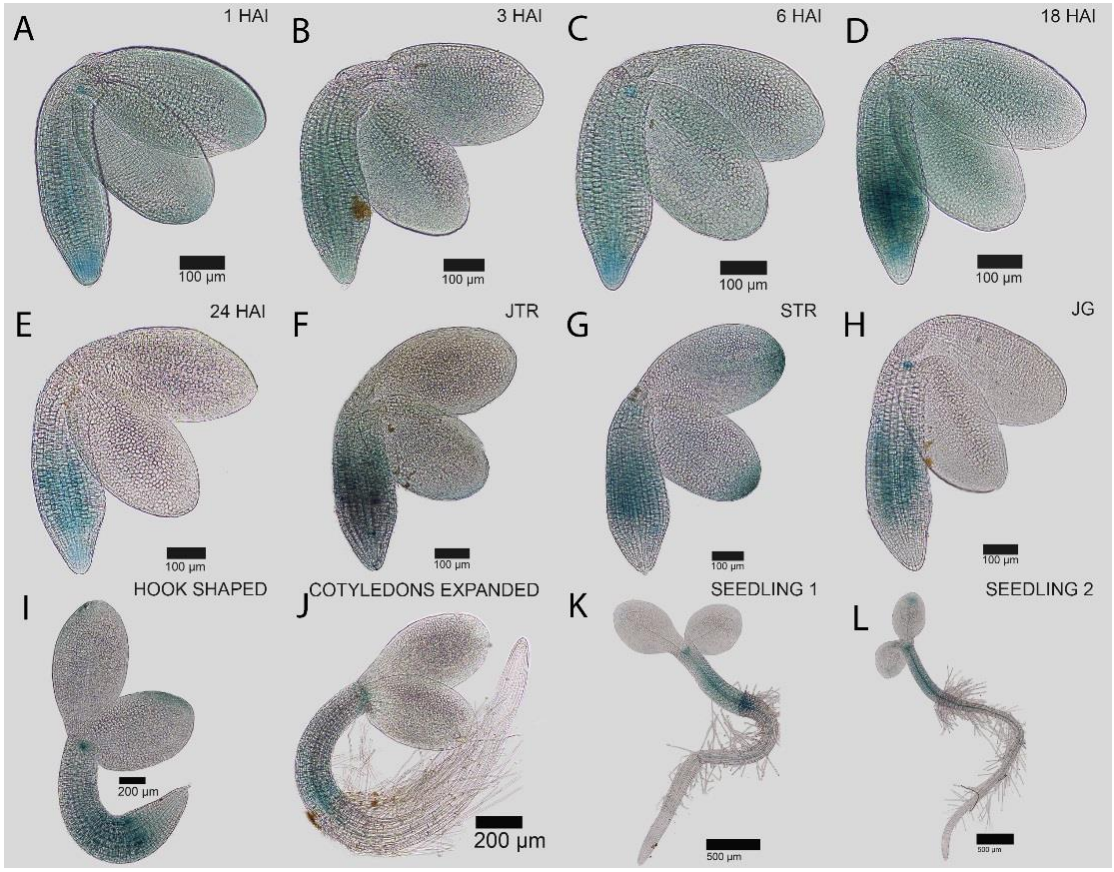


Figure 3.5: Time series images for the GUS expression of *GA3ox*1.The spatial and temporal distribution pattern of the *GA3ox1::GUS* promoter reporter activity during the transition of seed to seedling in *Arabidopsis thaliana* seeds. The blue staining indicates the GUS activity was expressed in the seed. The different stages of seeds: A: 1 h of imbibition; B: 3 h of imbibition; C: 6 h of imbibition; D: 18 h of imbibition; E: 24 h of imbibition; F: just testa ruptured; G: super testa ruptured; H: just germinated; I: hook shaped; J: cotyledons expanded; K: seedling 1 and L: seedling 2.

3.6.1.1.2 *GA3ox2::GUS*

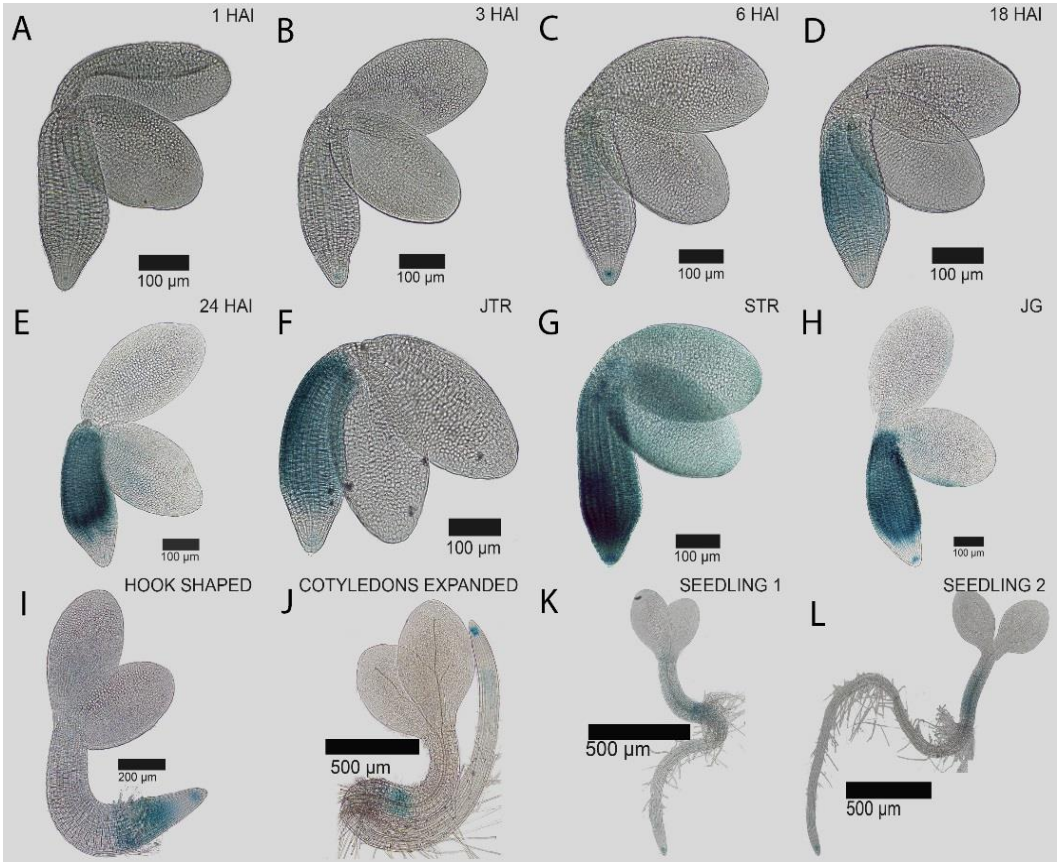


Figure 3.6: Time series images for the GUS expression for *GA3ox2*. The spatial and temporal distribution pattern of the *GA3ox2::GUS* promoter reporter activity during the transition of seed to seedling in *Arabidopsis thaliana* seed. The blue staining indicates the GUS activity expressed in the seed. The different stages of physical changes of seeds: A: 1 h of imbibition; B: 3 h of imbibition; C: 6 h of imbibition; D: 18 h of imbibition; E: 24 h of imbibition; F: just testa ruptured; G: super testa ruptured; H: just germinated; I: hook shaped; J: cotyledons expanded; K: seedling 1 and L: seedling 2.

3.6.1.2 GA signalling gene expression on GUS

The spatiotemporal expression of GA signalling genes was observed during the transition of seed toward germination to a seedling. Some of the cell types can perceive GA and produce a potential downstream response with the GA receptor *GID1A* and *GID1C* proteins' presence. According to the observations, the GID1A protein was shown generally scattered within the germinating embryo (Figure 3.7 A-G). *GID1C* protein also showed broadly dispersed expression in the cells of germinating embryo during the transition from seed to seedling. There was no expression at the root cop at the early stages of imbibition (Figure 3.7 A-C). However, expression occurred from 18 HAI until germination and seedling (Figure 3.7 D-L). Both *GID1A* and *GID1C* (Figure 3.7 I-L and Figure 3.8 1-L) showed that localised expression occured at the radicle and the endodermis after germination toward the seedling stages.

As well as *GID1A* and *GID1C*, the *SCL3* gene was also involved in the signalling pathway in *A.thaliana* seeds. During the early stages of imbibition (Figure 3.9, A), the *SCL3* signalling pathway was localised at the radicle. Then the expression concentrated at the quiescent area (Figure 3.9 B-C). Following the germination progression, expression does not occur at the columella and the lateral root cap cell for the rest of the germination process, until the seedling. (Figure 3.9, D-L).

For the *SLY:: SLY-GUS* translation fusion reporter *SLY 7* (Figure 3.10) and *SLY 12* (Figure 3.11), neither were involved in any GUS expression during the seed germination until seedling. There is no expression activity of GUS during the germination process. The previous research done by (Topham *et al.*, 2017) noted that a lack of protein accumulation in the gene encoded for *SLY* results in no expression.

3.6.1.2.1 *GID1A:: GID1A-GUS*

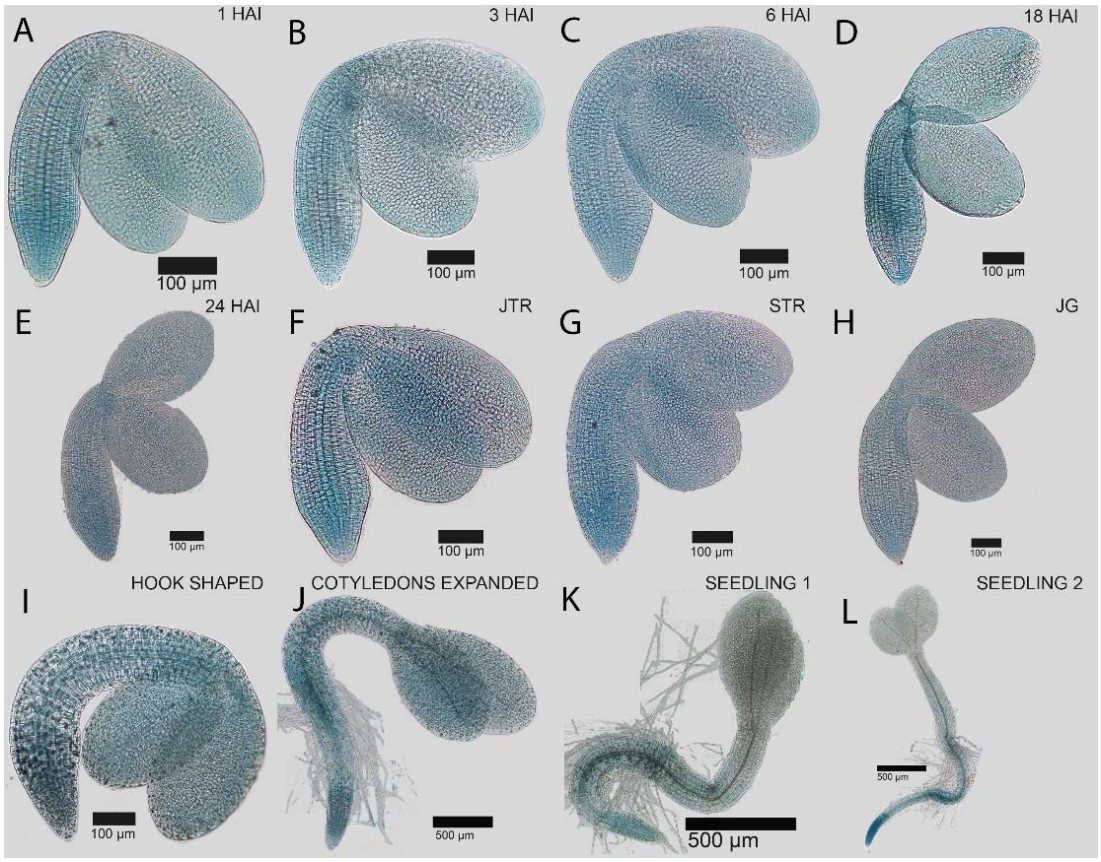


Figure 3.7: Time series images for the GUS expression for *GIDIA.* The spatial and temporal distribution pattern of the *GID1A::GID1A-GUS* translation fusion reporter activity during the transition of seed to seedling in *Arabidopsis thaliana* seed. The blue staining indicates the GUS activity was expressed in the seed. The different stages of physical changes of seeds: A: 1 h of imbibition; B: 3 h of imbibition; C: 6 h of imbibition; D: 18 h of imbibition; E: 24 h of imbibition; F: just testa ruptured; G: super testa ruptured; H: just germinated; I: hook shaped; J: cotyledons expanded; K: seedling 1 and L: seedling 2.

3.6.1.2.2 *GID1C::GID1C-GUS*

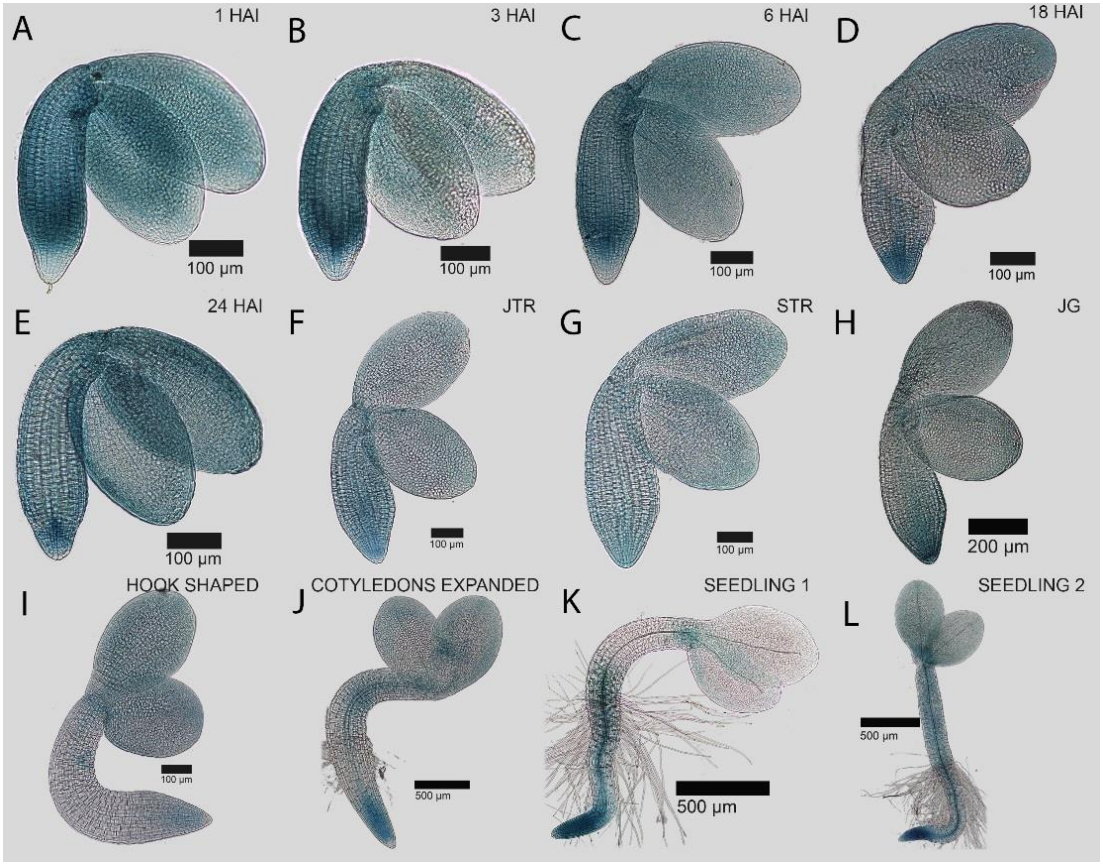


Figure 3.8:Time series images for the GUS expression for *GIDIC*. The spatial and temporal distribution pattern of the *GID1C::GID1C-GUS* translation fusion reporter activity during the transition of seed to seedling in *Arabidopsis thaliana* seed. The blue staining indicates the GUS activity was expressed in the seed. The different stages of physical changes of seeds: A: 1 h of imbibition; B: 3 h of imbibition; C: 6 h of imbibition; D: 18 h of imbibition; E: 24 h of imbibition; F: just testa ruptured; G: super testa ruptured; H: just germinated; I: hook shaped; J: cotyledons expanded; K: seedling 1 and L: seedling 2.

3.6.1.2.3 *SCL3::GUS*

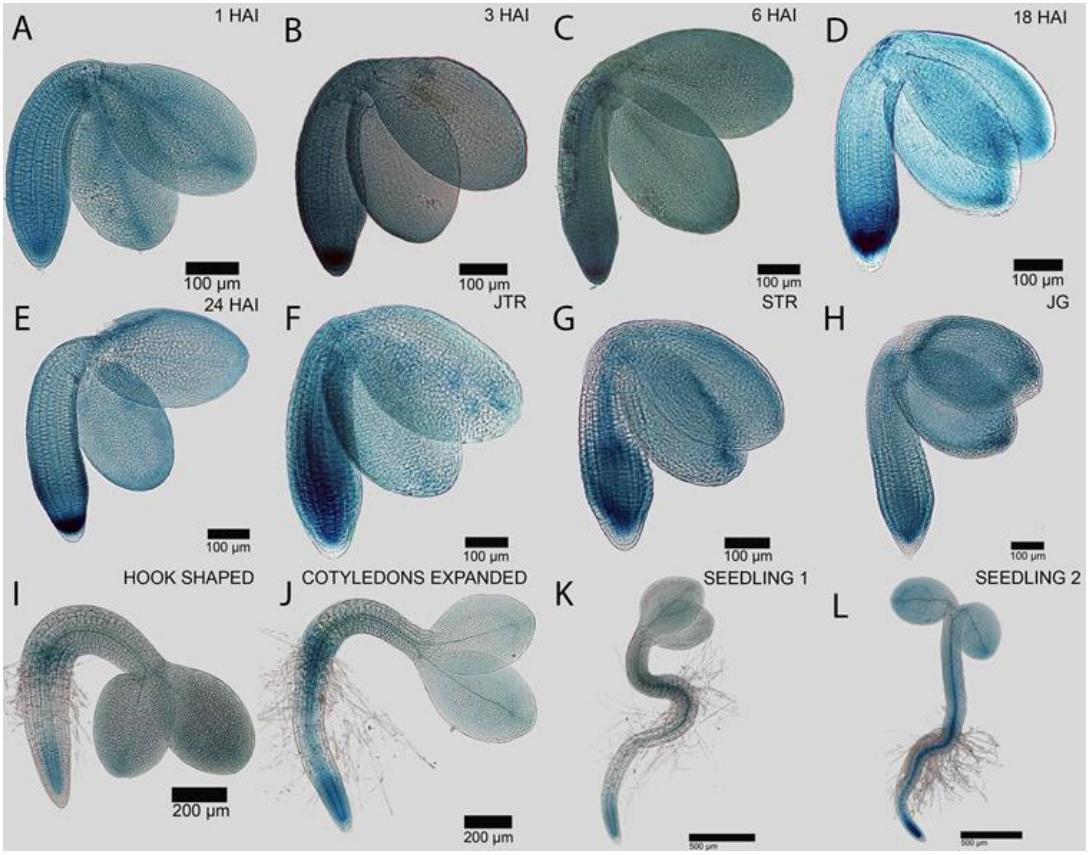


Figure 3.9: Time series images for the GUS expression for *SCL3*. The spatial and temporal distribution pattern of the *SCL3::GUS* promoter reporter activity during the transition of seed to seedling in *Arabidopsis thaliana* seeds. The blue staining indicates the GUS activity was expressed in the seed. The different stages of physical changes of seeds: A: 1 h of imbibition; B: 3 h of imbibition; C: 6 h of imbibition; D: 18 h of imbibition; E: 24 h of imbibition; F: just testa ruptured; G: super testa ruptured; H: just germinated; I: hook shaped; J: cotyledons expanded; K: seedling 1 and L: seedling 2.

3.6.1.2.4 *SLY:: SLY-GUS (7)*

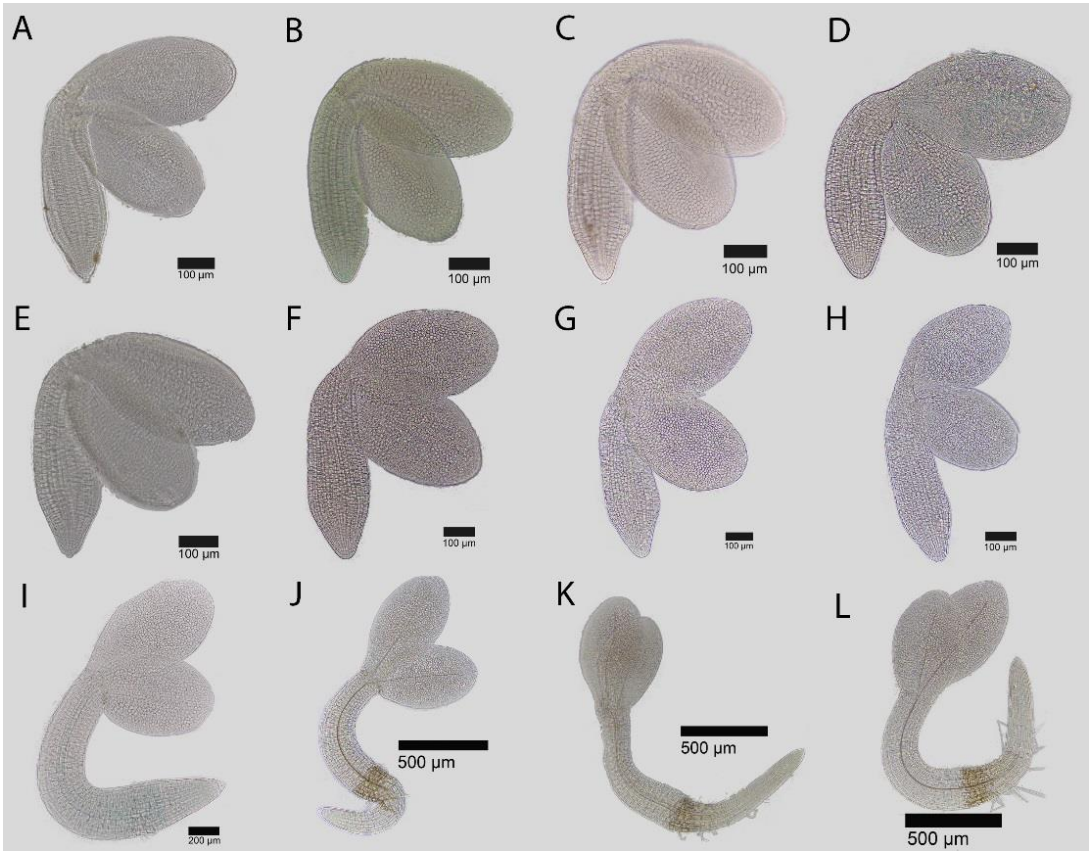


Figure 3.10: Time series images for the GUS expression for *SLY 7*. The spatial and temporal distribution pattern *SLY::SLY-GUS (7)* translation fusion reporter activity during the transition of seed to seedling in *Arabidopsis thaliana* seeds. There was no blue staining of the GUS activity expressed in the seed. The different stages of physical changes of seeds: A: 1 h of imbibition; B: 3 h of imbibition; C: 6 h of imbibition; D: 18 h of imbibition; E: 24 h of imbibition; F: just testa ruptured; G: super testa ruptured; H: just germinated; I: hook shaped; J: cotyledons expanded; K: seedling 1 and L: seedling 2.

3.6.1.2.5 *SLY:: SLY-GUS (12)*

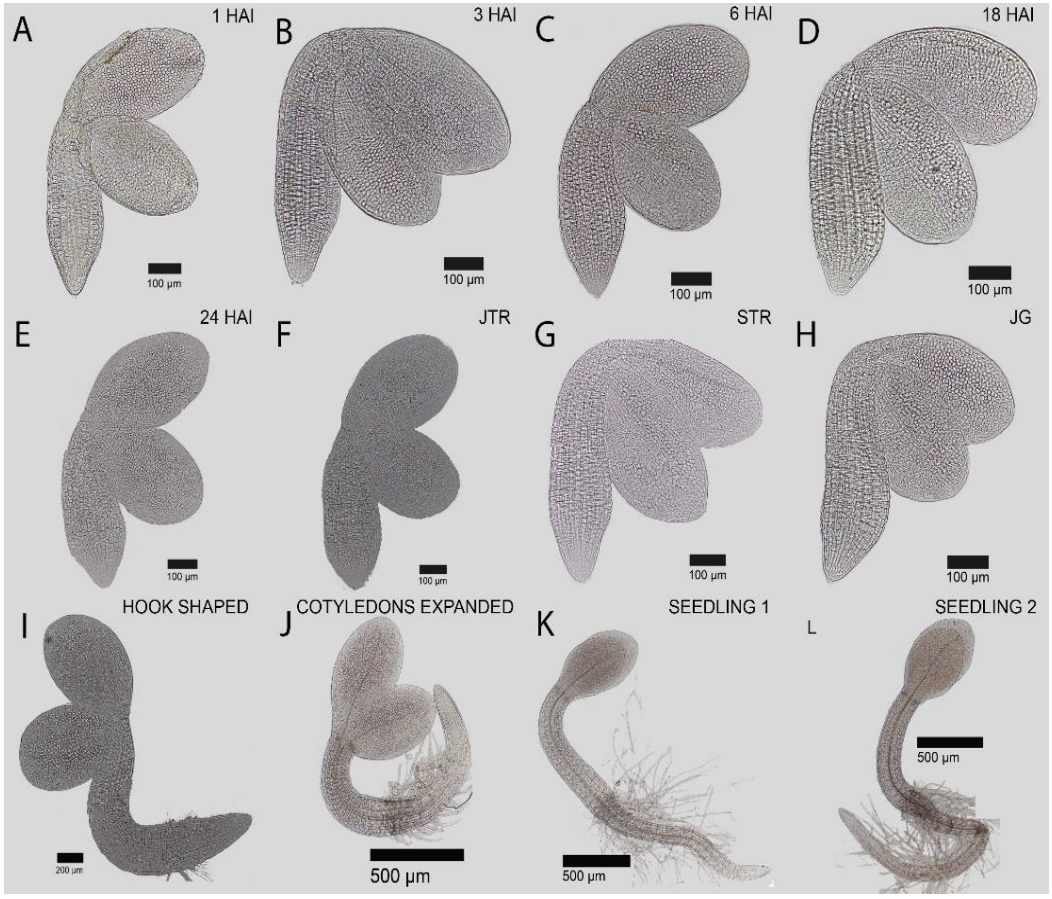


Figure 3.11: Time series images for the GUS expression for *SLY 12*. The spatial and temporal distribution pattern *SLY::SLY-GUS (12)* translation fusion reporter activity during the transition of seed to seedling in *Arabidopsis thaliana* seesd. There was no blue staining of the GUS activity expressed in the seed. The different stages of physical changes of seeds: A: 1 h of imbibition; B: 3 h of imbibition; C: 6 h of imbibition; D: 18 h of imbibition; E: 24 h of imbibition; F: just testa ruptured; G: super testa ruptured; H: just germinated; I: hook shaped; J: cotyledons expanded; K: seedling 1 and L: seedling 2.

3.6.1.3 GA responses gene expression on GUS

The GA response also involves the cell wall expansion in cell development before germination. The cell wall expansion promotes the cell wall weakening and loosens the cells because the cells are firmly glued together through their cell wall. This is to allow for the

cell to enlarge (Cosgrove, 2005). At the early stage of imbibition, the *EXPA 1::GUS* activity was induced within the radicle (Figure 3.12 A-C) and had a strong signalling expression of EXPA::GUS reporter at the root cap for 1 HAI to 6 HAI. The GUS activity was distributed within both hypocotyl and cotyledons (Figure 3.12 D-H). The expression at the root cap came back, started at hooked shaped towards the seedling (Figure 3.12 I-L).

Overall, for the *EXPA 9::GUS* promoter, there was no expression (Figure 3.13). However, there was a low expression at the early stage of imbibition at 1 HAI to 6 HAI (Figure 3.14 A-C). At the initial phase of imbibition, the expression of *EXPA 10::GUS* promoter activity was seen starting from 1 h until 24 HAI. The expression was at the connection of hypocotyl and cotyledons (Figure 3.14 A-E). Then, no expression activity was observed at the cotyledons' expanded stage to seedlings (Figure 3.14 F-H).

Nevertheless, *EXPA 10* promoter activity expression returned at the connection of hypocotyl and cotyledons at stage 1 to L (Figure 3.14 I-L). Overall, for *EXPA 15::GUS* reporter activity, there was no specific expression for the rest within the embryo. However, the expression was at the hypocotyl for stages A to G (Figure 3.15 A-G), and the rest of the stages did not have any expression activity of the *EXPA 15::GUS* reporter.

Corresponding to the *EXPA* gene, the *XTH* gene family was also involved in the GA response, in line with the *EXPA* genes. The *XTH* gene family is upregulated during seed germination. The *XTH 9::GUS* reporter was observed consistently along the hypocotyl and cotyledons. However, there was no expression at the radicle, lateral root cap and the columella (Figure 3.16 A-H). During the germination stage, the expression started to disappear from the cotyledon at I (Figure 3.16). The expression was still in the columella

area (Figure 3.16 J-L). There was no activity of GUS expression *XTH 18::GUS* for A-G (Figure 3.17 A-G).

Nevertheless, at 3 HAI and STR, there was low expression at the connection of hypocotyl and cotyledons (Figure 3.17 C and G). At the germination stage, JG, there was strong expression at the radicle tip and lateral root cap (Figure 3.17 H). Then the expression starts to leave the radicle and moved to the hypocotyl until the seedling (Figure 3.17 I-L). For the *XTH 19::GUS* reporter, the expression activity of *XTH 19::GUS* was seen at the initial imbibition at 1 h to 3 h. There is expression at the intersection of hypocotyl with cotyledons and the columella area (Figure 3.18 A-C), then the expression starts to move along the hypocotyl for the rest of the germination phase to seedling (Figure 3.18 D-H).

3.6.1.3.1 *EXPA 1::GUS*

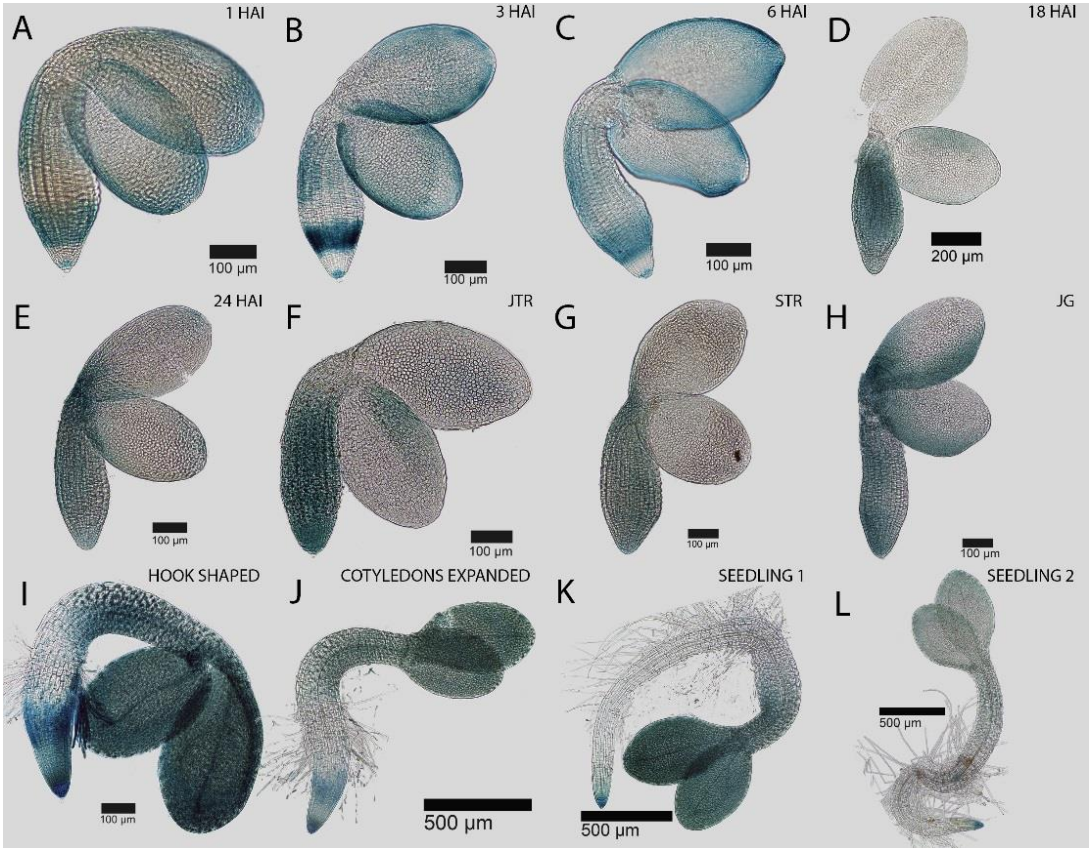


Figure 3.12: Time series images for the GUS expression for *EXPA 1*. The spatial and temporal distribution pattern of the *EXPA 1::GUS* promoter reporter activity during the transition of seed to seedling in *Arabidopsis thaliana* seeds. The blue staining indicates the GUS activity was expressed in the seed. The different stages of physical changes of seeds: A: 1 h of imbibition; B: 3 h of imbibition; C: 6 h of imbibition; D: 18 h of imbibition; E: 24 h of imbibition; F: just testa ruptured; G: super testa ruptured; H: just germinated; I: hook shaped; J: cotyledons expanded; K: seedling 1 and L: seedling 2.

3.6.1.3.2 *EXPA 9::GUS*

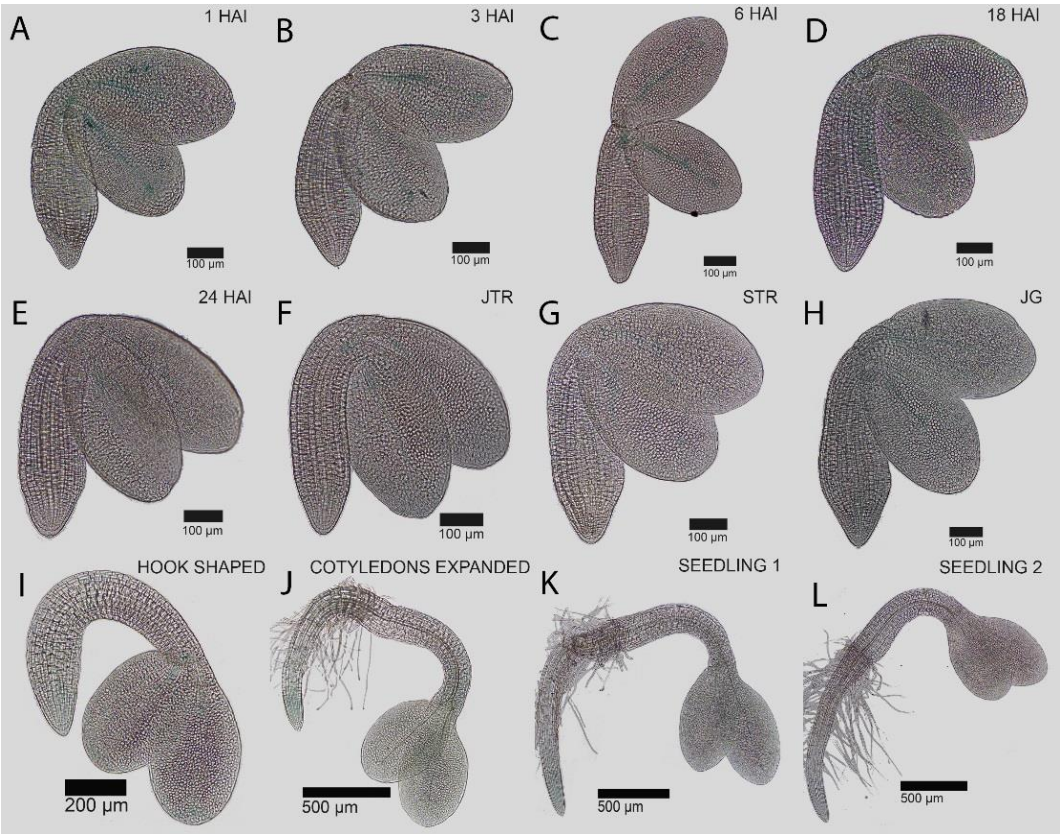


Figure 3.13: Time series images for the GUS expression for *EXPA 9*. The spatial and temporal distribution pattern of the *EXPA 9::GUS* promoter reporter activity during the transition of seed to seedling in *Arabidopsis thaliana* seeds. The blue staining indicates the GUS activity was expressed in the seed. The different stages of physical changes of seeds: A: 1 h of imbibition; B: 3 h of imbibition; C: 6 h of imbibition; D: 18 h of imbibition; E: 24 h of imbibition; F: just testa ruptured; G: super testa ruptured; H: just germinated; I: hook shaped; J: cotyledons expanded; K: seedling 1 and L: seedling 2.

3.6.1.3.3 *EXPA 10::GUS*

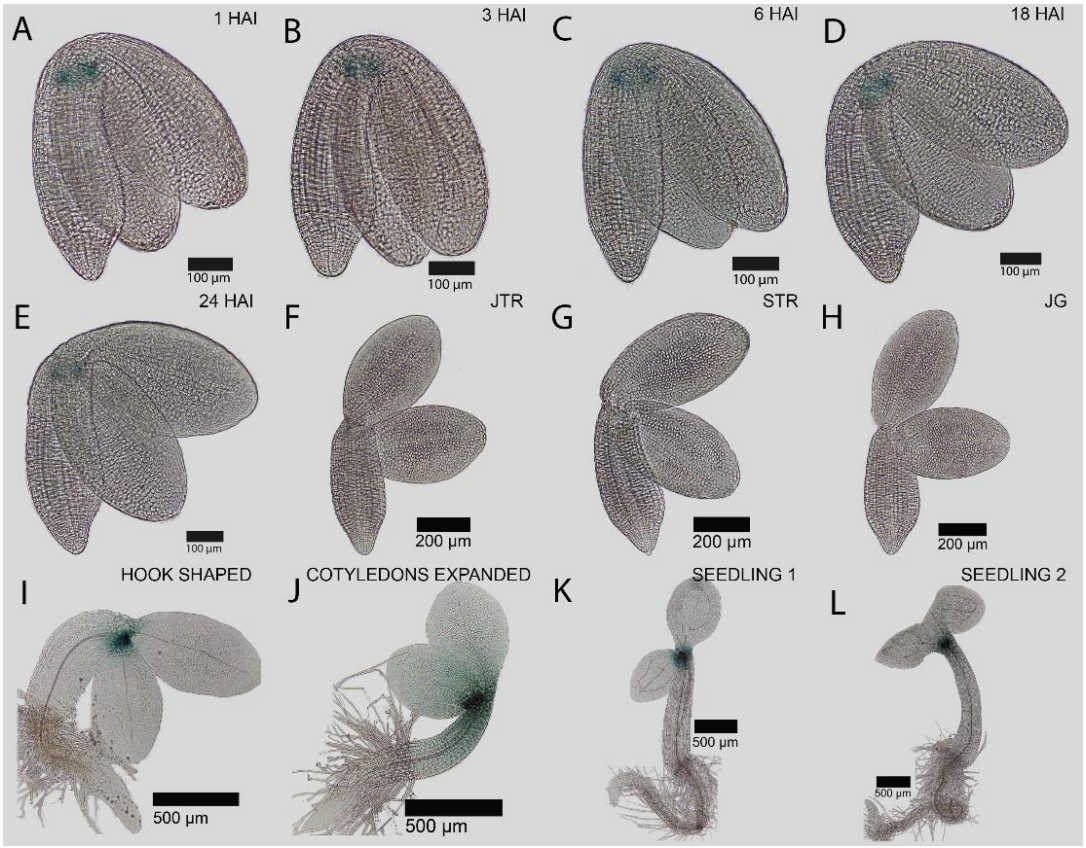


Figure 3.14: Time series images for the GUS expression for *EXPA 10*. The spatial and temporal distribution pattern of the *EXPA 10::GUS* promoter reporter activity during the transition of seed to seedling in *Arabidopsis thaliana* seeds. The blue staining indicates the GUS activity was expressed in the seed. The different stages of physical changes of seeds: A: 1 h of imbibition; B: 3 h of imbibition; C: 6 h of imbibition; D: 18 h of imbibition; E: 24 h of imbibition; F: just testa ruptured; G: super testa ruptured; H: just germinated; I: hook shaped; J: cotyledons expanded; K: seedling 1 and L: seedling 2.

3.6.1.3.4 *EXPA 15: GUS*

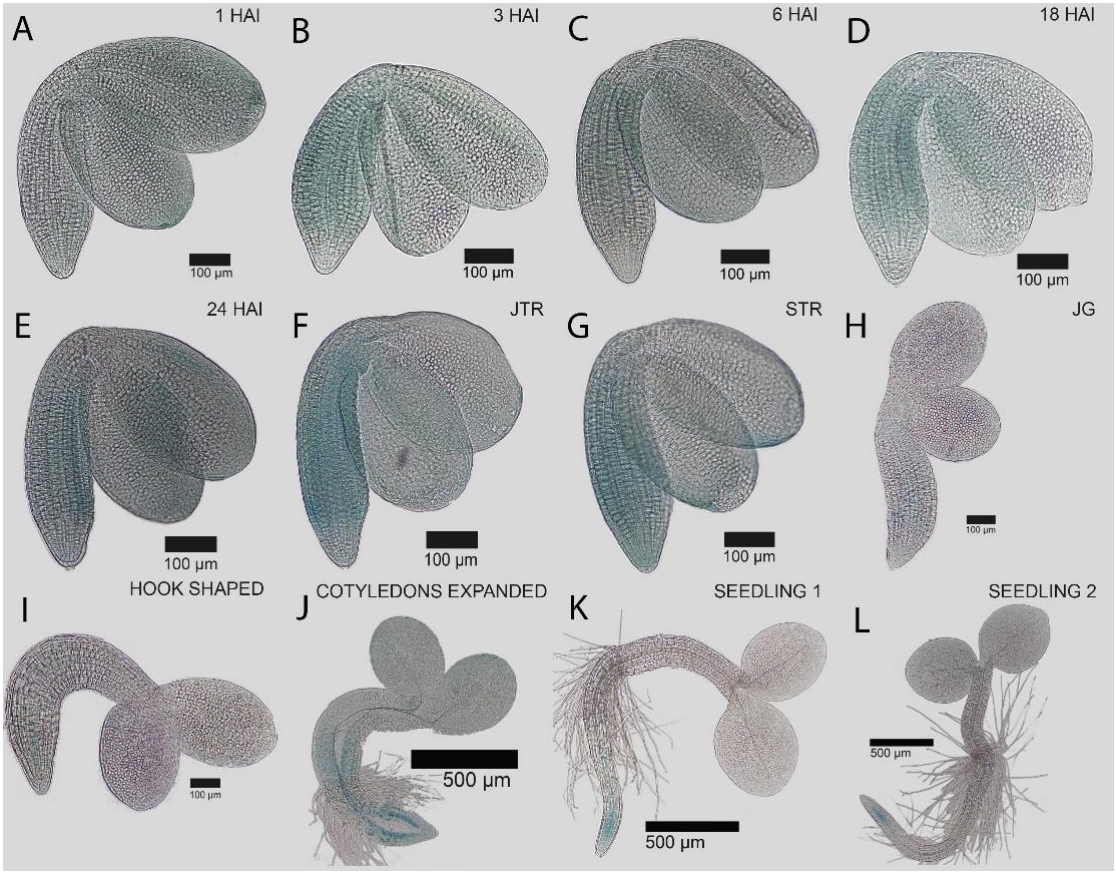


Figure 3.15: Time series images for the GUS expression for *EXPA 15*. The spatial and temporal distribution pattern of the *EXPA 15::GUS* promoter reporter activity during the transition of seed to seedling in *Arabidopsis thaliana* seeds. The blue staining indicates the GUS activity was expressed in the seed. The different stages of physical changes of seeds: A: 1 h of imbibition; B: 3 h of imbibition; C: 6 h of imbibition; D: 18 h of imbibition; E: 24 h of imbibition; F: just testa ruptured; G: super testa ruptured; H: just germinated; I: hook shaped; J: cotyledons expanded; K: seedling 1 and L: seedling 2.

3.6.1.3.5 *XTH 9::GUS*

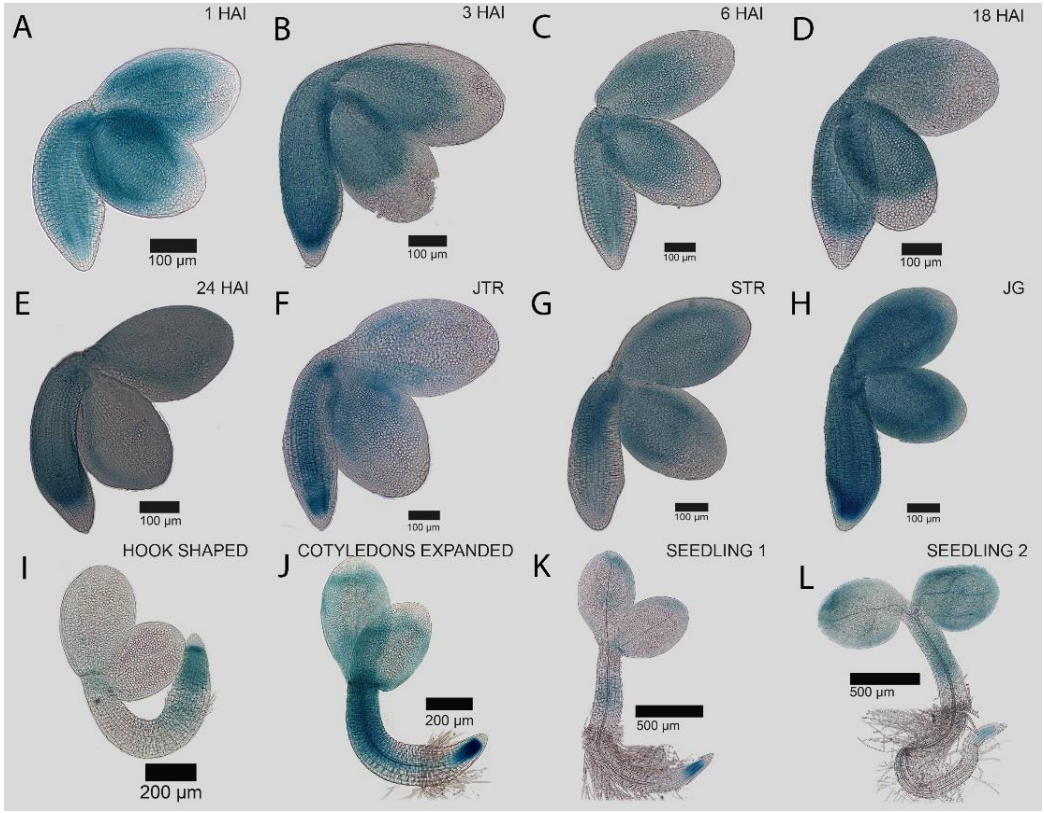


Figure 3.16: The time series images for the GUS expression for *XTH 9*. The spatial and temporal distribution pattern of the *XTH 9::GUS* promoter reporter activity during the transition of seed to seedling in *Arabidopsis thaliana* seeds. The blue staining indicates the GUS activity was expressed in the seed. The different stages of physical changes of seeds: A: 1 h of imbibition; B: 3 h of imbibition; C: 6 h of imbibition; D: 18 h of imbibition; E: 24 h of imbibition; F: just testa ruptured; G: super testa ruptured; H: just germinated; I: hook shaped; J: cotyledons expanded; K: seedling 1 and L: seedling 2.

3.6.1.3.6 *XTH 18::GUS*

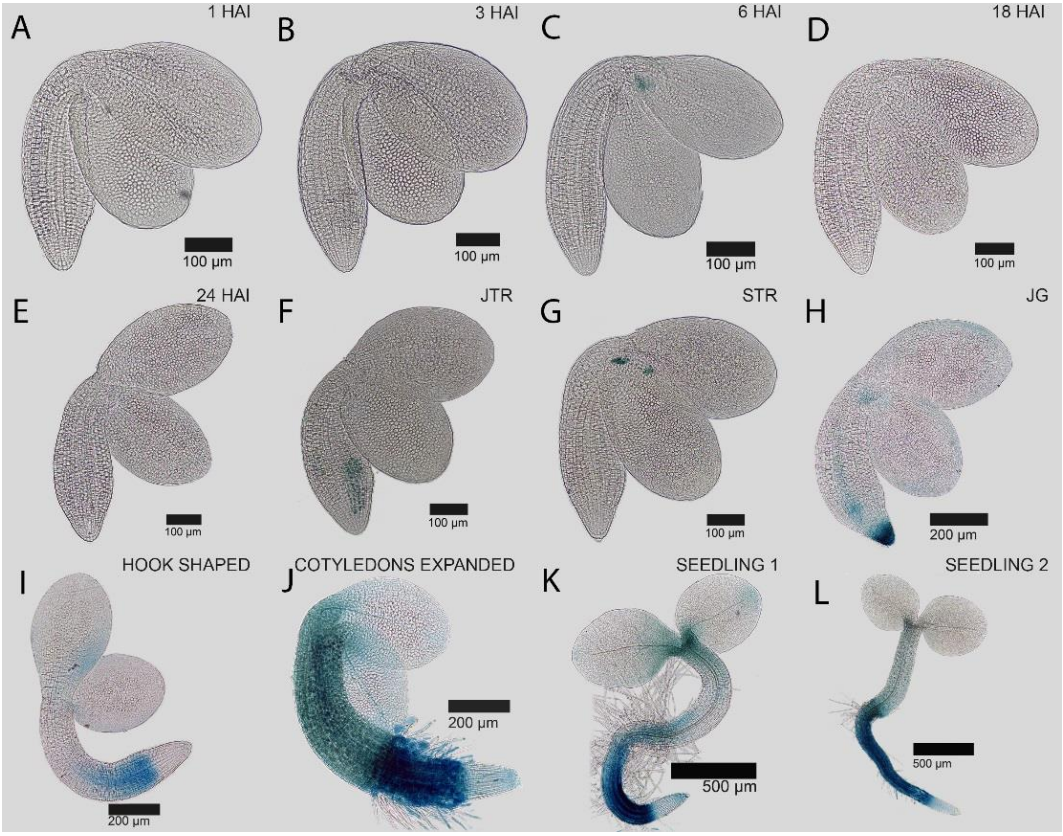


Figure 3.17: Time series images for the GUS expression for *XTH 18*. The spatial and temporal distribution pattern of the *XTH 18::GUS* promoter reporter activity during the transition of seed to seedling in *Arabidopsis thaliana* seeds. The blue staining indicates the GUS activity was expressed in the seed. The different stages of physical changes of seeds: A: 1 h of imbibition; B: 3 h of imbibition; C: 6 h of imbibition; D: 18 h of imbibition; E: 24 h of imbibition; F: just testa ruptured; G: super testa ruptured; H: just germinated; I: hook shaped; J: cotyledons expanded; K: seedling 1 and L: seedling 2.

3.6.1.3.7 *XTH 19::GUS*

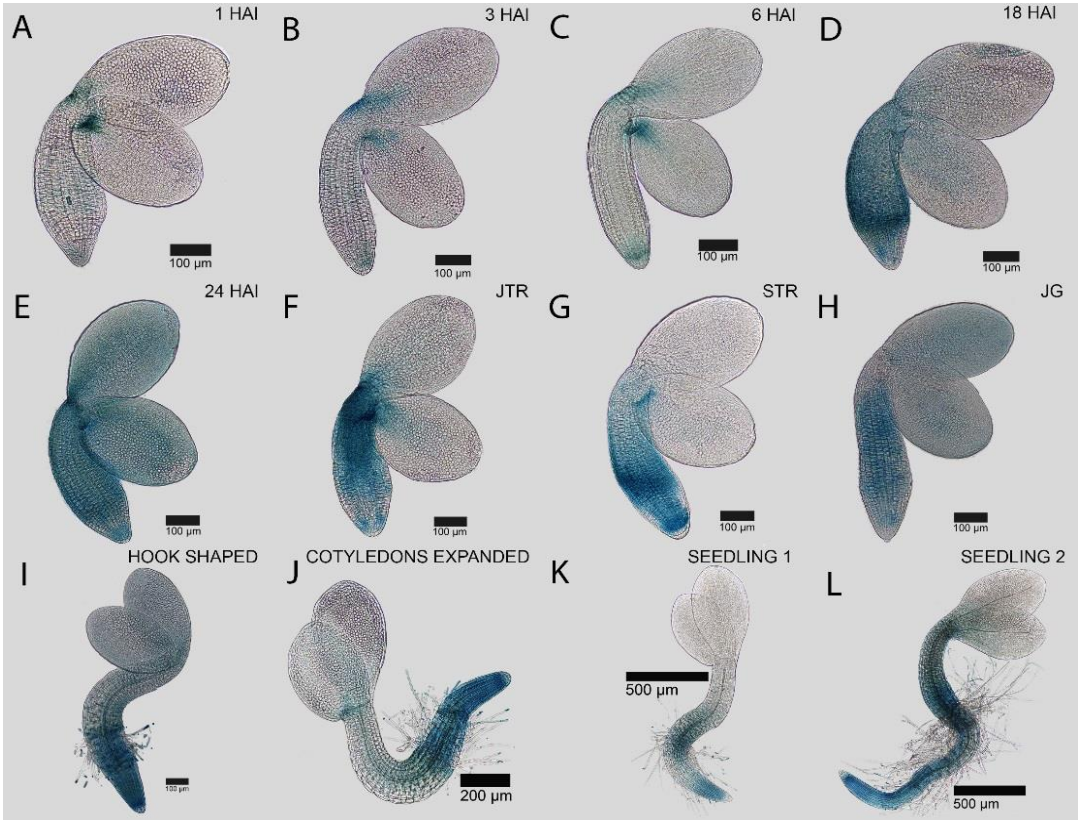


Figure 3.18: Time series images for the GUS expression for *XTH 19.* The spatial and temporal distribution pattern of the *XTH 19::GUS* promoter reporter activity during the transition of seed to seedling in *Arabidopsis thaliana* seeds. The blue staining indicates the GUS activity was expressed in the seed. The different stages of physical changes of seeds: A: 1 h of imbibition; B: 3 h of imbibition; C: 6 h of imbibition; D: 18 h of imbibition; E: 24 h of imbibition; F: just testa ruptured; G: super testa ruptured; H: just germinated; I: hook shaped; J: cotyledons expanded; K: seedling 1 and L: seedling 2.

3.6.1.4 DELLA protein expression on GUS

The DELLA protein is the transcription factor that inhibits the GA response. It can be considered a negative growth regulator of GA signal transduction (Dill, Jung and Sun, 2001).The *GAI::GAI-GUS* reporter shows a deficient staining expression at the early stage of imbibition at 1 h to 3 h (Figure 3.19 A-B) at the radicle and hypocotyl. However, no expression exists as the germination progresses to seedling (Figure 3.19, C-L). The RGA-GUS reporter does not show any expression activity for the whole stage of seed transition to seedling (Figure 3.20 A-L). While for *RGL2::RGL2-GUS* reporter and *RGL2(8): RGL2(8)-GUS*, they do not show any expression activity of GUS (Figure 3.21 and Figure 3.22, respectively). For the *RGL2::GUS*, there is no expression because it lacks an *RGL2* protein, with is consistent with the results of (Topham *et al.*, 2017).

3.6.1.4.1 *GAI::GAI-GUS (10)*

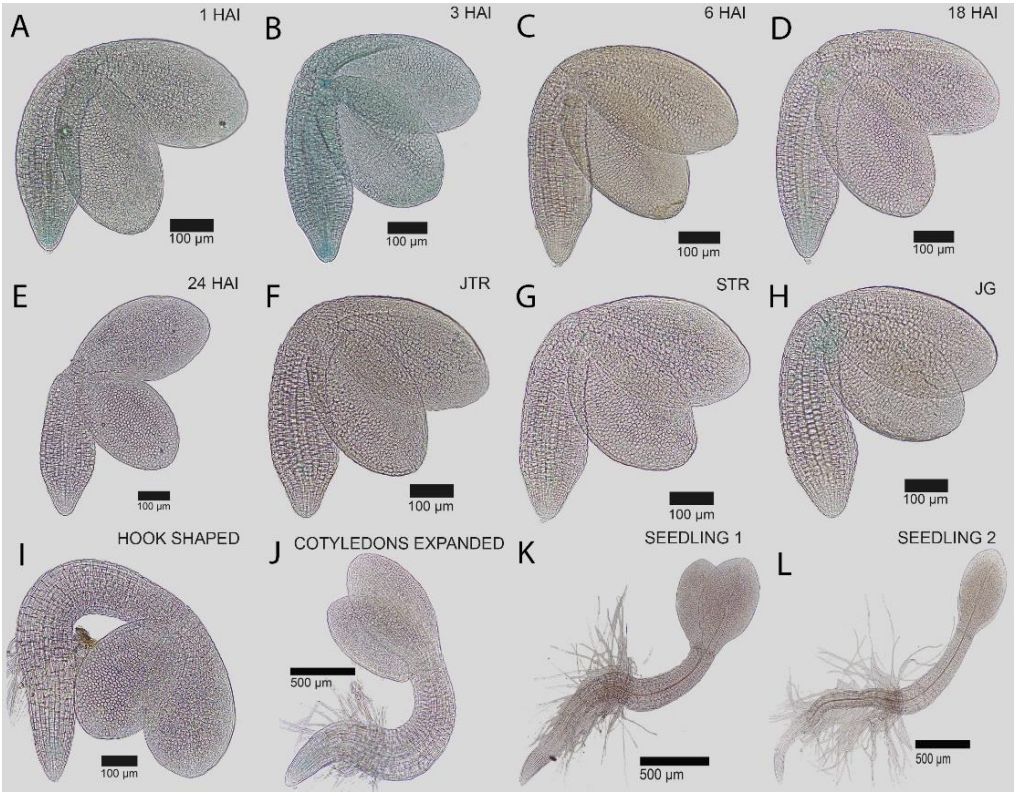


Figure 3.19: Time series images for the GUS expression for *GAI 10*. The spatial and temporal distribution pattern *GAI::GAI-GUS (10)* translation fusion reporter activity during the transition of seed to seedling in *Arabidopsis thaliana* seeds. There was no blue staining of the GUS activity expressed in the seed. The different stages of physical changes of seeds: A: 1 h of imbibition; B: 3 h of imbibition; C: 6 h of imbibition; D: 18 h of imbibition; E: 24 h of imbibition; F: just testa ruptured; G: super testa ruptured; H: just germinated; I: hook shaped; J: cotyledons expanded; K: seedling 1 and L: seedling 2.

3.6.1.4.2 *RGA:: RGA-GUS (3)*

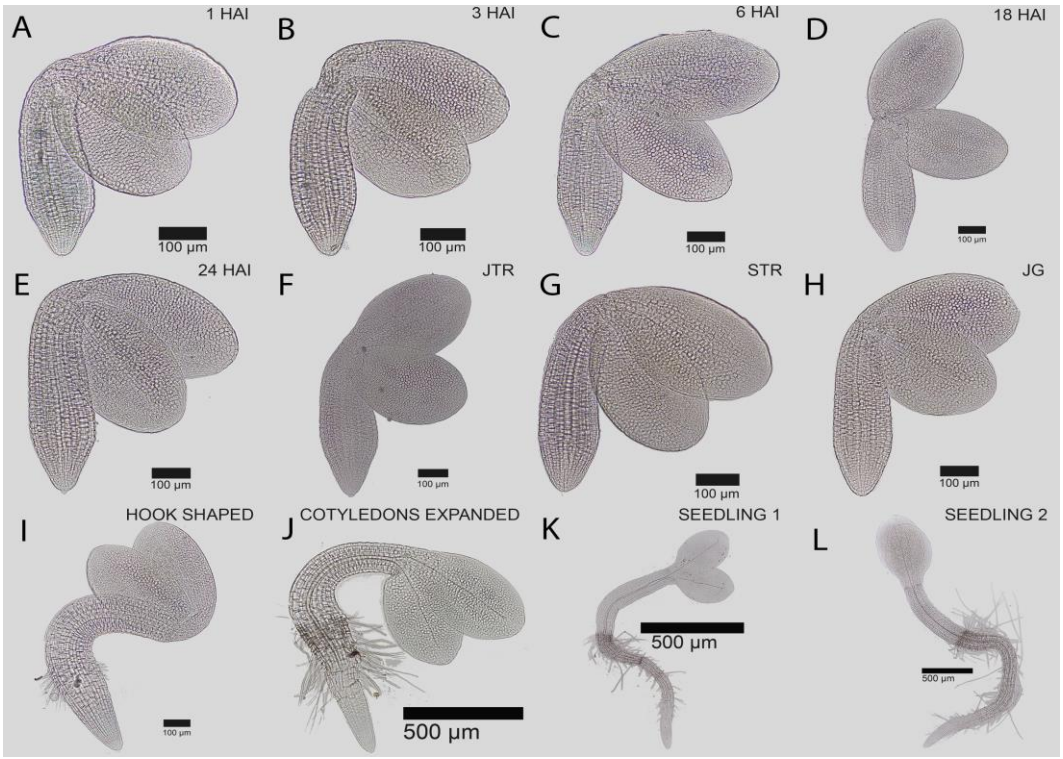


Figure 3.20: The time series images for the GUS expression for *RGA 3*. The spatial and temporal distribution pattern *RGA::RGA-GUS (3)* translation fusion reporter activity during the transition of seed to seedling in *Arabidopsis thaliana* seeds. There was no blue staining of the GUS activity expressed in the seed. The different stages of physical changes of seeds: A: 1 h of imbibition; B: 3 h of imbibition; C: 6 h of imbibition; D: 18 h of imbibition; E: 24 h of imbibition; F: just testa ruptured; G: super testa ruptured; H: just germinated; I: hook shaped; J: cotyledons expanded; K: seedling 1 and L: seedling 2.

3.6.1.4.3 *RGL2: RGL2-GUS (2)*

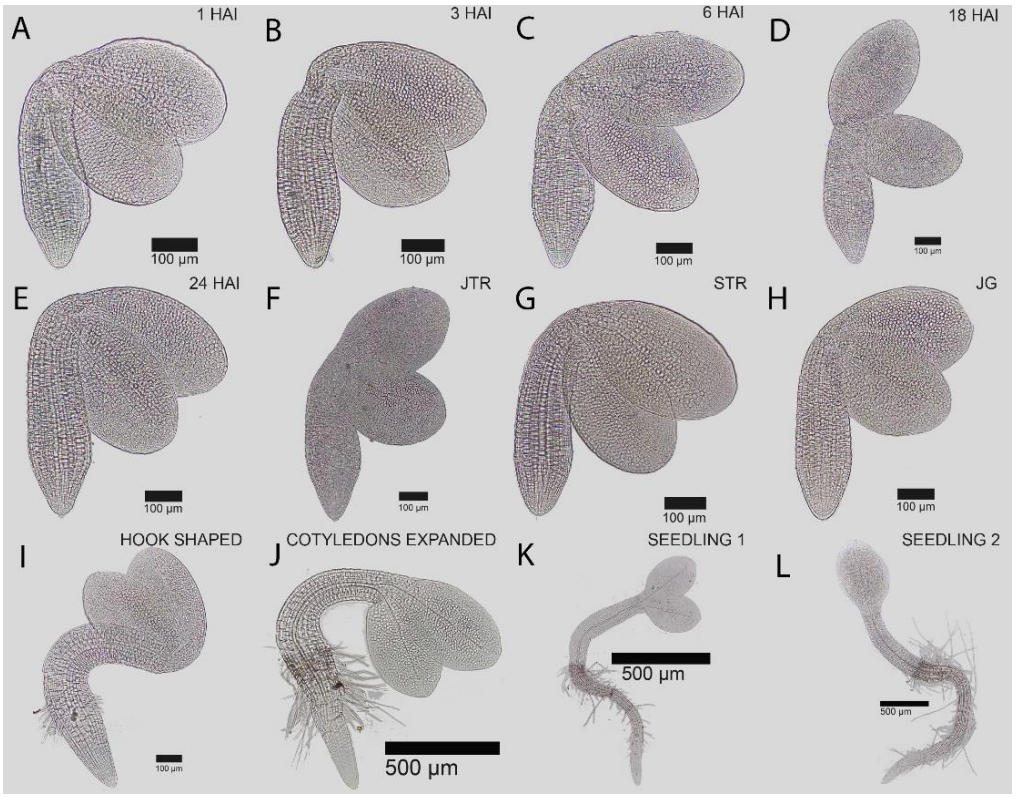


Figure 3.21: Time-series images for the GUS expression for *RGL2 (2)*. The spatial and temporal distribution pattern of the *RGL2::RGL2-GUS (2)* translation fusion reporter activity during the transition of seed to seedling in *Arabidopsis thaliana* seeds. There was no blue staining of the GUS activity expressed in the seed. The different stages of physical changes of seeds: A: 1 h of imbibition; B: 3 h of imbibition; C: 6 h of imbibition; D: 18 h of imbibition; E: 24 h of imbibition; F: just testa ruptured; G: super testa ruptured; H: just germinated; I: hook shaped; J: cotyledons expanded; K: seedling 1 and L: seedling 2.

3.6.1.4.4 *RGL2: RGL2-GUS (8)*

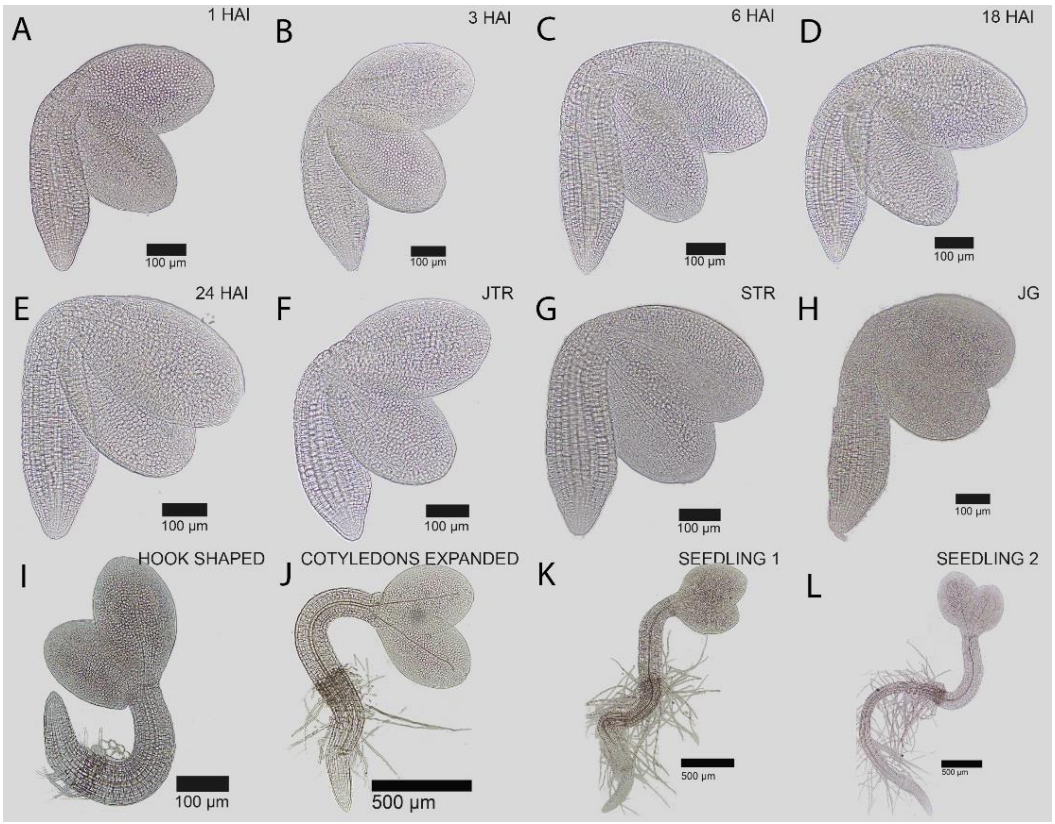


Figure 3.22 The time-series images for the GUS expression for *RGL2 (8)*. The spatial and temporal distribution pattern of the *RGL2::RGL2-GUS (8)* translation fusion reporter activity during the transition of seed to seedling in *Arabidopsis thaliana* seeds. There was no blue staining of the GUS activity expressed in the seed. The different stages of physical changes of seeds: A: 1 h of imbibition; B: 3 h of imbibition; C: 6 h of imbibition; D: 18 h of imbibition; E: 24 h of imbibition; F: just testa ruptured; G: super testa ruptured; H: just germinated; I: hook shaped; J: cotyledons expanded; K: seedling 1 and L: seedling 2.

3.6.1.5 ABA biosynthesis gene expression on GUS

The *AAO3* and *ABA* genes are from the ABA biosynthesis reporter gene. At the early imbibition to just testa rupture (Figure 3.23 A-F). The *AAO3::GUS* reporter has a strong expression of protein localisation along the hypocotyl. However, the expression was excluded from the radicle tip. At the super testa rupture (Figure 3.23 G), the protein's localisation was at the radicle tip and along the hypocotyl. Then the expression starts to leave the radicle tip at stage H to I (Figure 3.23 H-J). Finally, for the rest of the seedling stage, the expression came back at the cotyledons as it expands to seedling (Figure 3.23 J-L).

The *ABA::GUS* reporter activities were observed. The expression is at the hypocotyl and cotyledons, except at the radicle tip at the first hour of imbibition (Figure 3.24 A-D). As the germination progressed, the expression in cotyledons disappeared at 24 HAI to JTS (Figure 3.24 E-F). The expression is concentrated in the radicle tip and hypocotyl at the stages of STR to hook-shaped (Figure 3.24 G-I), then the expression was maintained at the hypocotyl and disappeared at the cotyledon (Figure 3.24 J-L).

3.6.1.5.1 *AAO3::AAO3-GUS*

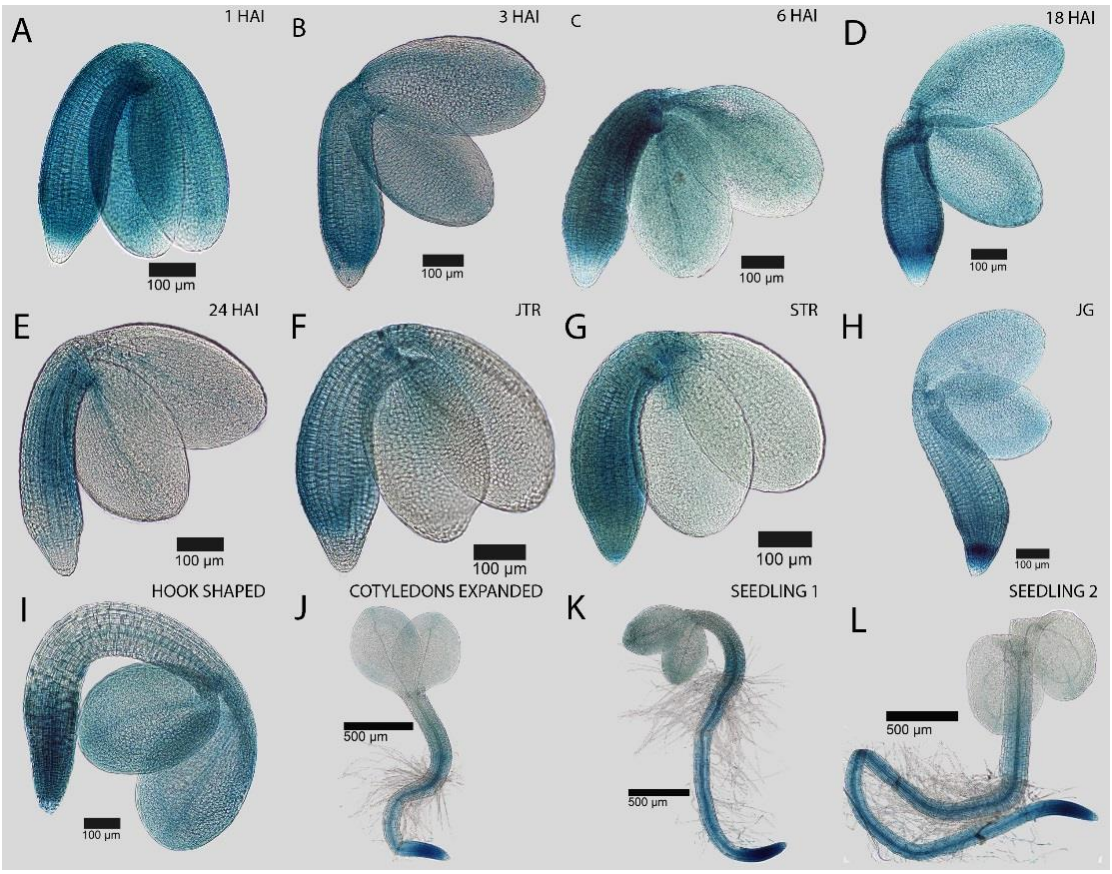


Figure 3.23: The time series images for the GUS expression for *AAO3*. The spatial and temporal distribution pattern of the *AAO3: AAO3-GUS* translation fusion reporter activity during the transition of seed to seedling in *Arabidopsis thaliana* seeds. The blue staining indicates the GUS activity was expressed in the seed. The different stages of physical changes of seeds: A: 1 h of imbibition; B: 3 h of imbibition; C: 6 h of imbibition; D: 18 h of imbibition; E: 24 h of imbibition; F: just testa ruptured; G: super testa ruptured; H: just germinated; I: hook shaped; J: cotyledons expanded; K: seedling 1 and L: seedling 2.

3.6.1.5.2 *ABA2::ABA2-GUS*

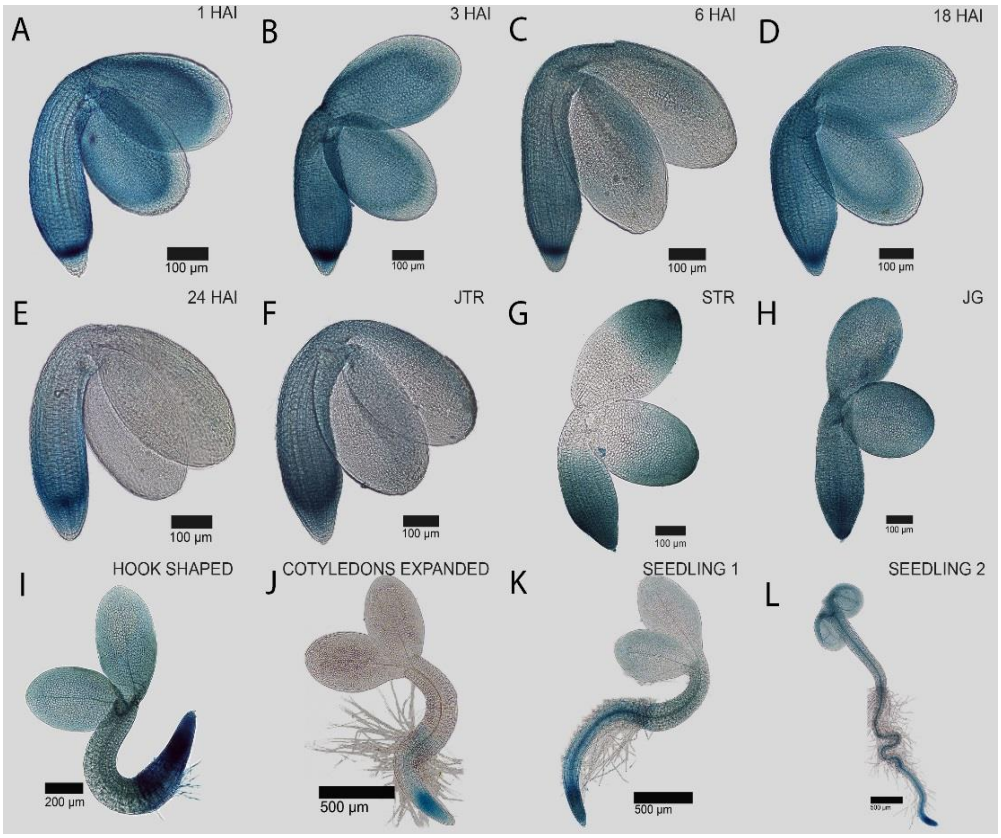


Figure 3.24: The time series images for the GUS expression for *ABA2*. The spatial and temporal distribution pattern of the *ABA2::ABA2-GUS* translation fusion reporter activity during the transition of seed to seedling in *Arabidopsis thaliana* seeds. The blue staining indicates the GUS activity was expressed in the seed. The different stages of physical changes of seeds: A: 1 h of imbibition; B: 3 h of imbibition; C: 6 h of imbibition; D: 18 h of imbibition; E: 24 h of imbibition; F: just testa ruptured; G: super testa ruptured; H: just germinated; I: hook shaped; J: cotyledons expanded; K: seedling 1 and L: seedling 2.

3.6.1.6 ABA response gene expression on GUS

RAB 18 gene is the ABA response gene that determines the spatiotemporal pattern during seed to seedling transition in *A. thaliana.* During the first germination time course, the expression of *RAB 18* was at the hypocotyl and cotyledons. However, there was no expression at the root cap and columella (Figure 3.25 A-I). As the cotyledon expands to the seedling phase, the GUS activity of *RAB 18* was visible at the radicle tip and hypocotyl (Figure 3.25 J-L).

3.6.1.6.1 *RAB 18::GUS*

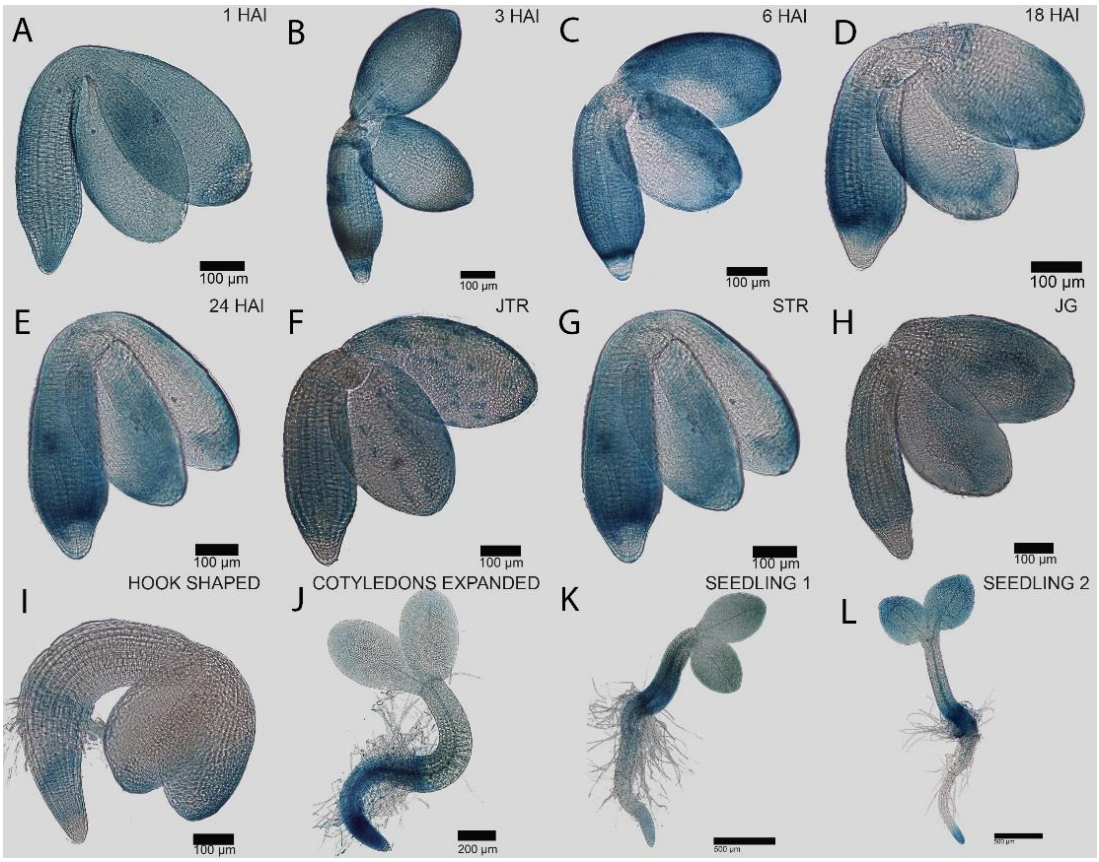


Figure 3.25: The time series images for the GUS expression for *RAB 18.* The spatial and temporal distribution pattern of the *RAB 18::GUS* promoter reporter activity during the transition of seed to seedling in *Arabidopsis thaliana* seeds. The blue staining indicates the GUS activity was expressed in the seed. The different stages of physical changes of seeds: A: 1 h of imbibition; B: 3 h of imbibition; C: 6 h of imbibition; D: 18 h of imbibition; E: 24 h of imbibition; F: just testa ruptured; G: super testa ruptured; H: just germinated; I: hook shaped; J: cotyledons expanded; K: seedling 1 and L: seedling 2.

3.6.2 2D embryo and seedling images

The gene expression images were summarised into a 2D cartoon model (the whole embryo) and seedling model, following the 2D embryo model, figure 3.26 (the different colours were used to plot the pattern of the gene expression to represent the genes).

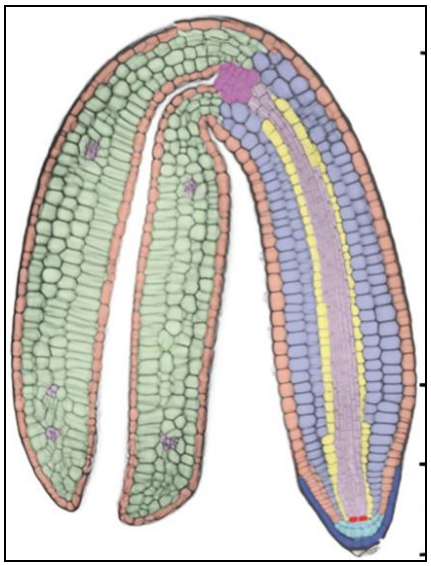


Figure 3.26:2D whole embryo *Arabidopsis thaliana* sample model. The 2D model is used to represent the whole results of the staining pattern of the genes. The different colours show the different segmentation within the cell types of *A. thaliana* seed.

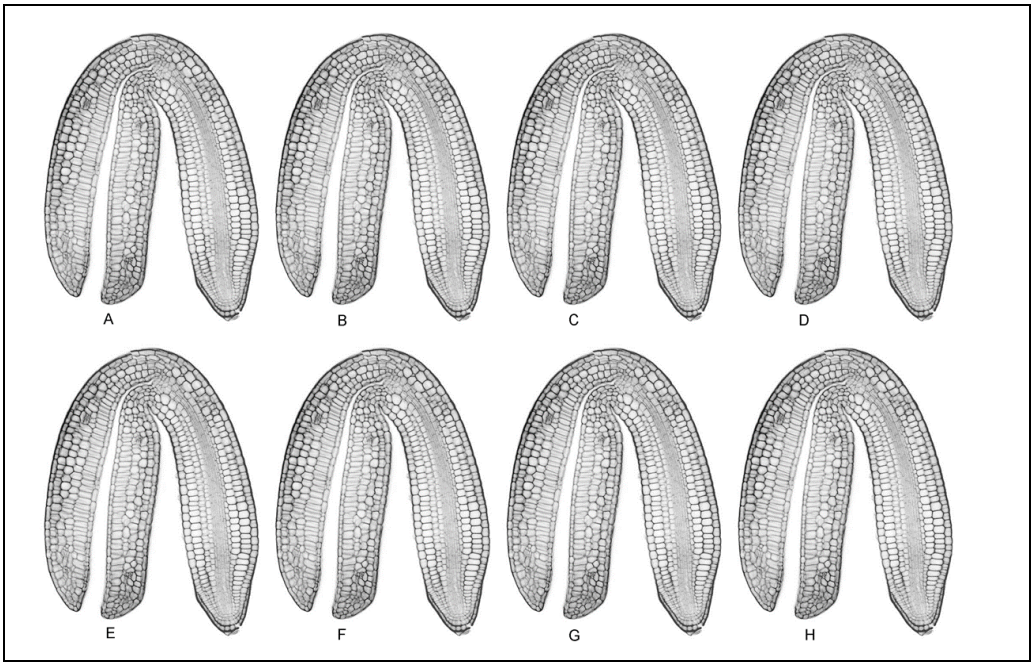
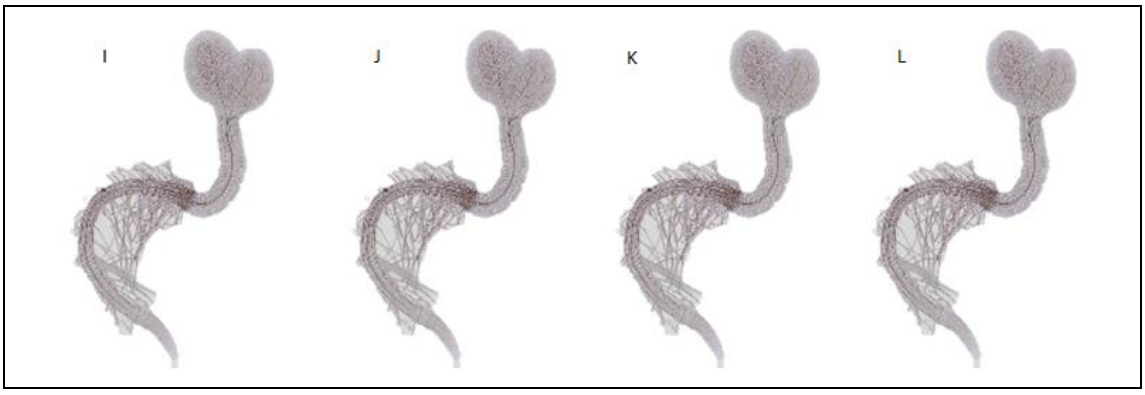


Figure 3.27: The embryo model used to represent the combination of the GUS expression activity during the transition of seed to germination. The letters represent the embryo at each stage: A: 1 HAI; B: 3 HAI; C: 6 HAI; D: 18 HAI; E: 24 HAI; F: JTR; G: STR and H: JG.



3.28: The seedling model was shown for the GUS activity after the post-germination event. The model used to represent *Arabidopsis thaliana* seeds during the localisation of GUS expression during seedling. The letters represent the seedling stage: I: HOOK SHAPED; J: COTYLEDONS EXPANDED; K: SEEDLING 1 and L SEEDLING 11.

3.6.2.1 GA synthesis

The expression of GUS were plotted into the embryo and seedling model. GA biosynthesis genes *GA3Ox1* and *GA3Ox2* were distributed during the transition of the seed to seedling. The expression activity for both *GA3Ox1* and *GA3Ox2* was correlated to each other along the embryo's hypocotyl, which starts at 24 HAI to JG. Whereas for the seedling model, the expression is focused along the hypocotyl and the germinating embryo.

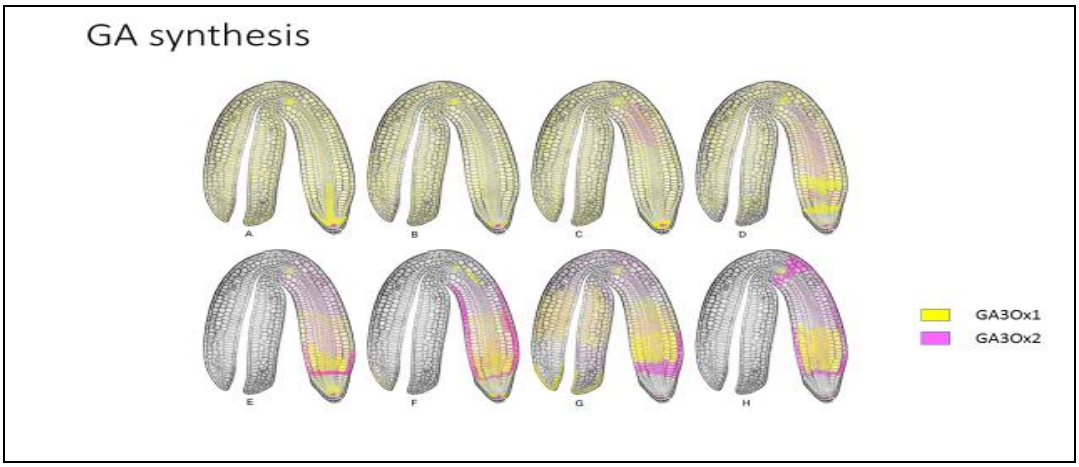


Figure 3.29: Giberellin (GA) synthesis for embryo model.The locations were plotted with different colours to represent GA synthesis expression in the germinating embryo. The letters represent the embryo at the following stages: A: 1 HAI; B: 3 HAI; C: 6 HAI; D: 18 HAI; E: 24 HAI; F: JTR; G: STR and H: JG.

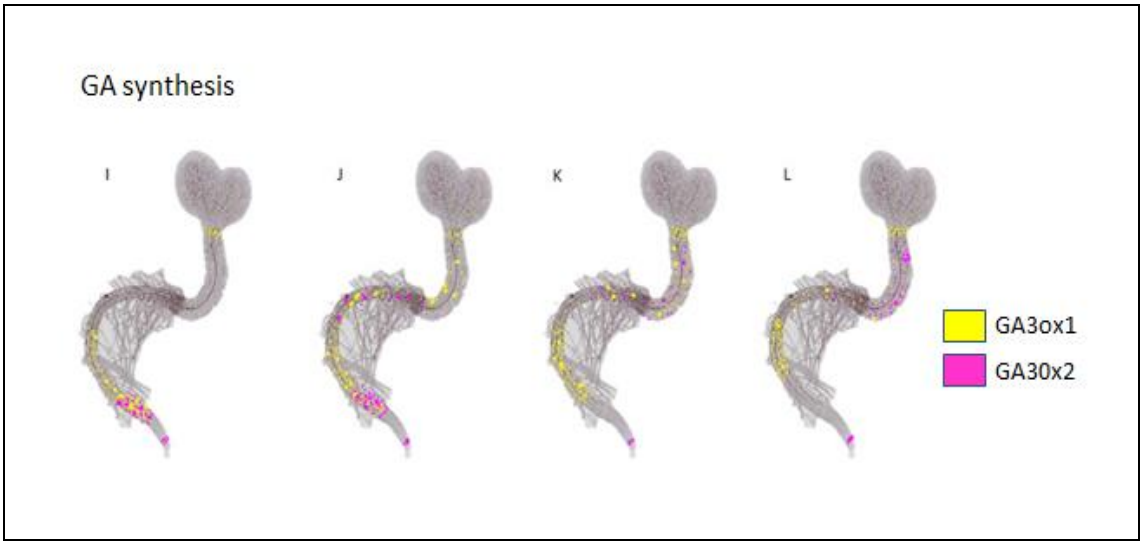


Figure 3.30: Giberellin (GA) synthesis for the seedling model. The location was plotted with different colours to represent GA synthesis expression in the seedling. The letters represent the seedling stages: I: HOOK SHAPED; J: COTYLEDONS EXPANDED; K: SEEDLING 1 and L: SEEDLING 11.

3.6.2.2 GA signalling

The spatiotemporal distribution of GA signalling acts differently based on the different genes of GA signalling. Overall, the GA signalling gene is active along the hypocotyl, but at a separate space. It is believed that GA signalling acts during the hypocotyl as it pushes the cotyledon to expand and grow upon early imbibition. From 18 HAI until JG, the *GID1C* and *SCL3* were interrelated and had the same expression above the columella at the embryo (Figure 3.8 and Figure 3.9).

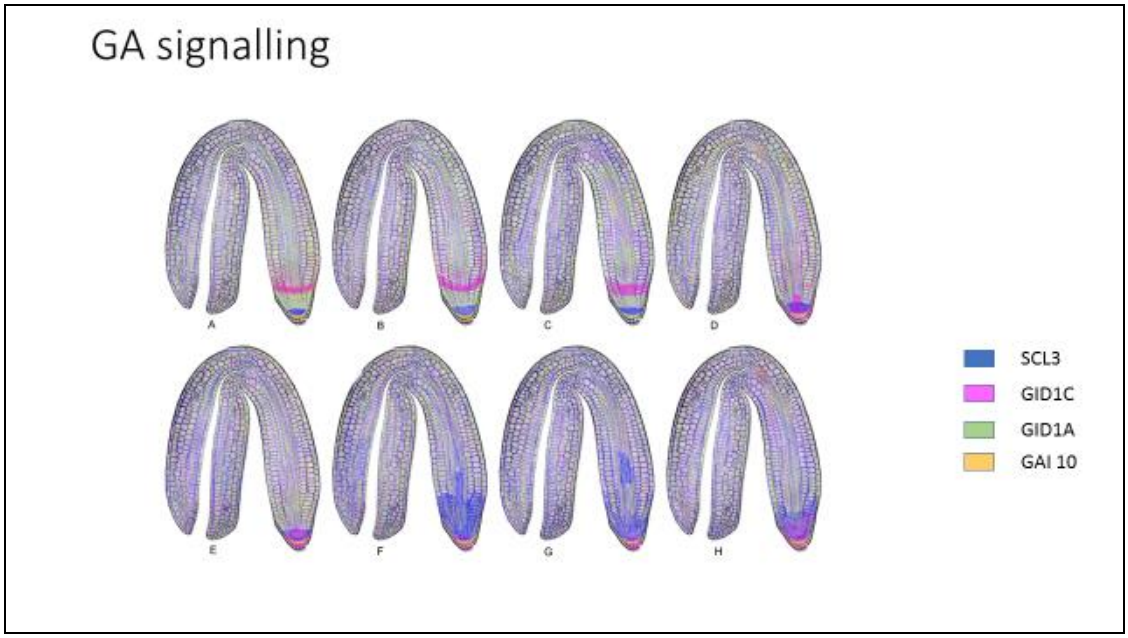


Figure 3.31: Gibberellin (GA) signalling for embryo model. The location was plotted with different colours to represent the expression of the GA signalling in the germinating embryo. The letters represents the embryo in stages: A: 1 HAI; B: 3 HAI; C: 6 HAI; D: 18 HAI; E: 24 HAI; F: JTR; G: STR and H: JG.

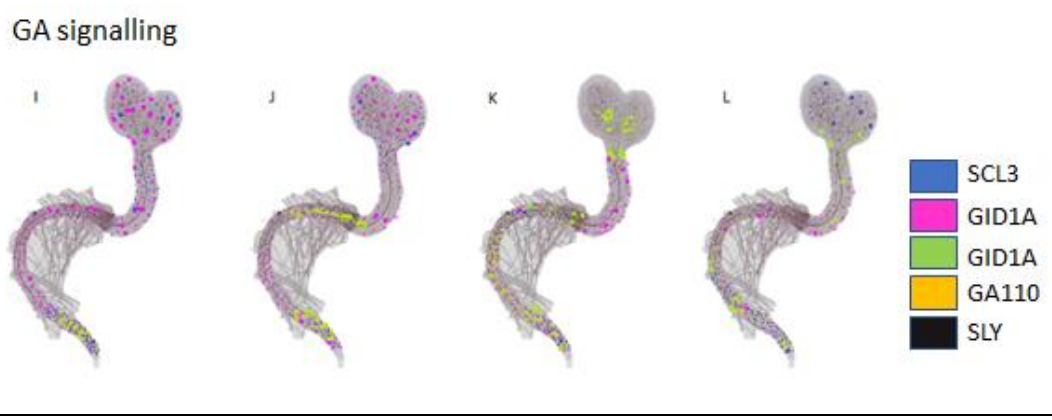


Figure 3.32: Giberellin (GA) signalling for the seedling model. The location was plotted with different colours to represent the expression of the GA signalling in the seedling. The letters represent the seedling stages: I: HOOK SHAPED; J: COTYLEDONS EXPANDED; K: SEEDLING 1 and L SEEDLING 11.

3.6.2.3 GA responses

During the early hours of imbibition to JTR (Figure 3.33 A-E), the expression of *EXPA 10* and *XTH 19* were almost at the same location, which was at the end of hypocotyl and the intersection of hypocotyl and cotyledons, respectively. This expression suggests that GA actively responds to loosen the cell wall by pushing the cotyledons by hypocotyl. However, the expression was also concentrated at the radicle tip area to allow the root's growth.

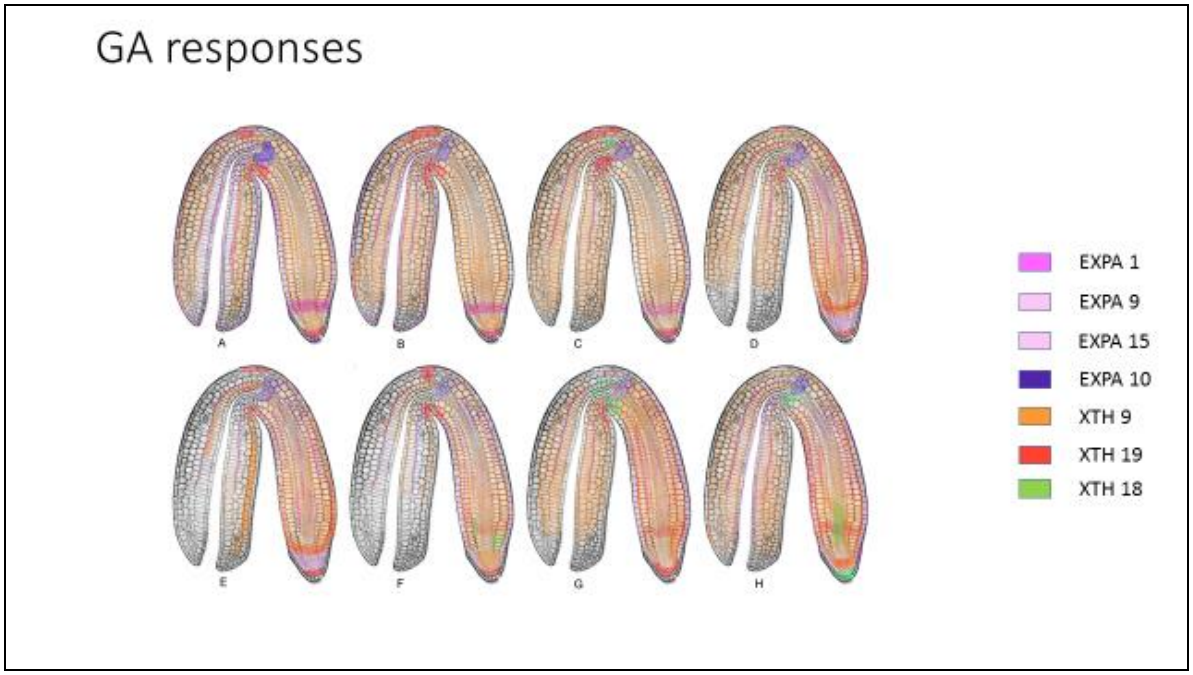


Figure 3.33: Gibberellin (GA) response for the embryo model. To reflect the location of gene expression for GA responses in the embryo, the colours were plotted. The letters represent the embryo in stages: A: 1 HAI; B: 3 HAI; C: 6 HAI; D: 18 HAI; E: 24 HAI; F: JTR; G: STR and H: JG.

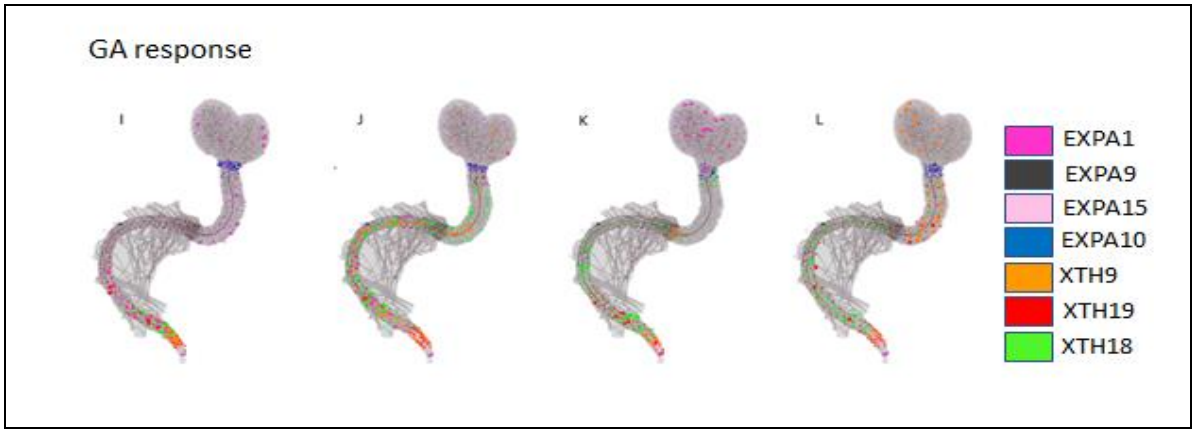


Figure 3.34:Gibberellin (GA) response for the seedling model.The locations were plotted with a different colour to represent the GA response expression in the seedlings. The letters represent the seedling stages: I: HOOK SHAPED; J: COTYLEDONS EXPANDED; K: SEEDLING 1 and L SEEDLING 11.

3.6.2.4 ABA synthesis

The expression activity of both *ABA2* and *AAO3* were correlated to each other along the columella area, started at 24 HAI to JTR (Figure 3.35 A-F). In contrast, the different active location of both genes was at the hypocotyl. In the seedling model, the expression was focused along the hypocotyl, as well as with the germinating embryo.

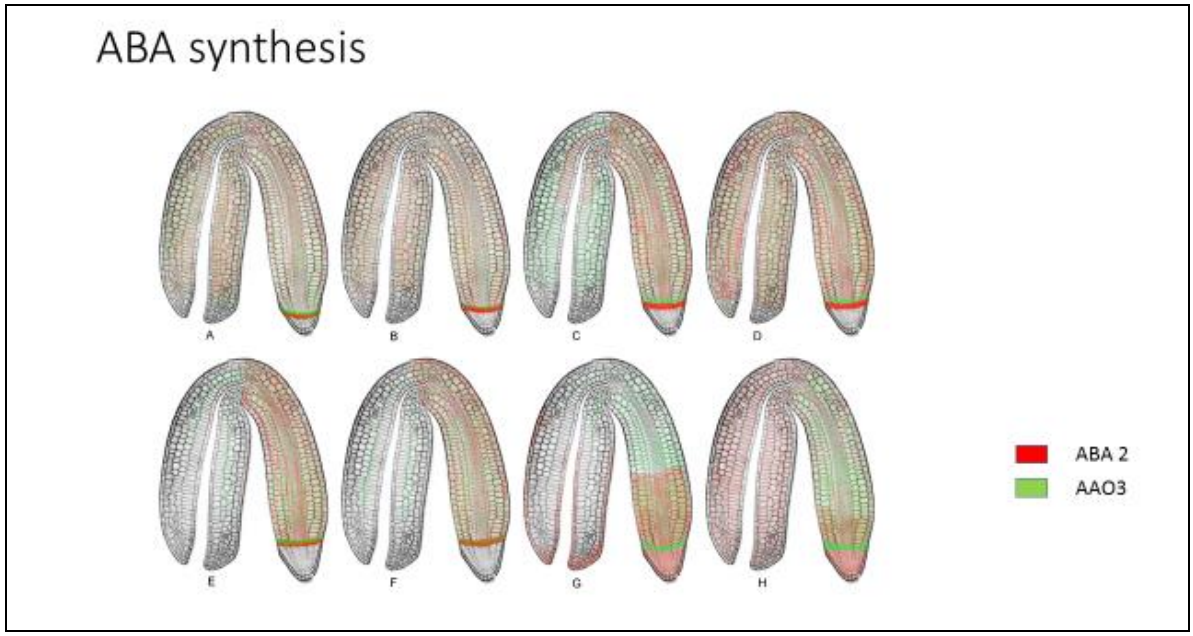


Figure 3.35: Abscisic acid (ABA) synthesis for embryos. The locations were plotted with different colours to represent the expression of ABA synthesis. The letters represent the embryo at stages: A: 1 HAI; B: 3 HAI; C: 6 HAI; D: 18 HAI; E: 24 HAI; F: JTR; G: STR and H: JG.

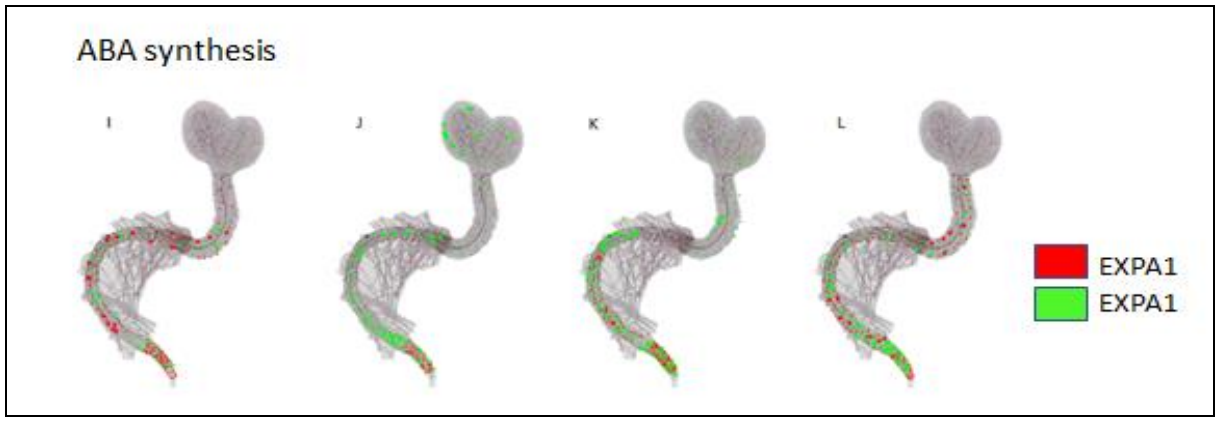


Figure 3.36: Abscisic acid (ABA) synthesis for the seedling model.The locations were plotted with different colours to represent the ABA synthesis expression in the seedlings. The letters represent the seedling stages: I: HOOK SHAPED; J: COTYLEDONS EXPANDED; K: SEEDLING 1 and L: SEEDLING 11.

3.6.2.5 ABA response

For the activity response to ABA 18 (*RAB 18*), the expression of GUS activity was used to determine the presence of ABA within the plant cell. ABA is involved in the development growth process and promotes seed dormancy.

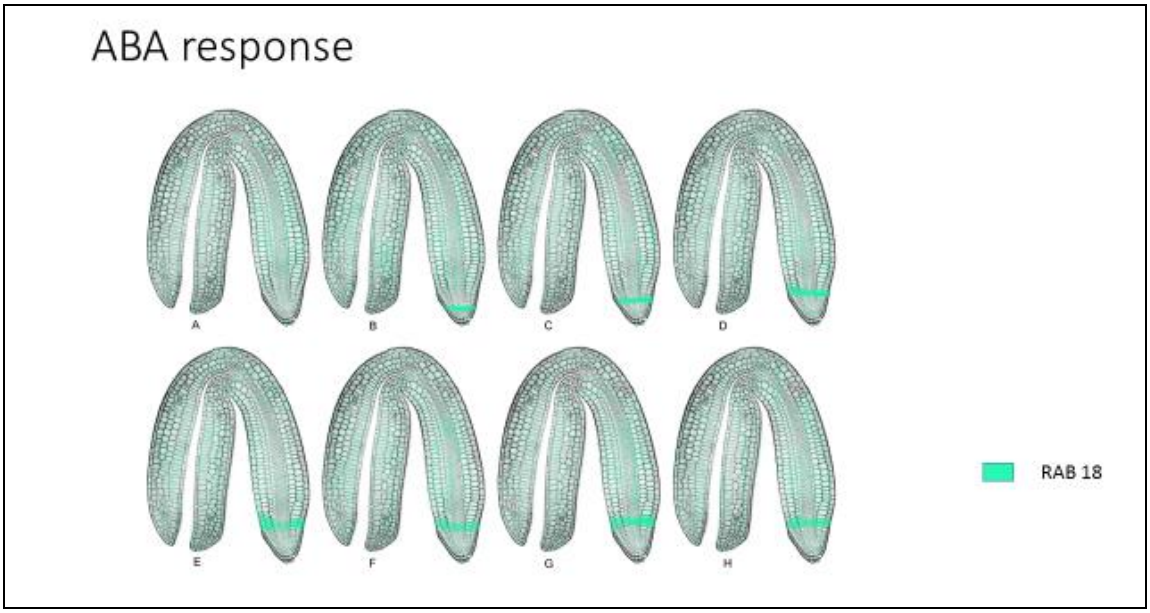


Figure 3.37: Abscisic acid (ABA) response for the embryo model. To reflect the position of the gene expression for ABA responses, the colours were plotted. The letters represents the embryo at stages: A: 1 HAI; B: 3 HAI; C: 6 HAI; D: 18 HAI; E: 24 HAI; F: JTR; G: STR and H: JG.

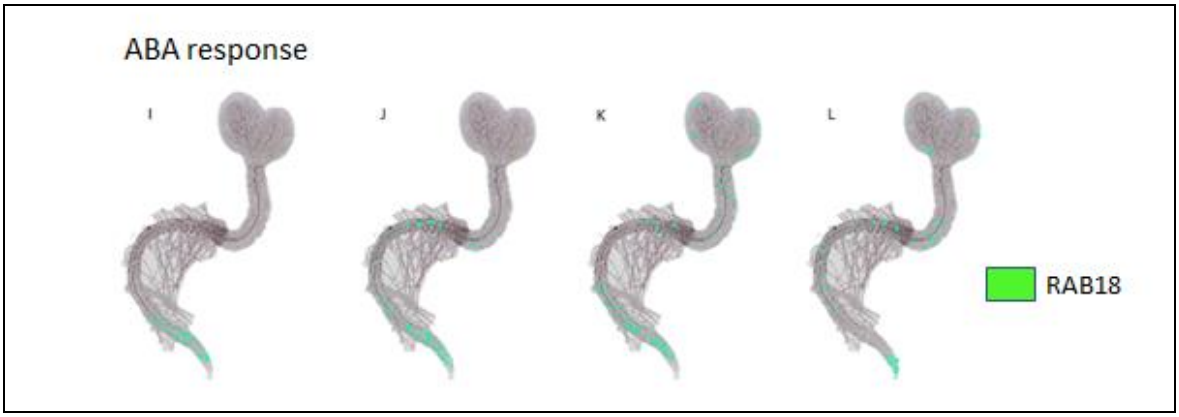


Figure 3.38: Abscisic acid (ABA) response for the seedling model. The locations were plotted with different colours to represent the expression of the ABA response in the seedling. The letters represent the seedling stages: I: HOOK SHAPED; J: COTYLEDONS EXPANDED; K: SEEDLING 1 and L: SEEDLING 11.

3.6.3 Starch GUS Reporter

The GUS reporter line of starch are presented separately based on the time series, starting from imbibition to just germinating (JG). The results are presented separately for each treatment. The screening of the seed lines showed 19% PEG after treatment. Used for further care were the final seed lines. The seed lines were tested for another homozygous plant screening. The other outcomes were the transgenic seed line PCR to detect gene expression using treatment with oestradiol. The phenotyping test was then followed by treatment with PEG and NaCl.

3.6.3.1 Starch GUS Reporter *APL*

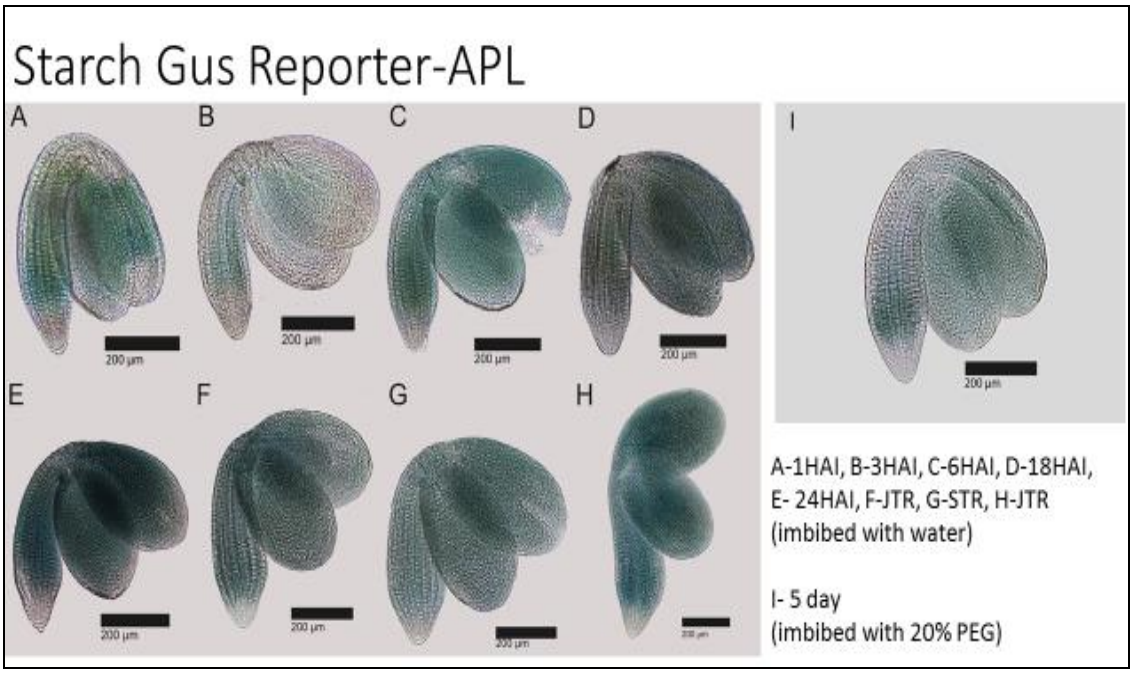


Figure 3.39: The time series for the starch GUS expression of *APL*. The spatial and temporal distribution pattern of *APL::GUS* reporter activity during the imbibition until JG. The blue staining indicates the GUS activity expressed in the seed. The various stages of seed changes are: A: 1 h imbibition; B: 3 h imbibition; C: 6 h imbibition; D: 18 h imbibition; E: 24 h imbibition; F: just testa rupture; G: super testa rupture; H: just germinate and J: 5 days imbibed in 20% PEG.

3.6.3.2 Starch Gus Reporter *APS*

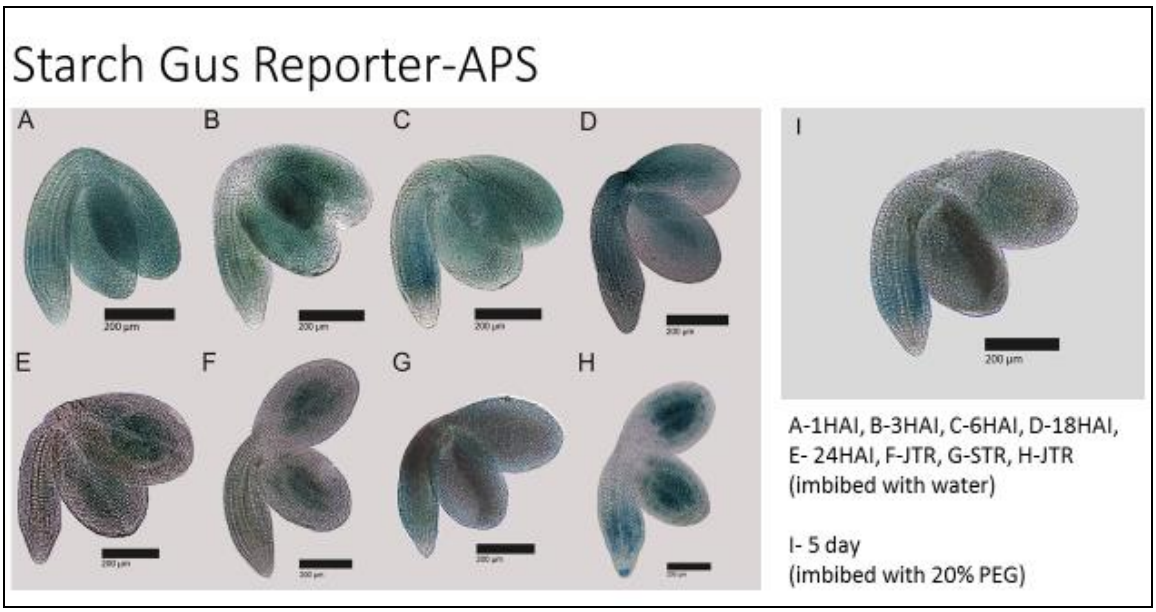


Figure 3.40: The time series for the starch GUS expression for *APS*. The spatial and temporal distribution pattern of *APS::GUS* reporter activity during the imbibition until JG. The blue staining indicates the GUS activity expressed in the seed. The various stages of seed changes are: A: 1 h imbibition; B: 3 h imbibition; C: 6 h imbibition; D: 18 h imbibition; E: 24 h imbibition; F: just testa rupture; G: super testa rupture; H: just germinate and J: 5 days imbibed in 20% PEG.

3.6.3.3 Starch Gus Reporter *PGM*

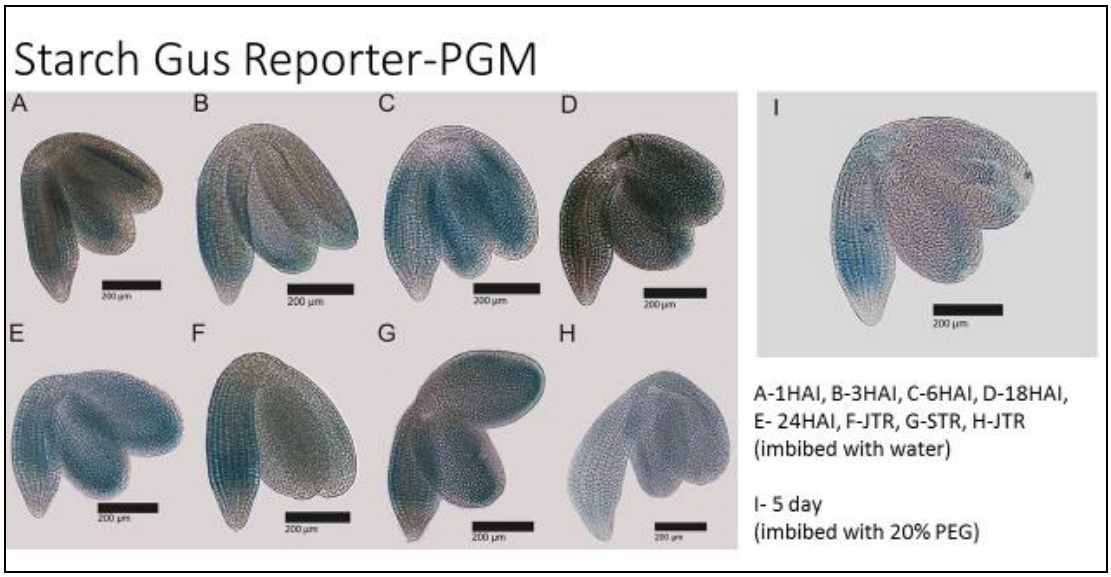


Figure 3.41: The time series for the starch GUS expression of *PGM*. The spatial and temporal distribution pattern of *PGM::GUS* reporter activity during the imbibition until JG. The blue staining indicates the GUS activity expressed in the seed. The various stages of seed changes are: A: 1 h imbibition; B: 3 h imbibition; C: 6 h imbibition; D: 18 h imbibition; E: 24 h imbibition; F: just testa rupture; G: super testa rupture; H: just germinate and J: 5 days imbibed in 20% PEG.

3.6.3.4 Starch Gus Reporter *PICL*

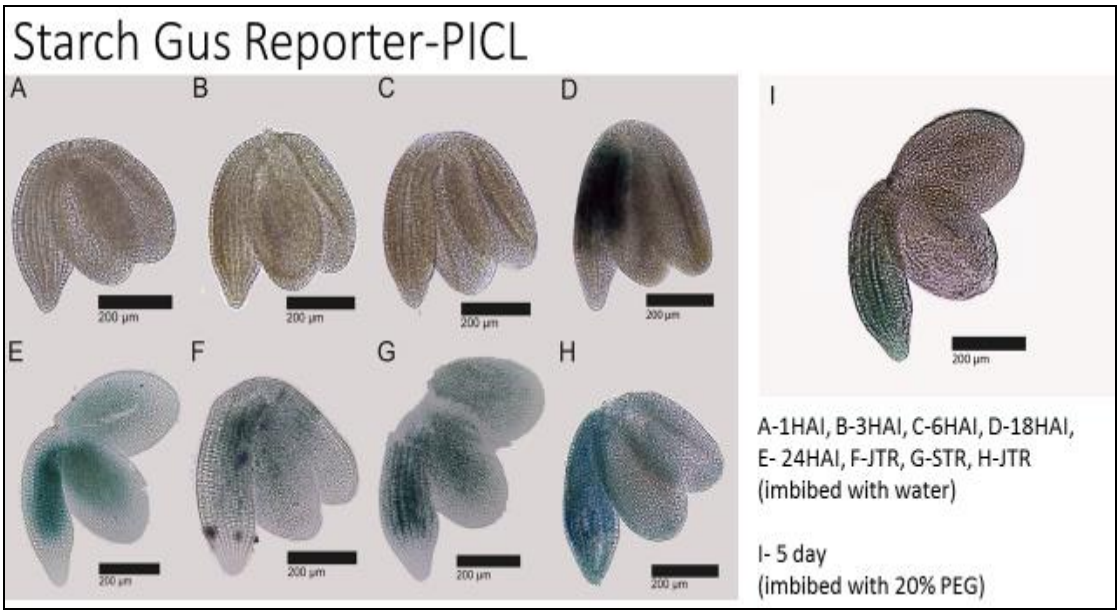


Figure 3.42: The time series for the starch GUS expression for *PICL*. The spatial and temporal distribution pattern of *PICL::GUS* reporter activity during the imbibition until JG. The blue staining indicates the GUS activity expressed in the seed. The various stages of seed changes are: A: 1 h imbibition; B: 3 h imbibition; C: 6 h imbibition; D: 18 h imbibition; E: 24 h imbibition; F: just testa rupture; G: super testa rupture; H: just germinate and J: 5 days imbibed in 20% PEG.

3.6.3.5 Starch Gus Reporter *pKAT*

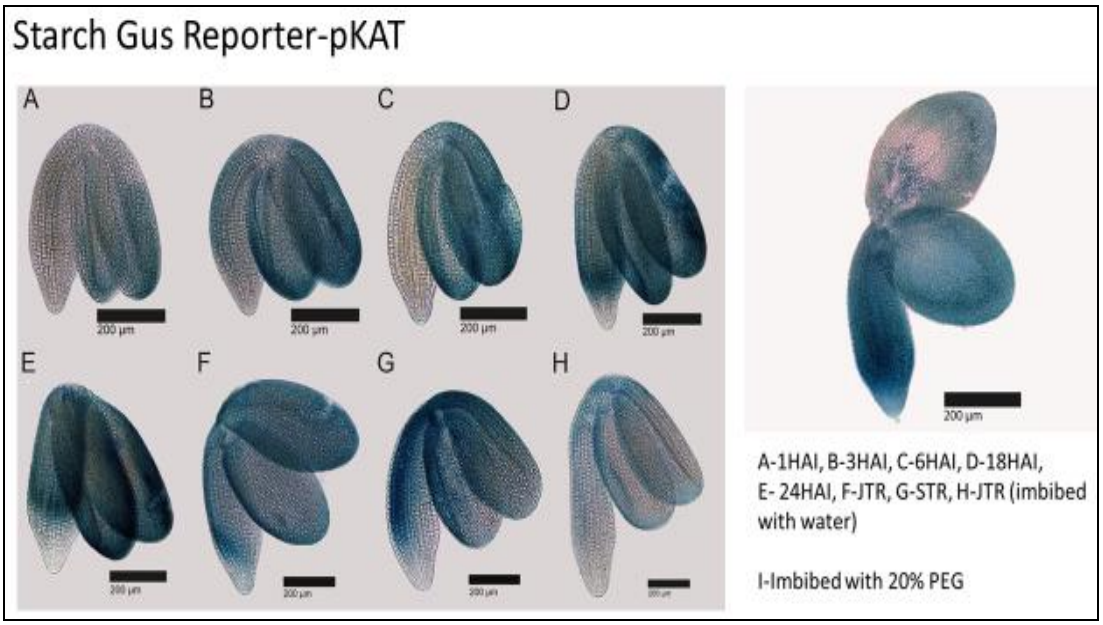


Figure 3.43: The time series for the starch GUS expression for *pKAT*. The spatial and temporal distribution pattern of *pKAT::GUS* reporter activity during the imbibition until JG. The blue staining indicates the GUS activity expressed in the seed. The various stages of seed changes are: A: 1 h imbibition; B: 3 h imbibition; C: 6 h imbibition; D: 18 h imbibition; E: 24 h imbibition; F: just testa rupture; G: super testa rupture; H: just germinate and J: 5 days imbibed in 20% PEG.

3.6.3.6 Starch Gus Reporter *SSI*

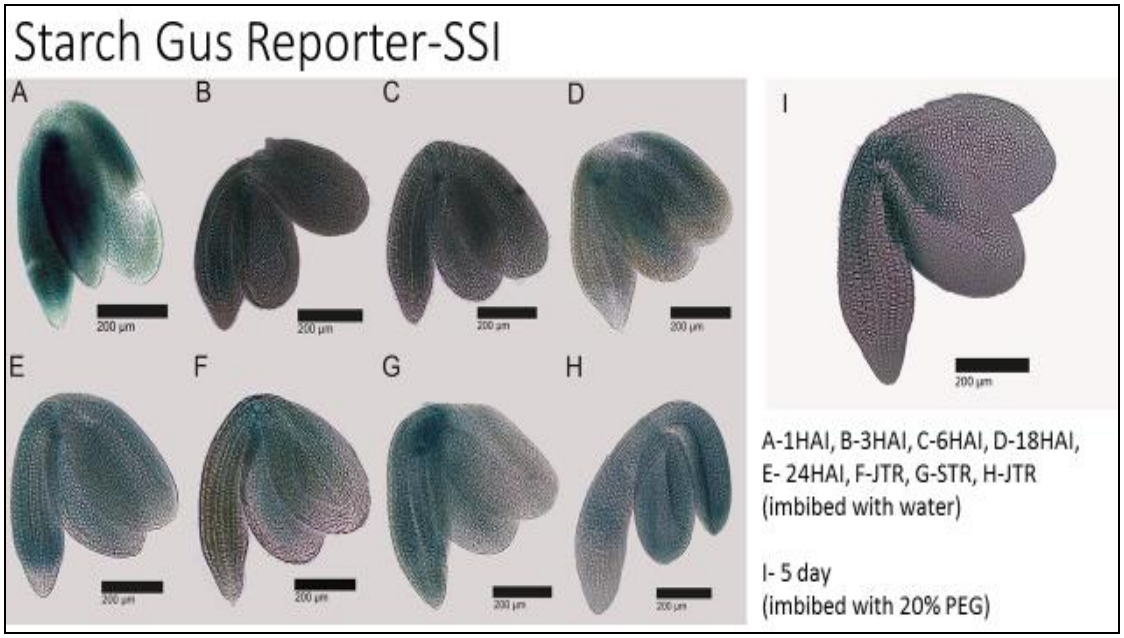


Figure 3.44: The time series for the starch GUS expression for *SSI.* The spatial and temporal distribution pattern of *SSI::GUS* reporter activity during the imbibition until JG. The blue staining indicates the GUS activity expressed in the seed. The various stages of seed changes are: A: 1 h imbibition; B: 3 h imbibition; C: 6 h imbibition; D: 18 h imbibition; E: 24 h imbibition; F: just testa rupture; G: super testa rupture; H: just germinate and J: 5 days imbibed in 20% PEG.

3.6.3.7 Starch Gus Reporter *Lacs6-1/7-1*

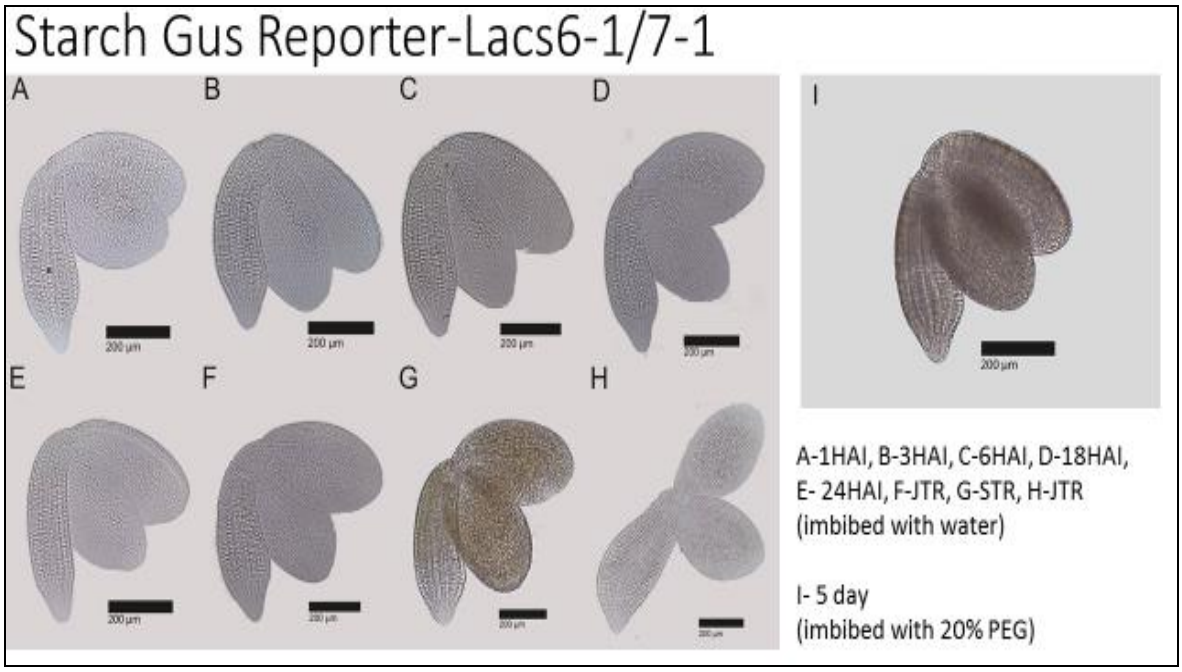


Figure 3.45: The time series for the starch GUS expression for *Lacs6-1/7-1*. The spatial and temporal distribution pattern of *Lacs6-1/7-1::GUS* reporter activity during the imbibition until JG. The blue staining indicates the GUS activity expressed in the seed. The various stages of seed changes are: A: 1 h imbibition; B: 3 h imbibition; C: 6 h imbibition; D: 18 h imbibition; E: 24 h imbibition; F: just testa rupture; G: super testa rupture; H: just germinate and J: 5 days imbibed in 20% PEG.

3.7 Discussion

Global seed production is highly interrelated with the world's population. The global population was over 7.6 billion in 2018 and is predicted to reach 9.9 billion by 2020 (world0meter, 2018). Thus, the global demand for food is likely to increase due to rapid population growth. Seed treatment, like the activity of losing the dormancy, can be applied to increase the germination synchronisation of seed populations and improve the uniformity and productivity of the seed during germination. The use of improved seeds from effective technology would contribute to higher agriculture production and increase farmers' returns. Thus, rural poverty would positively be impacted.

The transgenic seed gene expression in *A. thaliana* for the GUS reporter system was observed at different stages of germination, from the early hours of seed imbibition, through testa rupture, germination and post-germination to the seedling. At different periods, such as 1 HAI, 3HAI,6 HAI,18 HAI,24 HAI, JTR, STR, JG, HOOKSHAPED, COTYLEDONS Extended, SEEDLING 1 and SEEDLING II, the GUS activity was assayed at different stages of transformation. The expression of all the genes showed GUS activity in the image findings, gene expression was seen via their staining. The GUS blue stain showed that the gene was described in the region where the locations were actively reacting to X-Gluc solution.

GA biosynthesis (gibberellin acid made from *GA3Ox1* and *GA3Ox2*) is a complex plant process. GA signalling improves an embryo's growth potential. It also contributes to cell wall loosening and size expansion.

GA3Ox1 and *GA3Ox2* (gibberellin biosynthesis genes) are involved in the upcoming stage of bioactive GA biosynthesis (Delouche, 1969) (Olszewski *et al*., 2002). The GUS reporter

genes, *GA3Ox1: GUS* and *GA3Ox2: GUS* were used to identify the existence of GA in the embryo during the transition of seed to seedling. According to the previous finding by (Mitchum *et al.*, 2006), the *GA3Ox1* and *GA3Ox2* gene expression pattern was found to overlap spatially during the germination process. This finding was consistent with the previous result by (Mitchum *et al.*, 2006).

Following the observation of GUS expression, the cell layer has an essential role in GA perception and signalling response to seed germination. According to Ueguchi-Tanaka *et al.* (2005), GID1 receptor proteins require the downstream responses to GA. During the germination progression for *GID1A* and *GID1C*, the protein was present and localised within the hypocotyl and radicle. At the early germination for GID1s, GUS expression is restricted at the hypocotyl but excluded from the radicle. In contrast, this pattern was inverted until the protrusion of the radicle at 18 HAI. During the progression of germination, the conversion of the transition of expression was believed a GA perception shifted within the embryo cells to the radicle tip.

The *EXPA 1* images (Figure 3.12) show that the staining patterns on the tip of the radicle were identical, starting from the early stage of 1 HAI to the seedling process. It demonstrated the same pattern staining on columella and quiescent core at the first stage of 1 HAI to 6 HAI. The staining begins to shift backwards along the axis from 18 HAI onwards, which suggests that the gene reacted and regulated the hypocotyl. After four days of GUS staining, the slow staining of X-Gluc was visible in all stages of mesophyll. *EXPA 9* (Figure 3.16) showed no staining in the cotyledon region of mesophyll. The expression implies that during germination and development, *EXPA 9* managed the formation of leaves. During the germination progression, the gene activity of *EXPA 1* was observed at the radicle

tip and the lower hypocotyl. However, according to the study done by (Bassel *et al.*, 2014), the *EXPA 1* was localised at the cell expansion sites.

Figure 3.14 (*EXPA 10*) showed the same staining patterns at the shoot meristem for the early stages of 1, 3, 6, 18 and 24 HAI, but at JTR, STR and JG, the staining vanished, and then reappeared at the later stages of hook-shaped, extended cotyledons, and also at the shoot meristem, seedling 1 and seedling II. In conclusion, the shoot apical meristem was managed by *EXPA 10* at the early stages to regulate the development of leaves and at later stages to handle the rise of leaves and flowers.

Figure 3.15 (*EXPA 15*) showed that staining was scattered along the axis, starting from 1 HAI to JG at the early stage. It demonstrates the reaction to the radicular embryonic root after germination to the seedling stage. The genes were expressed at another location, in which the proteins react to that specific region. Except for the radicle edge, the *XTH 9* demonstrated the phrases that occurred along the hypocotyl. The term also appeared to govern the expansion of cotyledon for 1HAI until JG. For *XTH 9*, the GUS expression was positively active during the germination progression. The dynamic expression was believed to be caused by the active gene *XTH* reaction expansion of the cell during growth.

For *XTH 18* (Figure 3.17) at the early stages of 1 HAI until STR, no gene expression was seen. However, small staining confirmed the expression occurred at 24 HAI and STR. For JG, the expression shifted to the radicle tip before it moved back to seedling 2 after the radicle for the hook-shaped process. It can be concluded that during early imbibition, *XTH 18* was not expressed but was found at JG and after the cell began to expand. For *XTH 19* (Figure 3.18), the expression was seen near the provascular in the columella and cotyledon regions. At an early stage of germination, the low expression was seen at columella for 1

HAI, 3 HAI and 6 HAI. Then high expression was seen along the radicle before seedling for 18 HAI. The gene was shown to react to the stem elongation radicle for germination and development. As for *XTH 19* overall, during the germination progression, the GUS expressions were successful. Xyloglucan endotransglucosylase/hydrolase (XTH) is a protein modifying cell wall class. Both *XTH 9* and *XTH 19* showed the expression of GUS and this finding is similar to a previous study done by (Bassel *et al.*, 2014). In conclusion, both *EXPA* and *XTH* gene families play an essential role in propelling the cell expansion over cell-wall loosening (McQueen-Mason *et al.*,1992; Cosgrove, 2000).

According to the findings, numerous genes displayed different staining patterns and locations of the expressed gene. The stained area showed that the genes are controlled in that segment in Figure 3.14 (*EXPA 10*), the gene containing the shoot apical meristem controlled the development of the leaves at the early stages and controlled the rise of the leaves and flowers at the later stages. DELLAs are a GRA subfamily which mediate the signalling of GA. DELLA is a negative signalling regulator for GA and acts as a germination buffer. DELLAs for the GID1-GA-DELLA complex have been eliminated through *SLY* binding. This then allows germination to occur. According to the findings, the DELLA proteins are the negative regulator of GA signalling. *Gibberellin-insensitive* (*GAI*), gaI-3 (GAI-3) repressor, RGA-like 1 (*RGL1*), RGA-like (*RGL2*) and RGA-like 3 (,*k*) is the DELLA protein (Peng *et al*., 1997; Silverstone *et al*., 2001). According to the results, the category of DELLAs (*GAI 10, RGA 3, RGL2 (2)* and *RGL2 (8))* and *SLY* (*SLY 7, SLY 18*) do not stain after being put in a GUS solution that has been incubated for four days at 37°C. From early seed imbibition up to the seedling stage, they were not stained. According to the research of (Bassel *et al.*, 2004), after radicles, *RGL2*, indicates decreased expression of the radicles' DELLA gene. Also, during the seed-to-seedling transition, the DELLA gene

protein and *SLY* were not expressed. It can be inferred, based on this observation, that DELLA and *SLY* are the negative regulators of GA responses that function specifically to regulate the germination of the seed. Overall for DELLAs in this study, they did not show any reaction to the X-Gluc during the transition of seed to seedling. However, *GAI::GAI-GUS* showed low expression at the early stages of germination. This consistent result was found with other transgenic lines for other reported DELLAs. It showed a consistent effect, with no expression staining during the germination progression. This finding was supported by the previous study with the consistent results that DELLA protein reporters were not expressed in *Arabidopsis thaliana* (Stamm *et al.*, 2017; Topham *et al.*, 2017).

Following ABA biosynthesis, the *ABA2* and *AAO3* reporters used delegation to spot the ABA biosynthesis. During the final stage of ABA biosynthesis, the *ABA2* and *AAO3* genes encode the active enzyme. Moreover, the finding by (Topham *et al.*, 2017) was consistent with observing the localisation of *ABA* and *AAO3* at the outermost vasculature cells of the radicle. Whereas for the *RAB18* reporter, it was used as a delegation to regulate the ABA transcriptional response, as reported by (Lång and Palva, 1992). According to the observation, the expression was spatially overlapping with the *ABA2* and *AAO3* genes during the germination process. I believe that the ABA responses were induced at the cellular sites of ABA biosynthesis.

On the 2D embryo and seedling models, the images obtained for gene expression were plotted using different colours. To illustrate the genes at the particular time during the transition from seed to seedling on the embryo and seedling models, the gene expressions were combined on the model. Based on the reporter genes' function, the patterns of gene expression are present and behave differently. Some genes showed the same expression

during germination for all the stages, while some genes function differently depending on the time sequence. The precise time frame of early imbibition until the seedling stage was used to understand the exact time of gene expression. Some expression shared the same location.

Gene expression of seed holding of the starch GUS reporter gene was assessed at different stages of the germination sequence, starting from the early imbibition process to testa rupture, then onto the radicle protrusion. The seeds have been used for GUS activity at different stages to evaluate gene expression differences. This approach has been used to enable the spatiotemporal distribution analysis of gene expression with *A. thaliana* seeds. The seeds imbibed with 20% PEG or with water show a different expression pattern for each gene, depending on the effect. For this experiment, 20% PEG was used because during the pre-testing of seed germination, 20% PEG limited germination. The expressions were plotted into a 2D embryo model. The images display the staining pattern and the places where the genes have been expressed. The expression of seeds imbibed with water was the same as for *APL* (Figure 3.39), starting from A at 1 h of imbibition (1 HAI) to H only germinating (JG). Hypocotyl and cotyledons were expressed in the gene. That the term I is imbibed with 20% PEG suggests a similar expression position, which is also present in hypocotyl and cotyledons. *APL* is responsible for ADP-glucose phosphorylase activity and regulated the limiting step in starch biosynthesis.

APS (Figure 3.40) shows expression in A (1 HAI) and B everywhere during the early stages (3 HAI). From C to H (6 HAI to JG), the expression is in the hypocotyl and cotyledon. At H, expression at the tip of the radicle began to appear. The pattern of expressions imbibed with 20% PEG for I, showed similarity to G in both hypocotyl and cotyledons. The overall

staining pattern was the same for A to G. For H, the staining slowly disappears. Since the seed started to germinate, it is considered that the expression is gone, so the available starch is degraded as *PGM* results in starch degradation. The appearance is in cotyledons and less staining in hypocotyls.

In *PGM* (Figure 3.41) all stages, from A to H showed expression was active in the hypocotyl and cotyledon. Interestingly, the expression of I, which was imbibed with 20% PEG, also indicates the same pattern with water imbibition. The consistent finding for imbibition with 20% PEG and imbibition with water suggests that *PGM* acts the same at both conditions. *PGM* responses to drought results in starch degradation. At stage I, the cotyledon responses degrade the glucose, thereby making growth for epicotyl elongation. These finding highlights that there was no expression for *PICL* (Figure 3.42) starting at A to C. No post-germination expression is considered because the findings reveal that *PICL* is responsive to the plant abiotic stress hormone *ABA* in the post-germination growth response. The expression begins at hypocotyl D (18 HAI) then travels from E to H. Expression later occurs on cotyledons and hypocotyl. Stage I was identical to that of D, which is represented in the hypocotyl.

The expression of *pKAT* (Figure 3.43) is present in cotyledons. Expression is suggested in the cotyledons at phases A to C, then continues to shift toward hypocotyl, and at the same time is present for D to G at cotyledons. The staining also starts to disappear at H. It was assumed to be β-oxidation, due to *pKAT* involved in fatty acid degradation. This research shows that H has shown good staining on hypocotyls and cotyledons as well. The strong expression for I is demonstrated on hypocotyl, and low expression on cotyledons.

Figure 3.44 for *SSI* demonstrates regular expression during the transition of seed to germination. The starch synthesis genes were transcriptionally active in cells within hypocotyl and mesophyll of cotyledons, shown for A to H. The expression has focused on the shoot meristem for I. It has been suggested that this correlation of amylose is necessary for any further energy storage for plant activity. This correlation is related to *SSI*, which is required for amylose synthesis in the endosperm.

Based on the image (Figure 3.45), for the entire time course, starting from A to H, there was no expression for *Lacs6-1/7-1*. *Lacs6-1/7-1* is used to activate free fatty acids and plays a crucial role in fatty acid metabolism.

3.8 Conclusions

The physical and morphological modifications of *A. thaliana* seeds varied from time to time. Starting from early imbibition, germination and the seedling stages, there were many physical changes to the seed. There is specific gene expression after each physical and morphological shift of the seed. For each gene, the gene expression models for seed transfer to seedling were different: they show different patterns every time, from 1 HAI until early germination and seedlings, after treatment with X-Gluc solution. The staining portion of the seed of *A. thaliana* showed the position of the expressed gene. At each different time, multiple staining positions are present.

The hormonal signalling changes during the seed-to-seedling transformation are described in this study. The natural hormones GA and ABA work to regulate the germination and dormancy of seeds. During seed dormancy, the concentration of ABA increases, while during seed germination, it decreases, then GA sensitivity takes part in germination, resulting in the emergence of the radicle from the seed coat and seedling establishment. The

GA reaction of both *EXPA* and *XTH* genes results in the cell wall's remodelling enzyme (*CWRE*). The EXPAs function to make it possible for the cell wall to become unstable and allow the cell to expand further. The XTHs serve to break and reconnect the cell wall and assist in the cell elongation responsible for building the cell wall.

This research defines where and when GA signalling and downstream cell wall modifications occur in the seedling growth to drive seedling establishment. To know the exact time for the gene to be expressed, unique time frames, such as early imbibition to seedling stages were used. In this project, "Spatial-temporal distribution" was ideal for representing the time and place of gene expression. The combination of colours used to plot gene expression to visualise the location of GA and ABA signalling components, while combining the gene expression for synthesis, signalling and responses. Using microscopy, the spatiotemporal dynamics of each GA and ABA synthesis, signalling and reactions were investigated. During the seed transformation to a seedling, the precise period to see the differences was observed. The current location for GUS staining at the different locations was plotted in the 2D embryo model and models after the completion of seed germination. Potokina *et al*. (2002) also noted that “Due to the various functions of seed tissues and the different biochemical processes, these genes were expected to be coordinately regulated both spatially and temporally.” This research helps to explain the normal pattern of gene expression from the transition from seed to seedling, and to increase yield later on.

This study has shown that the morphological changes of the starch GUS reporter's expression in *A. thaliana* were different for different genes. The expression of the starch GUS reporter imbibed in water for the different stages showed different expression to seeds that were imbibed for five days with 20% PEG. Seeds imbibed in 20% PEG showed a delay

in the expression of GUS staining. In normal condition, if the seed id imbibed for five days, it has already grown into a new plant for Arabidopsis. However, in this situation, the seeds that had imbibed 20% PEG had delayed development and growth processes. The higher the concentration of the PEG limits the water that can be taken up by the seed. Thus, in this study, the seed imbibed in 20% PEG for five days delayed germination and caused a negative impact on seed germination. This is in line with research by (Kaydan and Yagmur, 2008). The availability of water is typically the limiting factor for the germination of non-dormant seeds, influencing the percentage, velocity and uniformity of emergence (Kaydan and Yagmur, 2008).

The different genes showed different expression every time. The expression profile analysis reveals that starch metabolism genes were active during the transition of seed toward germination. It can be concluded that starch synthesis in *A. thaliana* is achieved by a Spatial-temporal distribution pattern, regulated by the promotion of starch GUS reporter.

CHAPTER 4

STARCH AND GERMINATION

4.1 Introduction

In plants, starch and sucrose are the primary way of saving energy from the photosynthesis process. Starch consists of the plant's glucose green leaves from the photosynthesis process to provide growth power. Compared to glucose, starch is derived from many units together. Starch cannot be used straight away by the plant because it is a large molecule. Carbohydrate must be turned into specific units before it is utilised for energy: plants can only take starch in the simplest form, for example, in glucose form. During heterotrophic growth, starch represents the main ways plant storage carbohydrate. Within the plant, carbohydrates are stored mainly in the cotyledons, seed, leaves, fruit and tubers.

The primary chemical formula of starch is $(C_6H_{10}O_5)n$. Starch is produced in plant tissues and has numerous functions (Tetlow *et al.*, 2004). The short-term storage of carbohydrates called "transitory starch" is leaf starch. The transient polysaccharides are synthesized in chloroplasts during the daytime and are degraded at night to supply carbon for non-photosynthetic metabolism. The tuberous tissue starch will then serve as a carbon deposit, while the storage starches in seed production are long-term storage for next-generation storage (Tetlow *et al.*, 2004). The starch element is degraded into the simplest molecules and the plant will use this as fuel for energy. The enzyme that degrades the starch is amylase. For plants, starch is excellent storage because, when it is needed, the starch will be transformed into energy.

A seed is a small structure, and in unfavourable circumstances, it will survive. The seed embryo holds the food, which is then processed. While the embryo's stored food grows in a mature plant during germination, starch analysis is a relevant research study for the future since ~50% of our daily calories come from starch. Most of starch sources come from cereal crops (such as rice, wheat, barley and maize) and tuberous or root crops (such as cassava and potatoes). For many industrial applications, including paper, textiles and pharmaceuticals, starch is also an essential raw material. Recently, because of their use as raw materials to produce bioethanol, starch crops have become more relevant in economic terms. Increasing the starch content in the production of crops will help to compensate for the agricultural strain. Increasing the starch content of starch crops for non-food purposes, is important for industrial needs (Kossmann and Lloyd, 2000; Burrell and Road, 2003).

In the mid-1980s, a study of starch metabolism in *Arabidopsis thaliana* leaves begun (Caspar *et al*., 1985). The study was done by screening and altering the starch level content. The altered glucose content in the part of the plant was related to the control of respiration and iodine staining was used to access the altered starch structure in Arabidopsis (Caspar *et al*., 1985).

In agriculture, two critical environmental phenomena, salinity and drought, are declining day by day. This may influence crop productivity (Jamil *et al.*, 2006). Excess salt in soil will inhibit seed germination and prevent plant growth, initiating plant death. In growing a plant, seed germination is the primary factor. Producing a better plant is necessary. The salt content of the soil is soil salinity. Salt from the mineral weathering process happens naturally within the soil and water. The salt is collected by dust precipitation and is deposited. Salt may also be introduced artificially from irrigation and road salt. Some ions,

including Na^+, K^+, Ca^{2+}, Mg^{2+} and Cl are involved in salinisation. The sodium ion (Na^+) makes the soil soft and creates a weak soil structure, limiting water penetration and drainage.

The accumulation of salt in the soil allows salt to accumulate at the root. Salt typically gathers in the soil and root zone by saline irrigation, producing a negative effect on germination by preventing withdrawal of the surrounding water, thus decreasing the volume of water in the plant. Drought is also related to the soil's salinity and dryness: therefore, a high salt content in the soil prevents the water from spreading to the surrounding area and induces plant stress. To recover from salt stress, various plants have different abilities. In agriculture and the ecosystem, abiotic stressors, such as salinity and drought stress, is a severe issue. Salinity stress has a global impact on environmental degradation due to the loss of crops (Wang *et al*., 2003).

4.1.1 Starch metabolism in plant germinations

Carbohydrates and glucose are stored as starch in plants. Starch is a complex starch or polysaccharide. During the photosynthesis process, sugar is produced in a plant: in the presence of light, the green plant leaves make their food. Photosynthesis occurs with light in the daytime and involves sugar to form carbon dioxide and water. Sunlight energy is used to generate energy for the plant and processed as a source of starch. Stored starch is transformed directly into the simplest glucose used by the plant during the night, or days without sunlight. After the initiation of germination, starch degradation takes place. Degradation of starch stimulates carbon for the initial growth of seedlings (Lloyd and Kötting, 2016).

Hence, starch metabolism is vital for plant growth. Typically, the plant accumulates starch in transient and long-term reserves. Leaf starch (or transitory starch) is used as a reservoir of short-term carbohydrates. Transitory starch varies between species (Streb and Zeeman, 2012). In contrast, medium to long-term energy sources fuel plant development during germination. Starch also accumulates in the plant's non-photosynthesis tissues, such as in seed, roots and tuber. The non-photosynthetic cell type synthesises starch, but not much as in roots and tubers (Streb and Zeeman, 2012).

4.1.1.1 Starch is a complicated storage of carbohydrate

In plants, the storage form of glucose is starch. Starch is a complex starch or polysaccharide. Starch results from the photosynthesis process wherein the presence of light, the leaf of the green plant, produces its food. Photosynthesis occurs with light in the daytime and involves sugar to form carbon dioxide and water. The energy of sunlight has been used to produce energy for the plant and processed as a starch source. The stored starch is converted into the most straightforward glucose used straight away by the plant during the night or day without sunlight.

Starch consists of two glucose polymers: amylopectin and amylose. To form linear chains, A-1,4 glucosidic bonds join the glucose units, then connect to alpha-1,6-glucosidic bonds, amylopectinin branches (Streb and Zeeman, 2012). Amylose is a smaller molecule that has far fewer branch points, while amylopectin is denser because it has 70–90% of the granules' weight (Denyer *et al.*, 2001). Starch is usually stored in leaves, but starch also accumulates in plant seeds, roots and tubers for non-photosynthetic tissue (Streb and Zeeman, 2012). This storage starch is contained in heterotroph cell amyloplasts. It is used for the growth process for medium to long-term energy sources.

Carbohydrates are an essential source of energy for living organisms and are the central pathway for most cells. A carbohydrate consists of atoms of carbon (C), hydrogen (H) and oxygen (O). Simple carbohydrates, since they have a lower molecular weight, are often called simple sugars. Sugar is classified into monosaccharides, polysaccharides and disaccharides. Monosaccharides are single sugars, like glucose, fructose and galactose, since they are made from one sugar unit.

In comparison, two sugar units such as sucrose, lactose and maltose are formed from two sugars. Examples of starch, glycogen and cellulose include polysaccharides formed from more than 12 sugar units. For a plant, starch is a compact storage molecule, while glycogen is sugar storage for animals, and cellulose provides the cell wall's foundation for plants. In most living species, seeds play a vital role since it is a primary nutrition source. The command meal storage is starch in several crops. A seed is a compact structure, and carbohydrates, protein, lipids, vitamins and minerals are also a transportable source. Usually, seed crops' first storage product is carbohydrates and protein (Kigel, 1995).

4.1.2 Aims and hypothesis of this study

By converting simple sugars (glucose) into a complex carbohydrate, starch synthesis osmotically regulates cellular turgor (starch). Sugar can affect the cells' osmotic capacity and the ability of water to be absorbed and drive cell expansion. Lower osmotic water potential is caused by the development of a lot of starch, so that substances can move in because water (solute) absorption results in turgor, cell expansion and germination. Starch is present in the crop. It has been confirmed and disappeared after amylase treatment in confocal imaging.

4.1.2.1 Objectives

Several objectives are achieved at the end of this study, such as:

i. To comprehend the degradation of starch and the ability of cell water for seed germination in osmotic stress.
ii. To investigate the abundance of starch found in transgenic seeds.
iii. To examine the impact of the accumulation of starch and degradation during germination of cell turgor on seed phenotyping.

4.2 Arabidopsis as an oilseed

Arabidopsis thaliana is an oilseed, and is similar to a major oilseed flower, *Brassica napus* (rapeseed). As the central seed carbon reserve, Arabidopsis stores oil in the cotyledons. The triglycerides (TAGs) typically shape fats/oils, including seed and pollen, to store fat in the plant kingdom. The oil bodies contain lipids, which are still in a liquid state (Huang, 2003). TAGs are an energy storage medium, an ester derived from glycerol and three fatty acids. TAGs are actively mobilised to support development.

TAGs accumulate in animal and plant tissues. In *A. thaliana*, lipid storage is synthesised and used as fuel energy for other seed growth, such as germination. During seed maturation, TAGs accumulate and are stored in the seed until germination, then used to fuel seedling growth (Graham, 2008). Most oilseed storage is the same as with starchy seeds, which reserve carbon and energy for fuelling the germination and post-germination seed until seedlings, before photosynthesis is efficiently established (Baud and Lepiniec, 2009).

For *A. thaliana*, starch accumulates temporarily in the growth of Arabidopsis seeds. Whereas in wild-type plants, the main carbon store is a lipid (Andriotis *et al.*, 2010). Storage

compound (lipid storage) accumulation began at the maturation stage (Goldberg *et al*., 1994). Thus, the embryo development increases and the embryo can fill the seed sac. After this, the endosperm begins to decrease and decay into one layer around the embryo (Berger, 1999). During seed maturation, most endosperm cells die. The seed storage for lipid storage (*A. thaliana*) compound synthesis, according to studies by (Turnham and Northcote, 1983; Eastmond and Rawsthorne, 2000) ends such synthesis within a few days of seed maturation. These situations demonstrate and explain the seed's ability to remobilise lipid before germination by β-oxidation. (Chia *et al*., 2005).

4.2.1 Seed storage oil mobilisation

There is a substantial decrease in most sugars, organic acids and amino acids during seed maturation (Fait *et al.*, 2006), indicating their successful absorption into storage reserves. The efficiency of oil mobilisation depends on reserve accumulation during seed maturation in germination and seedling establishment. Several studies have shown that Arabidopsis' fatty acid composition differs depending on the part of the tissue. Around 1/10th of the fatty acid is found in the dissected seeds as a whole (Penfield *et al.*, 2004), while in the embryo contains ~60% of the total fatty acid within cotyledons for other plant tissue and ~30% of the fatty acid composition is in radicles and hypocotyls (Li *et al.*, 2006).

There are several biochemical pathways for the mobilisation of oil storage in *A. thaliana* to break up the seed's oil storage. The first step of lipid/oil degradation is catalysed by lipase (Huang, 2003). The enzyme that hydrolysate and catalyse the lipid is lipase. It hydrolyses TAGs on the water intersurface to create free fatty acid (FA) and glycerol. The FAs hit the glyoxysome, then (single-membrane organelles) β-oxidation stimulates acyl-CoA for

subsequent catabolism. Gluconeogenesis is a metabolic pathway that contributes to the generation of carbohydrate glucose.

4.2.2 Starch detection in germinating embryos

Some granules are detected under the confocal microscope, based on observations from the previous experiment. The granules were treated under the confocal microscope with amylase treatment and then imaged. After amylase treatment, the granules dissolved. The granules are known to be starch. According to previous studies conducted on alpha-amylase, the starch's initial degradation into the soluble form has been performed (Juliano and Varner, 1969). It is observed in cereal (Sun and Henson, 1990), in which the starch granules were degraded by alpha-amylase when the starch granules are germinated in barley (Hordeum vulgare L).

4.3 Abiotic stress

As a stress factor, environmental factors are known to reduce crop productivity. Abiotic stress occurs from drought, floods, salinity, chemical toxicity and excessive temperature caused by agricultural production. Abiotic stress can cause the natural environment and agriculture to deteriorate (Wang *et al*., 2003). The most common issues in agriculture are salinity and drought, which reduce crop production by approximately 50% (Boyer, 1982). Without any stress from the atmosphere, plants will grow healthier. The abiotic stressors, salinity and drought, contribute to the osmotic pressure**.** As a living organism, a plant doesn't move like an animal. The root is the most crucial part of the plant against abiotic stress (Brussaard *et al*., 2007). The root plays a vital role in ensuring that the plant has a high chance of survival and that the soil can keep the plant healthy plant growth. Most plants can withstand stress, but different tissues tolerate pressure differently. The tissue that includes stress during transcription is different from the tissue that relies on the stress

involved (Cramer *et al.,* 2011). Drought is the restriction of the cell's water uptake. Drought is one of the environmental factors that reduces agricultural outputs. Barley *(Hordeum vulgare L.)* is the most crucial cereal in developing countries, and is widely grown in dry and semi-arid areas. It is, therefore, facing severe drought conditions. Plants' drought tolerance is essential to obtain the best form of breeding for drought adaptation, to resolve this situation (Amini, 2013).

Salinisation is the condition in which salt deposition accumulates in the soil. This damages the plant because of the change in the potential gradient of osmosis. The osmotic pressure is changed by water-soluble salt and can prevent the cellular activity of the cell (De Azevedo Neto *et al*., 2004; Zhu, 2016). The ion ratio, such as Na^+/K^+, is disturbed during salination in the plant cell. This is related to the high salt concentration in the cytoplasm. The water uptake capacity decreases with a rise in Na+ and it can also alter the function of transporter enzymes (Conde *et al*., 2011).

4.3.1 Osmotic stress

Osmotic stress is identified when the absence and scarcity of water restricts water consumption and reduces plant production and growth (Zhu *et al*., 1997). The excessive salt in water and soil, and the drought effect that lead to the osmosis is the most common form of osmotic stress. The spontaneous movement of a solvent (water) through the cell membrane is osmosis in the plant. The diffusion of water molecules with a higher solvent concentration (higher water potential) progresses towards a lower solvent concentration (lower water potential) to create an environmentally stable and healthy equilibrium. Until equal pressure is produced, osmosis continues. The majority of plants can respond to osmotic stress, but not all species can withstand and adapt to this stress (Zhu *et al*., 1997).

Crop germination and plant growth have specific effects on osmotic stress. The state of the solution, with a sudden shift in the concentration of the solute, is osmotic stress. This contributes to rapid changes in the movement of cells surrounding water. High concentrations of salt (or any solvent in the solution) will cause an osmosis cycle to pull water out of the cells. The different solvent levels in the solution causes the water from the cell to migrate out to the lower solvent density.

Osmotic stress is a crucial factor in agriculture. Osmotic stress conditions have been used to assess genotype variations between transgenic seeds, in terms of salt and drought stress and to determine the germination rates for salt and drought stress conditions. Generally, germination and seedling growth are hindered by toxicity and osmotic pressure. The stress condition is the product of salinity, and drought will affect growth, reducing the percentage of germination.

4.3.2 Drought stress

In the soil, drought stress drains water. Drought stress is caused by a lack of water in the soil. The stress of drought will alter the physiological, morphological and molecular characteristics of the plant. Plants are sessile organisms. They are not the same as animals that can travel freely, but not for plants. The drought situation can vary with each area and specific plants have adapted for their particular water soil state (Farooq *et al.*, 2012). In this study, to assess an actual drought stress situation, polyethylene glycol (PEG) is used to induce drought stress in the plant. PEG is applied to produce drought stress since PEG-6000 does not penetrate the cell: producing an osmotic potential of the nutrient solution culture (Lagerwerff, 1961) and presenting a plant water deficit. Higher molecular weight PEG (4000 to 8000) was used to regulate drought stress in the 1970s to 1980s. Some papers have

researched and documented the distinct molecular weight of PEG. The theory of calculating osmotic pressure concentration for different molecular weights is also discussed (Kaufmann and Michel, 1973; Money, 1989).

4.3.3 Salt stress

Salt stress is a primary factor inhibiting the germination of seeds. The accumulation of excess salts in the soil results in the soil's poor structure and limited water drainage. Crop yield and plant growth capabilities have decreased because of salinity stress. The tolerance to salinity stress is different in plant organisms (Munns and Termaat, 1986). Many plants and seeds do not germinate and grow in saline soils. The abiotic stress of salinity is the world's worst problem. Salinity in semi-arid regions is affected, and crop production is reduced (Neumann, 1995). Salinity inhibits and prevents seed germination. In saline conditions, soil that has too much sodium can produce a toxic atmosphere for planting, then water absorption by the seed and plant from the soil is induced and plant growth decreases. According to (Munns, 2005) the amount of salinity-exposed agricultural land is approximately 8 million hectares globally. This resulted from inadequate irrigation, which has caused a decrease in agricultural productivity.

During germination, the seeds require a lot of water uptake in the saline salt tension because of the salt accumulation. This may increase the osmotic pressure due to the accumulation of solutes, induced by sodium around the seeds, which is harmful to the seed due to the excessive uptake of ions during water uptake. The high level of salt in the soil causes low seed germination. As a result, inadequate irrigation creates difficulty in moving nutrients and water to plant roots due to the massive salt concentrations. The plant can absorb the water, which makes the cell wall swell and increase in size.

The cell is usually turgid. To stand up under sunshine, the cell must be resilient, preventing the plant from wilting. If sugar t is applied to the soil, the plant's cell becomes lower in osmotic water potential, in contrast to the sugar solution. The supply of too much glucose will cause the plant cell to be flaccid and shrink due to water diffusion into the soil. Glucose allows the cell to have lower osmotic water potential if there is lots of glucose in the plant cell itself. The lower osmotic potential allows the solvent (water from the soil) to be absorbed by the cell, which has a higher osmotic action capability than the plant cell. It thus leads to cell and turgor enlargement.

4.4 Seed samples in the studies

The seeds used for this research come from various categories. The best-selected seed and traits were chosen for the experiment to obtain the best outcome.

4.4.1 Oestrogen-inducible systems

In most plants, inducible systems are implemented to express the expression of a particular gene in the plant. Inducible systems are widely applied to plant experiments, such as Arabidopsis, tomato, maize, soybean and many other model plants. Inducible systems are widely used to deregulate the degree of gene expression at certain stages of plant growth and in particular tissues of interest (Reynolds, 1999). The experiments are conducted with the application of the inducible method in a strictly isogenic sense.

The oestrogen-inducible mechanism was used by all the transgenic plants in this experiment. The oestrogen for phenotyping was added to the seed. To stimulate the gene expression to overexpress it, estradiol (oestrogen) was added. Oestrogen was used to cause gene expression and was detected phenotypically. Seeds are phenotypically wild before oestrogen is applied. The transgenic plant is made from an inducible oestrogen system,

using the expression of the gene of interest in this project to trigger it. The construct used to create transgenic plants was a chimeric transcription factor, XVE. XVE is produced to express the promoter via the addition of oestrogen to induce the gene. XVE is made up of the DNA binding domain (DBD) (X), the VP16 (V) acid transactivation domain and the human oestrogen controlling area (ER). The G10-90.0 promoter regulates such systems.

Figure 4.1: A diagram to represent the XVE-chimeric transcription activator involving oestrogen. The expression of the gene interest within the plant is induced by developing the oestrogen- inducible system for the transgenic plant. To allow gene expression, estradiol is activated. The level of expression grew after estradiol induction and had to fold more than average genes.

4.5 Initial seed lines screening

The seeds were screened to get the best seed, following the entire transgenic seed line. The seed lines that survived the PEG treatment are screened in an initial examination. The germination test for transgenic seeds (T2) was conducted with 19% PEG for initial seed line selection screening. Estradiol was used for the procedures for each treatment. The controlled study used ethanol to see the PEG effect on germination. Percentages of germination of seeds for treatment with 19% PEG is reported. The transgenic seed line that was used for the first initial screening is shown in Table 4.1.

Table 4.1: The initial seed phenotyping screening with 19% PEG for the transgenic seeds of *SEX1i, PGMi, β-amylase* and *α-amylase*.

A transgenic plant (line)	Treatment with 19% PEG	Treatment with 19% PEG + 30 µM EST
SEX1i (1,2,3,4,6,7,8,11)	/	/
*PGMi (*1,2,3,4)	/	/
β-amylase (1,2,3,4,5,6,7,9,10,13,14,15, 16,17,18)	/	/
α-amylase (1,2,3,4,5)	/	/
Wild Type Columbia	/	/

Based on these screenings, the line was selected and chosen to undergo a further test for homozygous screening, such as:

- *SEX1*i- lines 3, 7, 8, 11
- *PGMi*- lines 1, 2, 3, 4
- *β-amylase*- lines 1, 2, 3, 6, 15
- *α-amylase*- lines 2, 3, 4, 5

4.5.1 Planting homozygous plants

Plants with the same alleles are homozygous. These could be homozygous dominant, or homozygous recessive: the dominant phenotypes are expressed by the homozygous dominant, while the recessive phenotypes are expressed by the homozygous recessive. Homozygous seeds were treated with 10% bleach after recognising the homozygous rows, putting the treated seeds on 0.5 MS and 0.8% agar. Stratification in a cold room (4°C) was then followed by a move to a constant light room (22°C) for three days. It is better to pull

the root from the media for transplantation using 0.5 MS, 0.8% agar and a non-toxic MS agar, like HYG agar. For a large seed selection for phenotyping, the resistant seedlings are transplanted into soil in the glasshouse.

4.6 Results

The photos from the GUS reporter line of starch are presented separately, based on the time series from imbibition to (JG) only germinating. Results are presented separately for each treatment. The screening of the seed lines showed 19% PEG after treatment. The final seed lines were used for further treatment. The seed lines were tested for another homozygous plant screening after the screening of the seed lines. Other outcomes were the transgenic seed line PCR to detect gene expression using estradiol treatment. The phenotyping test was then followed by treatment with PEG and NaCl.

4.6.1 Confocal imaging to detect starch

Confocal imaging for the wild form of seed was observed using PAPI staining under confocal microscopy. Confocal imaging shows that no starch granules were present during imaging in Figure 4.2.

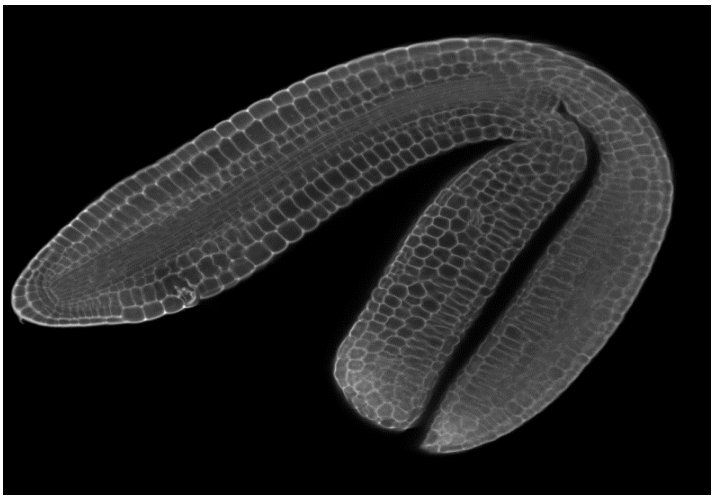


Figure 4.2: The WT Col of *Arabidopsis thaliana* on confocal imaging. The WT Col has been treated by PAPI staining but without the amylase treatment to prevent starch degradation. However, there were no starch granules present during the confocal imaging with PAPI staining.

4.6.2 Initial lines screening then homozygous lines for transgene seeds

By using 19% PEG treatment, the screening of the primary seed lines was applied to get the best germination rate, followed by the selection to get the homozygous plant. The screening phases of the transgenic lines to get the homozygous lines are shown in Table 4.2 below.

Table 4.2: The transgene seeds lines before and after the screening with hygromycin (HYG).

Transgenic plant	Lines before screening	Line after initial screening	The homozygous line based on HYG screening
SEX1i	1,2,3,4,6,7,8,11	3,7,8,11	*SEX1i 3-6* *SEX1i 7-5* *SEX1i 8-2* *SEX1i 11-1*
PGMi	1,2,3,4	1,2,3,4	*PGMi 1-7* *PGMi 2-4* *PGMi 3-3* *PGMi 4-2*
β-amylase	1,2,3,4,5,6,7,9,10,13,14, 15,16,17,18	1,2,3,6,15	*BAM 1-3* *BAM 2-3* *BAM 3-2* *BAM 6-8* *BAM 15-7*
α-amylase	1,2,3,4,5	2,3,4,5	*AAM 2-4* *AAM 3-1* *AAM 4-1* *AAM 5-3*

4.6.3 Final seed lines screening and homozygous plant

The transgenic seeds were tested with the HYG treatment for the last time to obtain the homozygous lines that were used in the experiments. Table 4.3 shows the final screening of seed transgenes after selection under treatment with HYG.

Table 4.3: The final screening of the transgenes seed of *SEX1i*, *PGMi*, *BAM* and *AAM* after screening treatments with hygromycin (HYG).

Transgenic plant	Lines before screening	Line after initial screening	The homozygous line based on HYG screening
SEX1i	1,2,3,4,6,7,8,11	3,7,8,11	*SEX1i 3-6* *SEX1i 7-5* *SEX1i 8-2* *SEX1i 11-1*
PGMi	1,2,3,4	1,2,3,4	*PGMi 1-7* *PGMi 2-4* *PGMi 3-3* *PGMi 4-2*
β-amylase	1,2,3,4,5,6,7,9,10,13,14,15,16,17,18	1,2,3,6,15	*BAM 1-3* *BAM 2-3* *BAM 3-2* *BAM 6-8* *BAM 15-7*
α-amylase	1,2,3,4,5	2,3,4,5	*AAM 2-4* *AAM 3-1* *AAM 4-1* *AAM 5-3*

4.6.4 RT PCR gel electrophoresis imaging on transgenic seeds

The RT PCR images are the output of RT PCR, after gel electrophoresis has been performed. This amplicon of PCR is used to validate the mRNA transcription level. *Actin 7* was used as a control, and was loaded beside the target gene to compare the level of expression with the target genes. The different genes display different effects and behaving differently. Figures 4.10 - 4.13 show the mRNA's amplicon level after RT PCR.

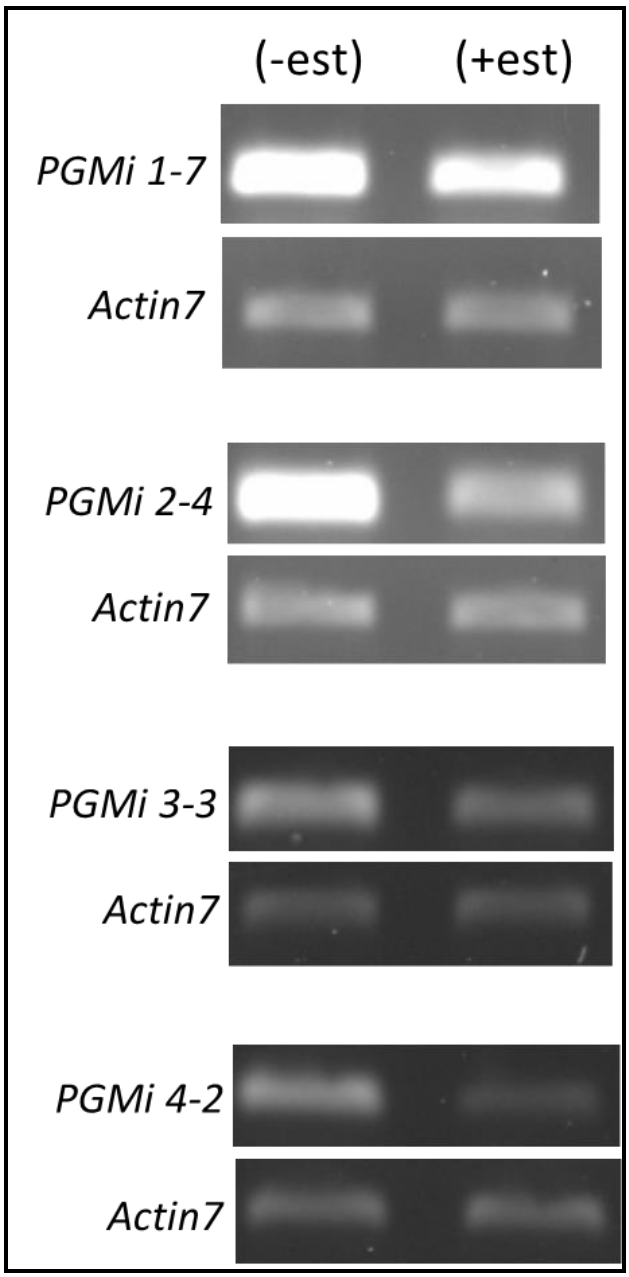


Figure 4.3: The gel electrophoresis images for *PGMi* after RT PCR. The line was *PGMi*, but the PCR amplicon was *PGM*. The positive result showed no induction or degradation of mRNA PGM on estradiol for *PGMi 1-7, PGMi 2-4, PGMi 3-3 and PGMi 4-2* treatment mRNA of PGM has degraded.

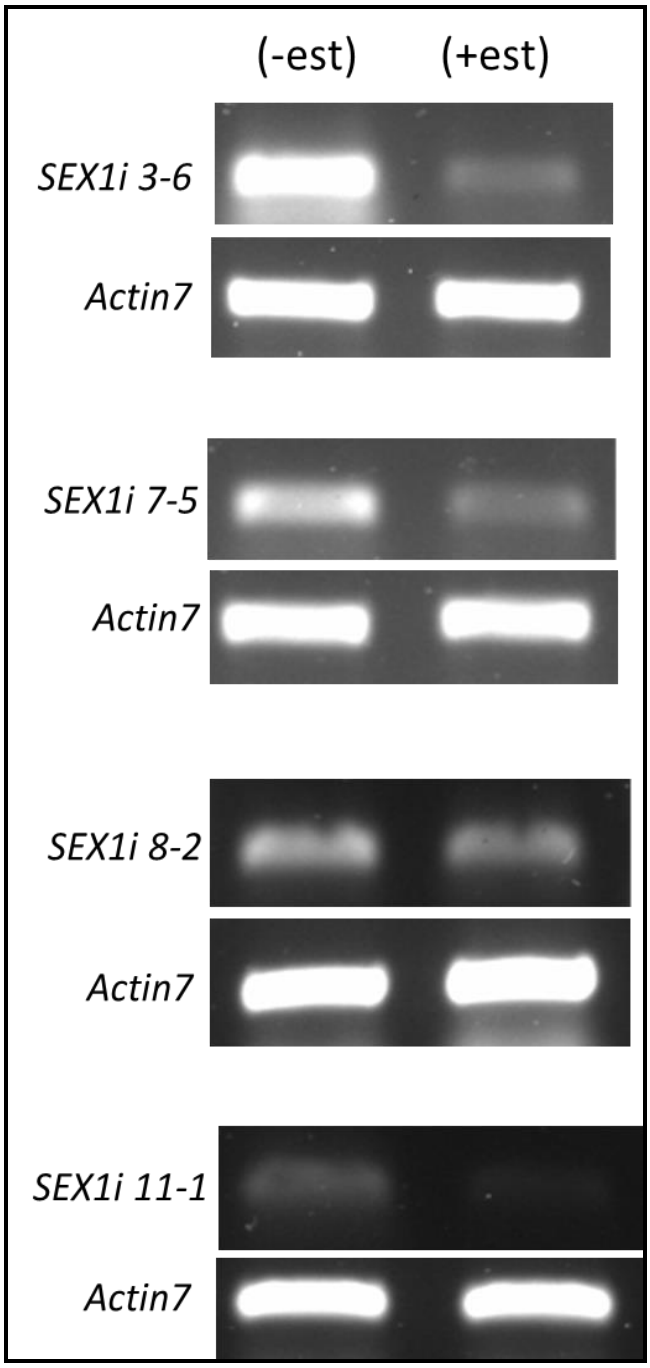


Figure 4.4: The gel electrophoresis images for *SEX1* after RT PCR. The line was *SEX1i*, but the PCR amplicon was *SEX1*. The positive result showed no induction or degradation of mRNA SEX1 on estradiol for *SEX1i 3-6, SEX1i 7-5, SEX1 8-2 and SEX1i 11-1* treatment because the mRNA of *SEX1* had degraded.

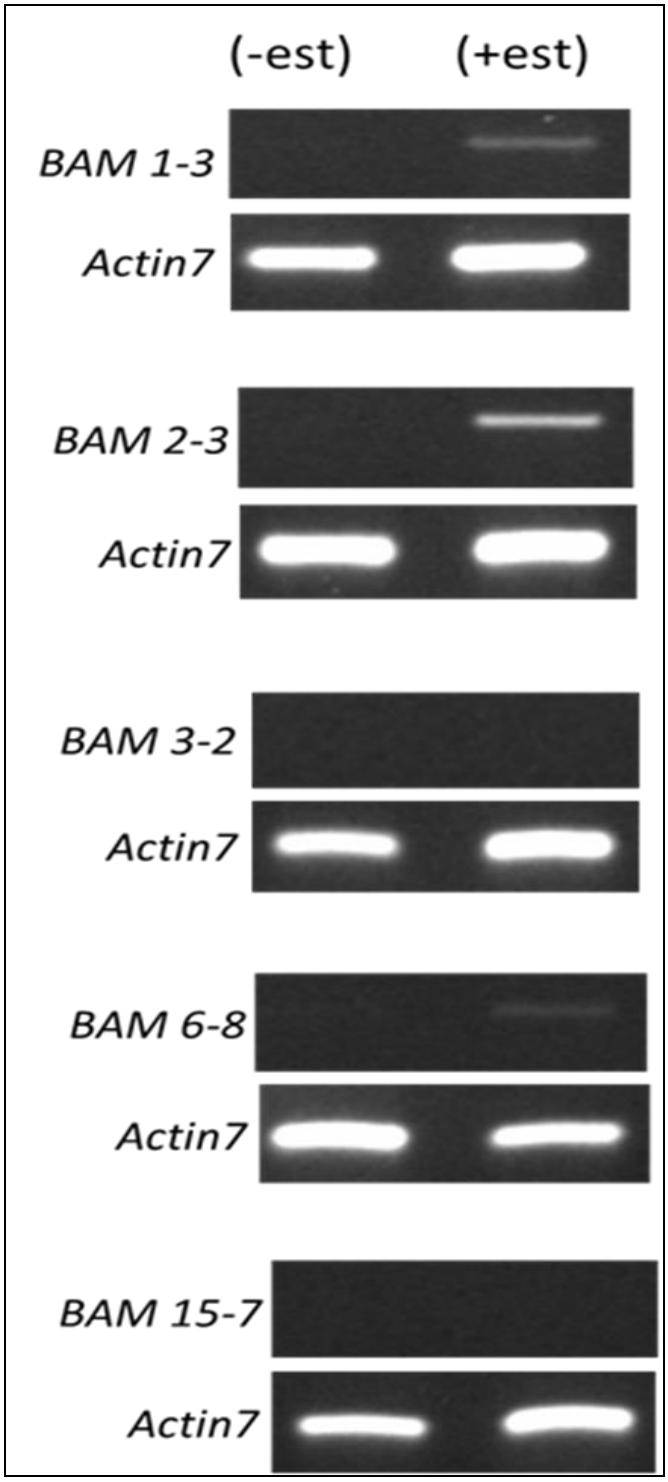


Figure 4.5: The gel electrophoresis images for *BAM* after RT PCR. The induction of *BAM*'s level of mRNA product present and showed a positive result for *BAM 1-3* and *BAM 2-3*. While for *BAM 3-2*, *BAM 6-8* and *BAM 15-7*, mRNA induction was absent.

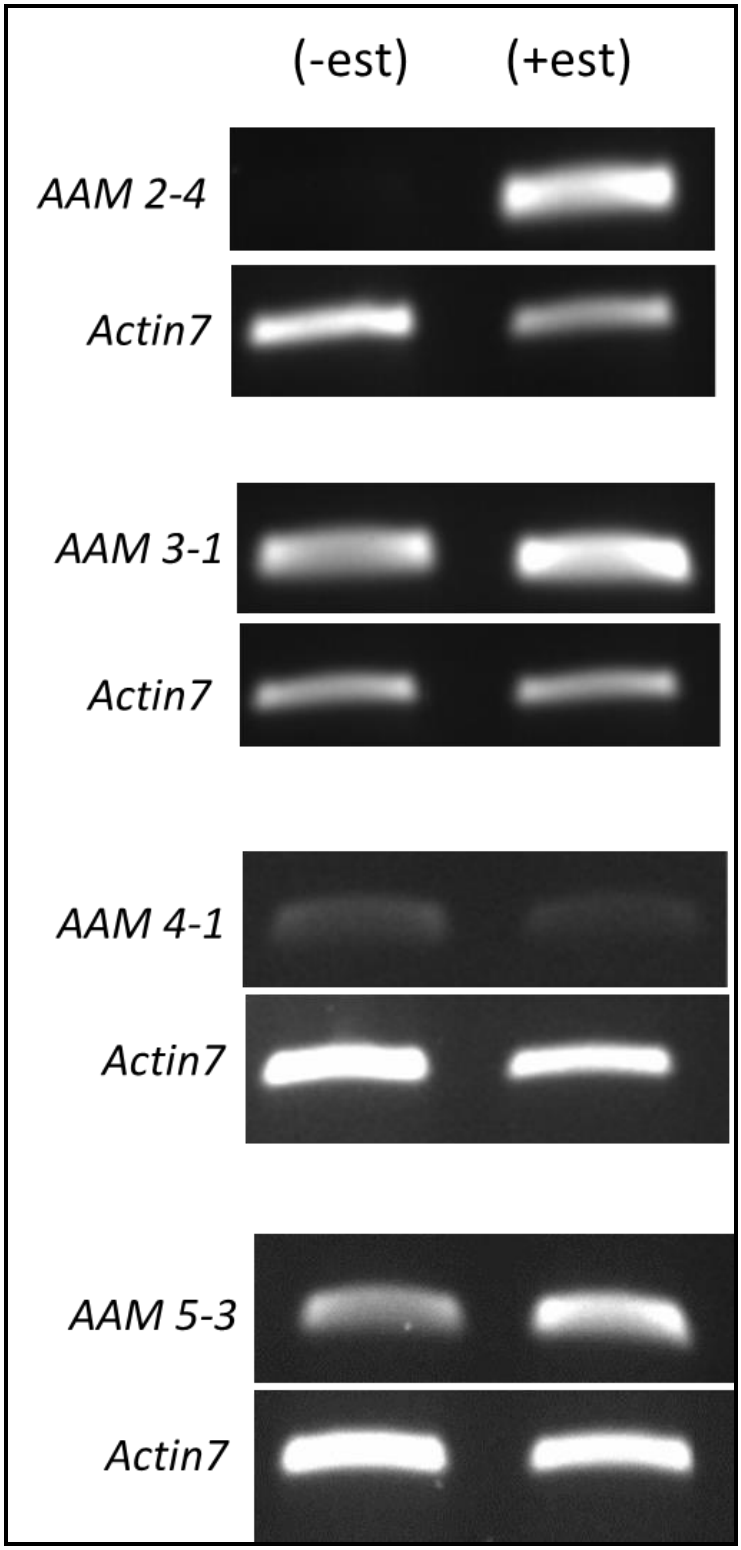


Figure 4.6: The gel electrophoresis images for *AAM* after RT PCR. The induction of the level of mRNA product of *AAM* present and showed the positive result for *AAM 2-4*, *AAM 3-1* and *AAM 5-3*. While for *AAM 4-1*, the induction of mRNA was absent.

4.6.5 Phenotyping with 20% PEG

The transgenic seed phenotyping with 20% PEG showed that the seed could live and germinate, with the strongest phenotyping compared to other PEG concentrations. The best germination of seeds was at 20% PEG when pre-testing transgenic seeds for treatment.

4.6.5.1 Germination for WT Col

For any experiment, germination of WT Col is the mandatory procedure. It will demonstrate no other effect of WT Col for treatment due to no active genes inside.

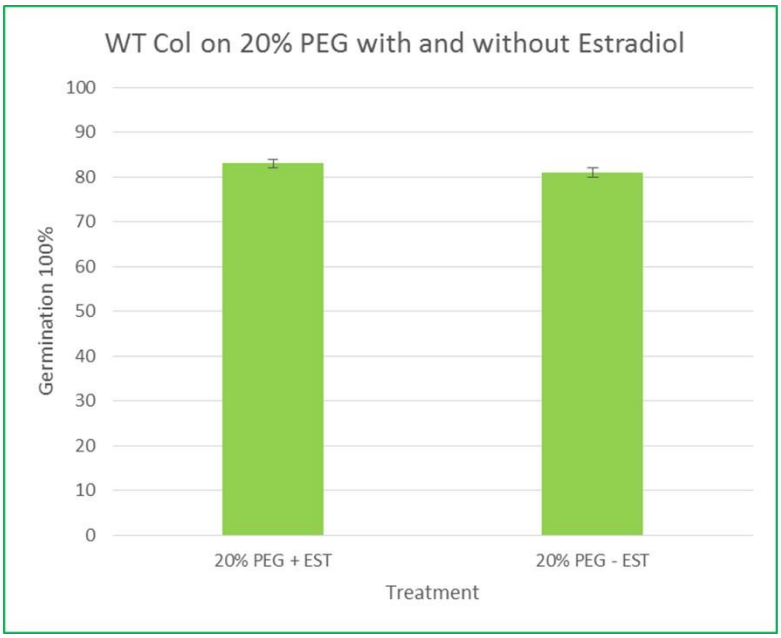


Figure 4.7: Germination of 20% PEG with and without estradiol for WT Col for seven days.There was no difference between both treatments (Figure 4.7): WT Col with 20% PEG without estradiol and treatment with 20% estradiol. Furthermore, there were no significant effects on the wild forms of estradiol therapy. The pre-testing of the wild type Col on estradiol and without estradiol treatment showed the germination is over 70% for both treatments. From this, it can be concluded the seed has been used, and it showed a positive effect in germination.

4.6.5.2 Germination for transgene seeds on 20% PEG

The germination on 20% PEG for transgenic seeds during treatment showed different results for each transgenic seed. For each seed, its germination gene functions are involved, and the other genes behave differently.

4.6.5.2.1 Germination for *PGMi*

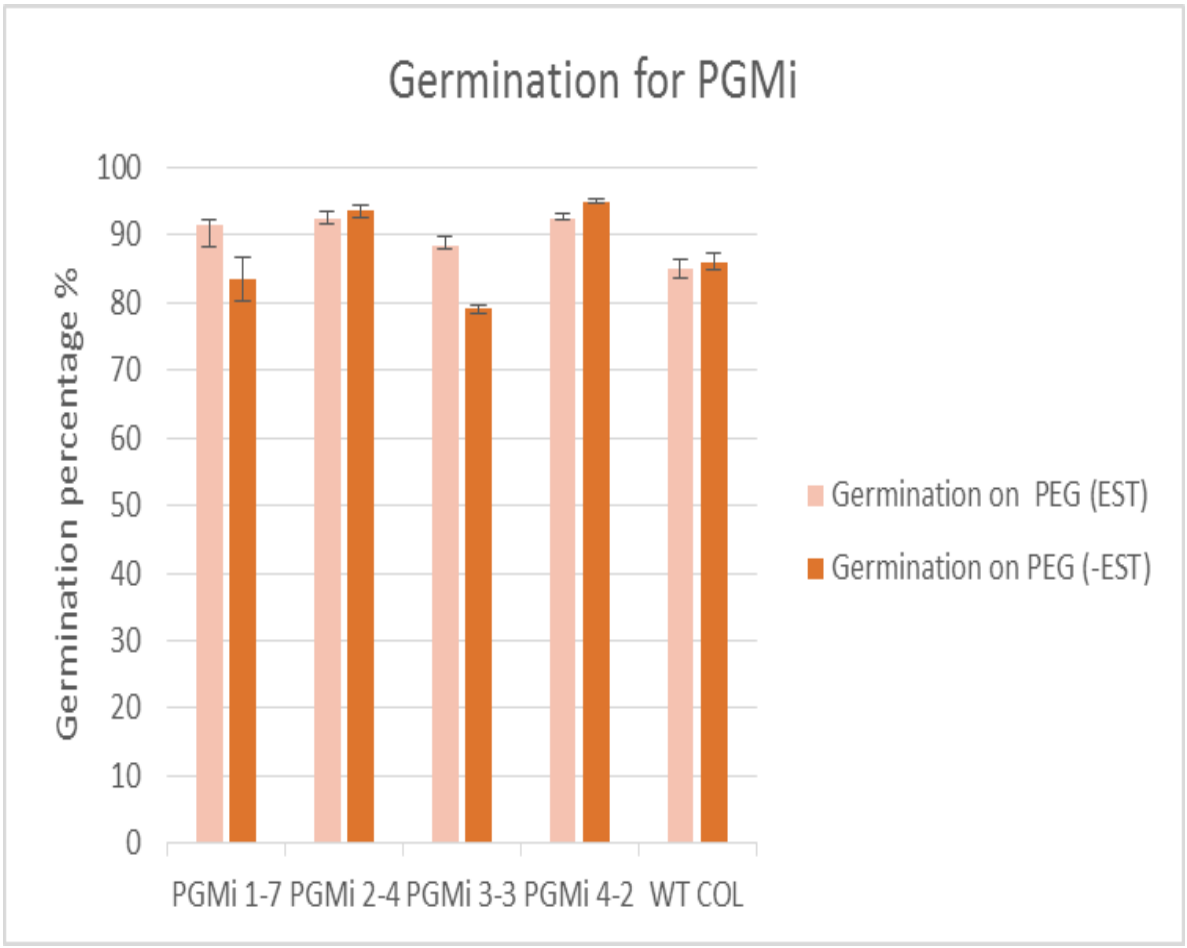


Figure 4.8: Germination for *PGMi* on 20% PEG. The effect of 20% PEG on germination of *Arabidopsis thaliana* after seven days imbibed in water for *PGMi* 1-7, *PGMi* 2-4, *PGMi* 3-3 and *PGMi* 4-4. The *WT Col* acts as a control in the experiment. Values are given as mean (n=50) and the vertical bars indicate ± SE.

4.6.5.2.2 Germination for *SEX1i*

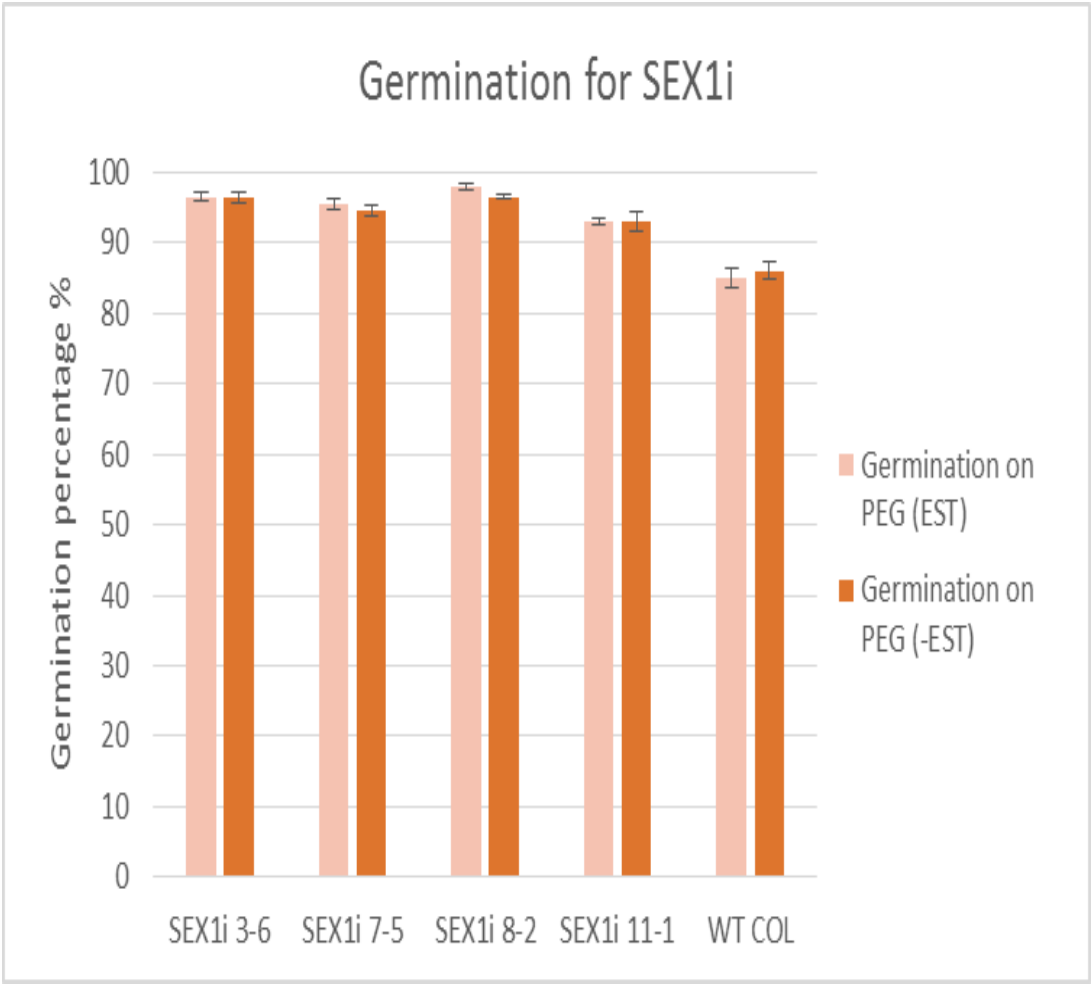


Figure 4.9: Germination for *SEX1i* on 20% PEG. The effect of 20% PEG on germination of *Arabidopsis thaliana* after seven days imbibed in water for *SEX1i* 3-6, *SEX1i* 7-5, *SEX1i* 8-2 and *SEX1i* 11-1. The *WT Col* acts as a control in the experiment. Values are given as mean (n=50) and the vertical bars indicate ± SE.

4.6.5.2.3 Germination for *AAM*

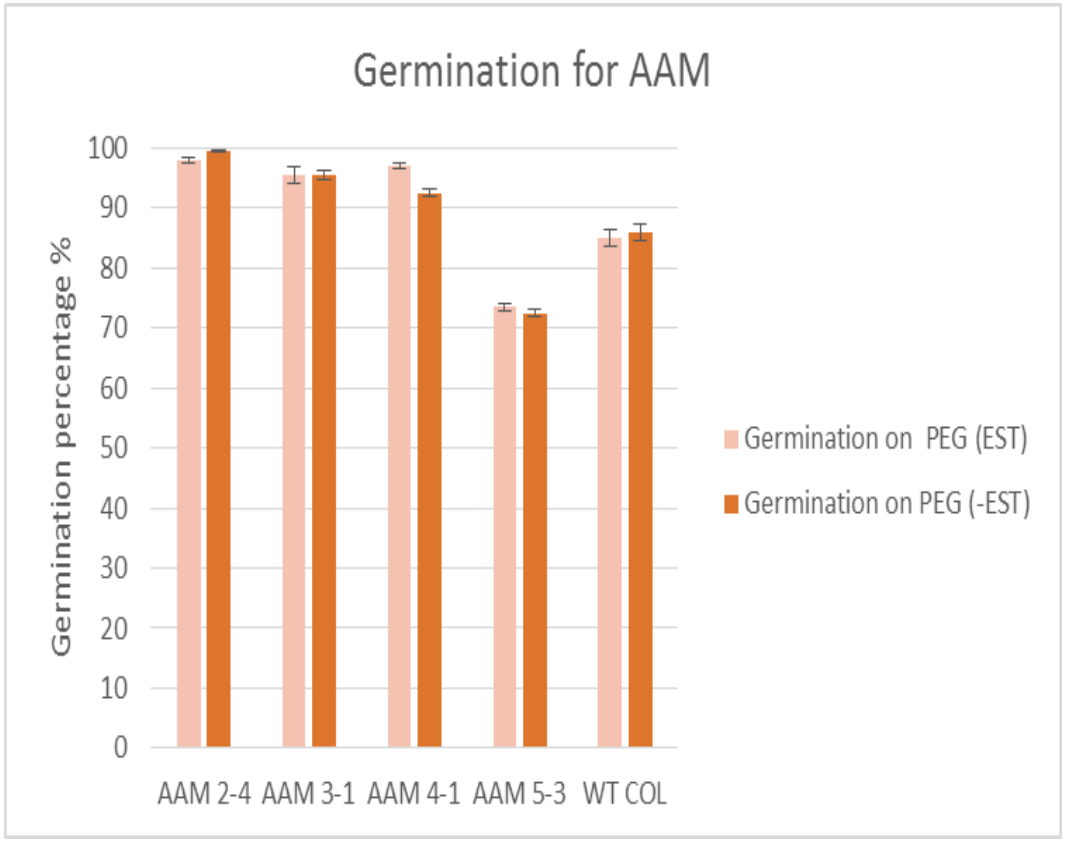


Figure 4.10: Germination for *AAM* on 20% PEG. The effect of 20% PEG on germination of *Arabidopsis thaliana* after seven days imbibed in water for *AAM* 2-4, *AAM* 3-1, *AAM* 4-1 and *AAM* 5-3. The *WT Col* acts as a control in the experiment. Values are given as mean (n=50) and the vertical bars indicate ± SE.

4.6.5.2.4 Germination for *BAM*

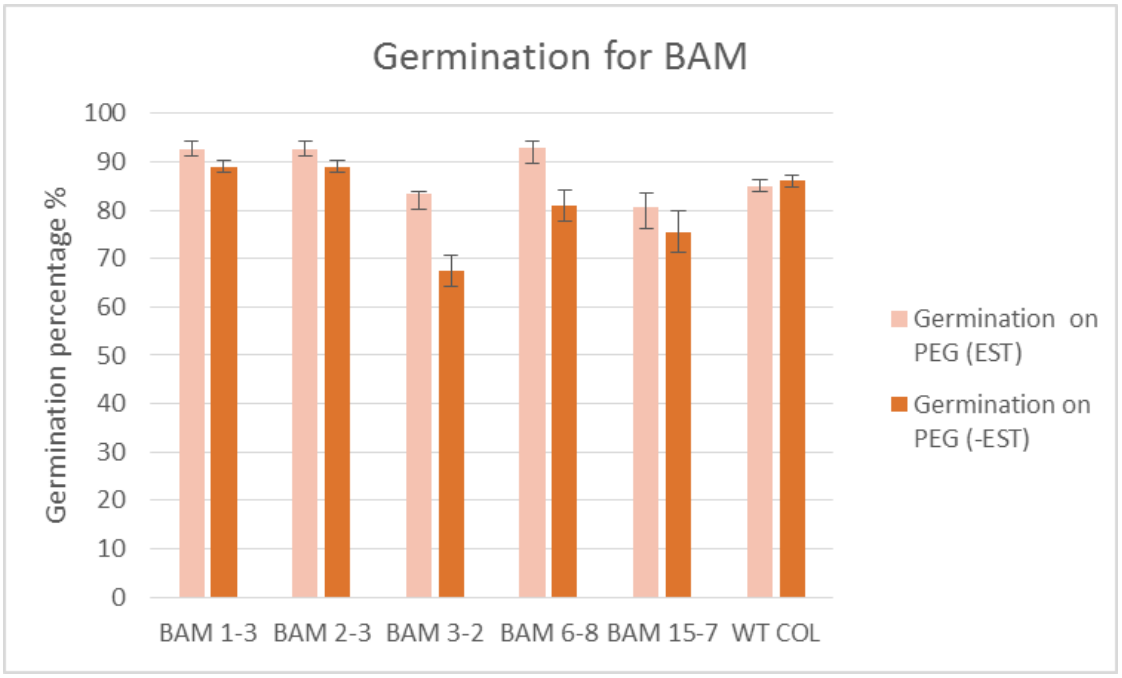


Figure 4.11: Germination for *BAM* on 20% PEG. Effects of 20% PEG on germination of *Arabidopsis thaliana* after seven days of imbibition in water for *BAM* 1-3, *BAM* 2-3, *BAM* 3-2, *BAM* 6-8 and *BAM* 15-7. The *WT Col* acts as a control in the experiment. Values are given as mean (n=50), and the vertical bars indicate ± SE.

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4.6.6 Phenotyping with salt stress (NaCl)

Salt stress caused by NaCl affects the germination of seeds. The various NaCl concentrations provide different results for seed germination, and for other plants, the ability of a seed to survive in salt stress varies.

4.6.6.1 Germination for WT Col

The germination of WT Col demonstrated the effect on the rate of seed germination at different concentrations. The highest NaCl level induced a low seed germination grade, and

the seeds were unable to thrive in high salt concentrations. The high salinity decreases the germination of seeds. The best germination rate occurred at 200 µM, following pre-treatment of WT Col (Figure 4.12) at different levels of NaCl.

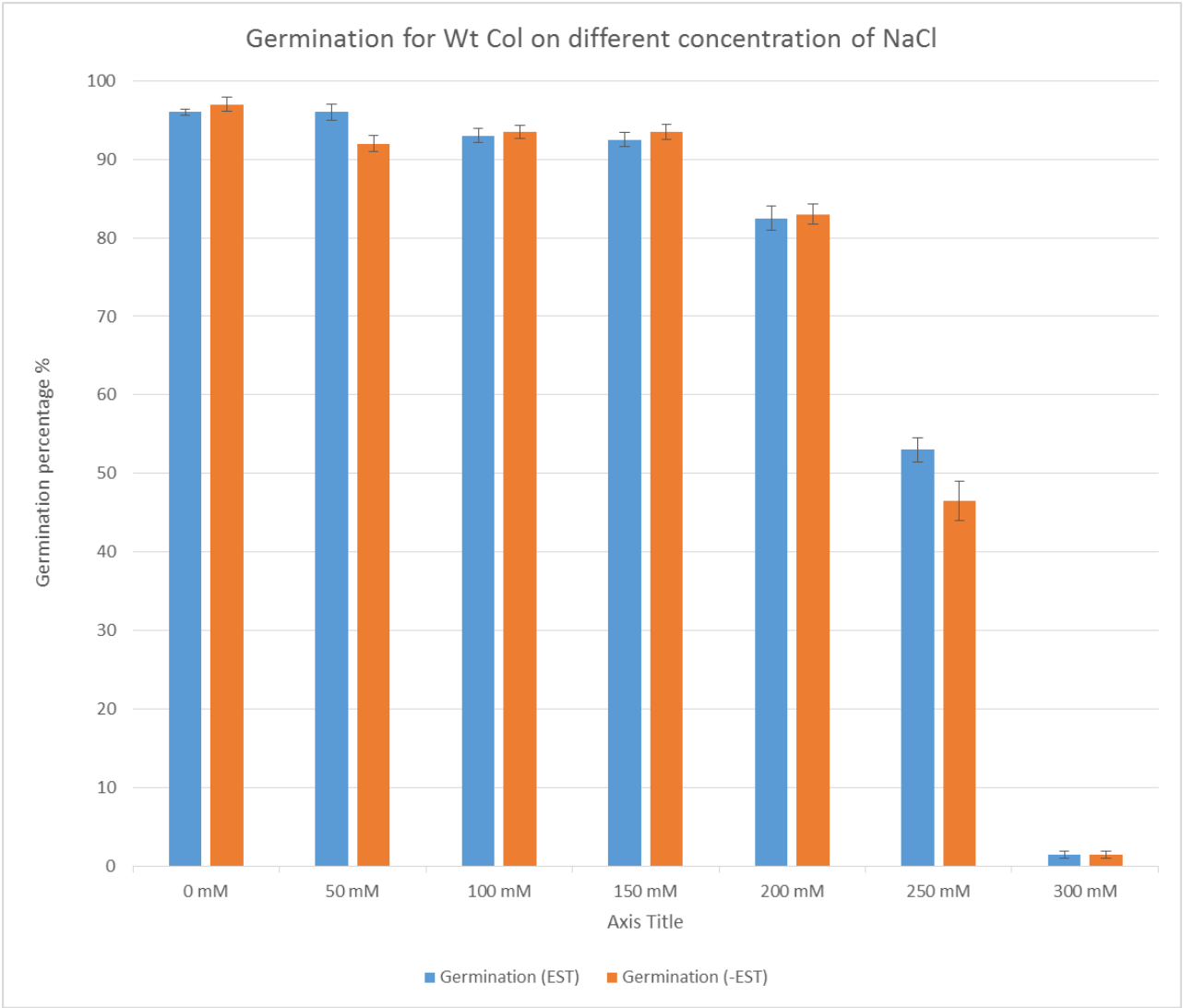


Figure 4.12: Pretesting of wild type *Arabidopsis thaliana* seeds germination on different concentrations of NaCl. At 0 mM to 150 mM, there was more than 90% germination, and it did not show any difference in salt stress compared with 200mM, 250mM and 300mM of salt stress. The germination at 200 mM demonstrated the best phenotype for salt stress.

4.6.6.2 Germination of transgene seeds on 200mM NaCl

Transgene seed germination at 200 µM NaCl was selected because it showed the best germination rate, and the seed can survive at that concentration. For other transgenic seeds, the transgenic seeds' phenotyping had a different impact on the germination rate.

4.6.6.2.1 Germination of *PGMi*

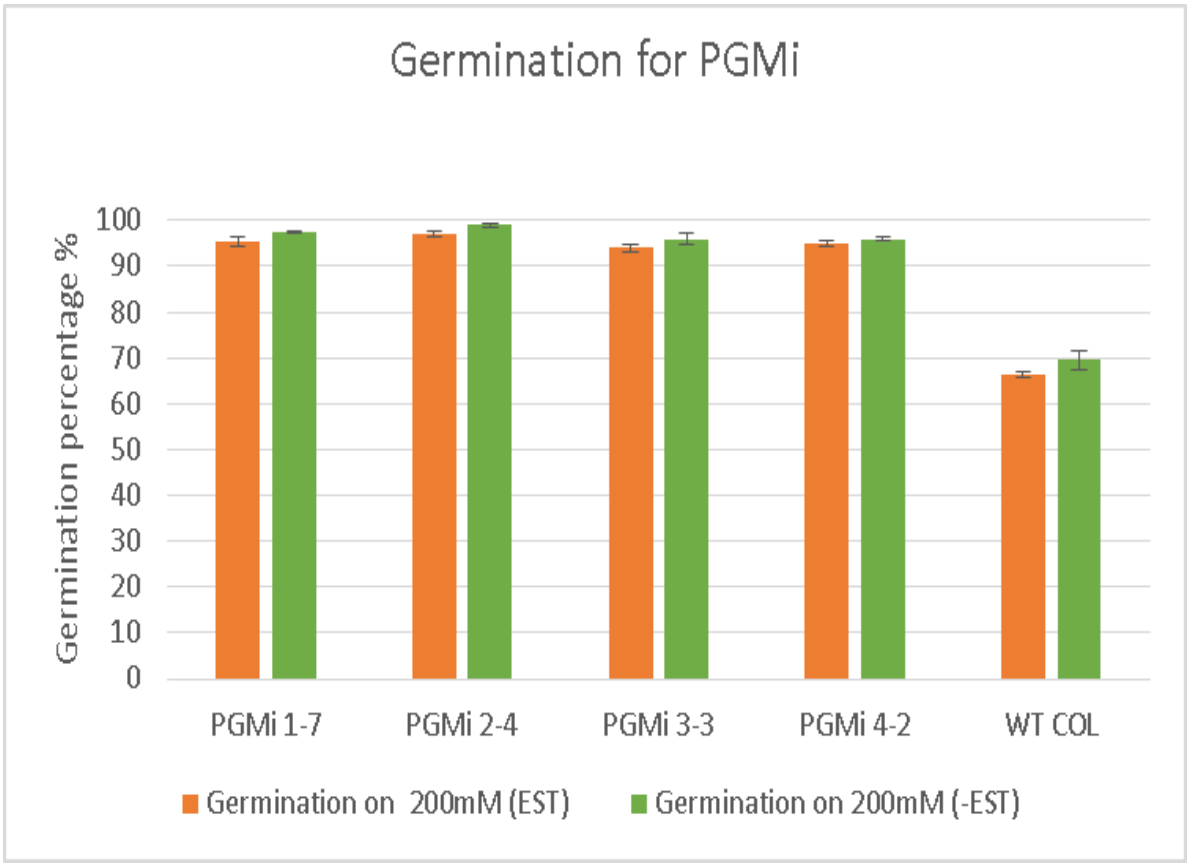


Figure 4.13: Germination of *PGMi* on 200 mM NaCl. The effect of 200 mM NaCl on germination of *Arabidopsis thaliana* after seven days imbibed in water for *PGMi* 1-7, *PGMi* 2-4, *PGMi* 3-3 and *PGMi* 4-4. The *WT Col* acts as a control in the experiment. Values are given as mean (n=50), and the vertical bars indicate ± SE.

4.6.6.2.2 Germination of *SEX1i*

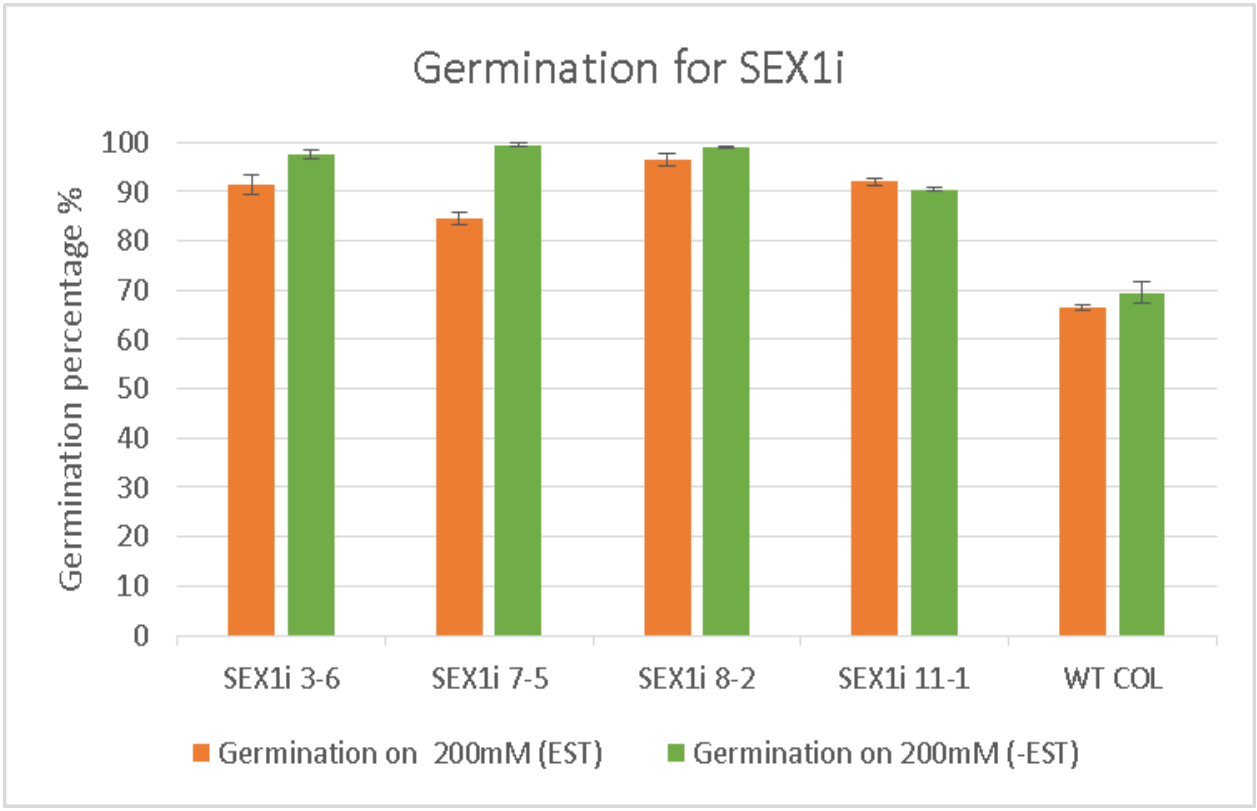


Figure 4.14: Germination of *SEX1i* on 200 mM NaCl. The effect of 200 mM NaCl on germination of *Arabidopsis thaliana* after seven days imbibed in water for *SEX1i* 3-6, *SEX1i* 7-5, *SEX1i* 8-2 and *SEX1i* 11-1. The *WT Col* acts as a control in the experiment. Values are given as mean (n=50), and the vertical bars indicate ± SE.

4.6.6.2.3 Germination of *AAM*

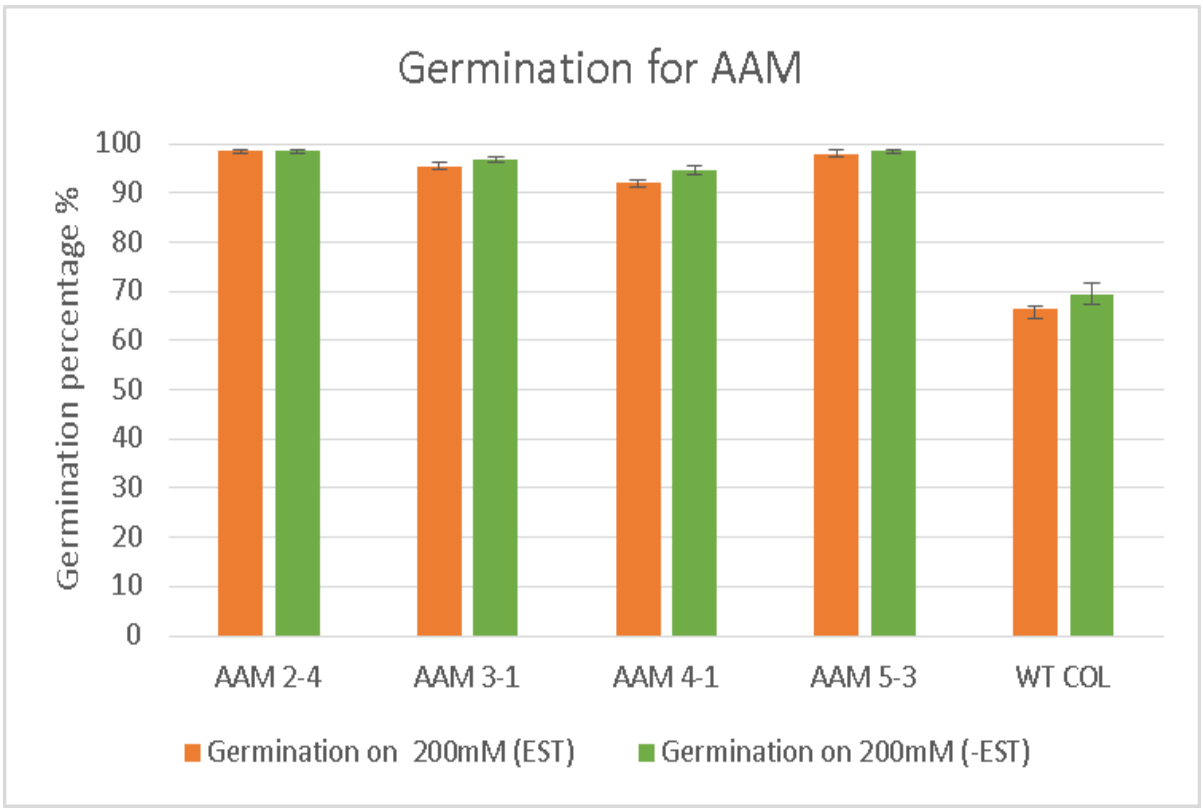


Figure 4.15: Germination of *AAM* on 200 mM NaCl. The effect of 200 mM NaCl on germination of *Arabidopsis thaliana* after seven days imbibed in water for *AAM* 2-4, *AAM* 3-1, *AAM* 4-1 and *AAM* 5-3. The *WT Col* acts as a control in the experiment. Values are given as mean (n=50), and the vertical bars indicate ± SE.

4.6.6.2.4 Germination of *BAM*

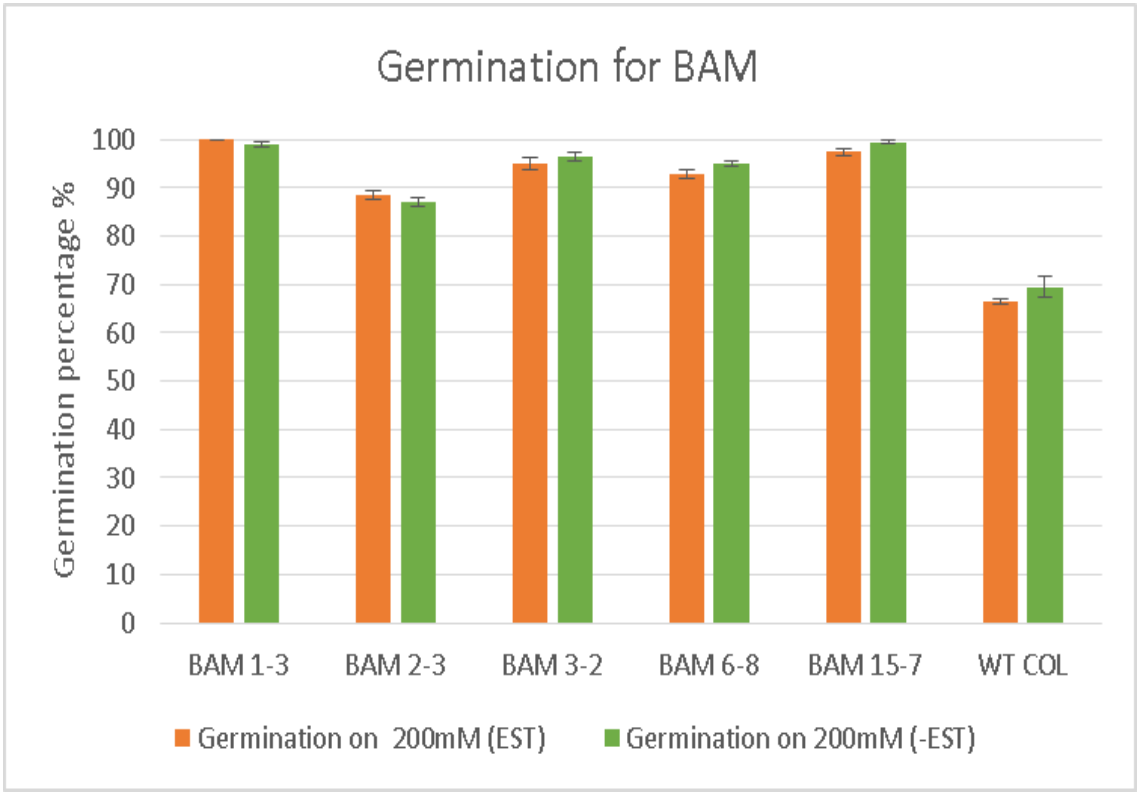


Figure 4.16: Germination of *BAM* on 200 mM NaCl. The effect of 200 mM NaCl on germination of *Arabidopsis thaliana* after it has been imbibed in water for seven days with *BAM 1-3*, *BAM 2-3*, *BAM 3-2*, *BAM 6-8* and *BAM 15-7*. The *WT Col* acts as a control in the experiment. Values are given as mean (n=50), and the vertical bars indicate ± SE.

Following the germination screening, transgenic lines with 20% PEG and 200 mM NaCl, such as *PGMi 1-7*, *PGM 3-3*, *SEX1i 3-6*, *SEX1i 7-5*, *BAM 3-2*, *BAM 6-8*, *AAM 4-1* and *AAM 5-3* were selected for transgenic seed after phenotyping screening.

4.7 Discussion

Seed germination is the main operation to grow a new plant in agriculture. At the same time, agriculture is the most important sector that plays a crucial role in a country's economic growth. The better the seed germination, the higher the yield of the crop produced. The understanding of seed germination and its return will give benefits for future agriculture development. As agriculture develops, production increases and becomes more market-oriented.

The seed samples from the oestrogen-inducible system were created by our previous lab member, Dr. Petra Stamm. The heterozygous seeds were screening to get the homozygous seeds, with a few treatments to identify the pure homozygous sources selected. The homozygous selection were planted in bundles to get seeds to use in the experiment. The seeds were then subjected to RT PCR to identify the mRNA induction levels. The strong induction level of mRNA during PCR imaging confirms the expression of genes in the seed samples.

The RT PCR confirmed the transcription level of mRNA in the seed lines. Following that, the Arabidopsis seed germination phenotyping under the stress conditions of PEG and NaCl was examined. Seed phenotyping on stress situations of drought (PEG) and salinity stress (NaCl) were both shown to be different in this study. Drought and salinity are common environmental problems in the agricultural sector that affect seed germination and plant growth development. Most seeds are sensitive to abiotic stress, especially during germination. This also leads to the loss of the yield regarding the failure of seed germination. Seed germination is essential in enhancing a plant. Salt is usually present in water irrigation, and the soil will hinder the germination process because the seed absorbs

the salt causing toxicity to the plant. In drought, the osmotic stress created by sodium chloride (NaCl) causes the water not to be absorbed by the seed.

4.7.1 Confocal imaging

The accumulation of starch granules to germinate embryo cells under confocal imaging is documented in the previous laboratory observations. These are proven to be starch granules because the granules are gone when amylase solution was applied. Amylase treatment has degraded the starch, meaning that the degradation of starch products from oil mobilisation is transformed into starch during seed germination. WT Col Arabidopsis seeds were imbibed and dissected following this study until JG (as the time after the gene was induced). By skipping the amylase injection to avoid starch degradation, the embryos were stained with PAPI staining. The captured embryo images under the confocal microscope revealed no granules in the seed's embryo, and there were no starches in the germinating embryo. Starch granules are thought to be abundant in plastids, mesophyll, vascular and root caps, but not much in the seed. Since no starch was present in WT Col of Arabidopsis, the starch had already converted during germination during the gene was induced, so there was no further study for GUS's embryo reporter lines of starch.

4.7.2 Initial seed lines screening and homozygous seed line screening for transgene seeds

The transgene seeds from the Bassel lab were at the T2 stage. For 80 seeds with 19% PEG, pre-testing was performed to screen the transgenic seeds to pick the seed from lines with the highest germination rate. The chosen seed lines were used for further experiments. The lines were tested with 19% PEG with 30 μM estradiol, and without estradiol as a control treatment. Based on the effect of the germination rate on estradiol, the lines were screened.

For more HGY screening for homozygous plants, the lines which showed more significant results were used.

Before collection, the seeds from the selected lines were sown with 30 μM HGY on MS agar. The resistant HGY seedling was individually transplanted into a container for eight plants. For homozygous screening, the harvested seeds were then re-plated on HYG agar. As a homozygous plant, 100% HYG resistant seedlings were used.

4.7.3 RT PCR gel electrophoresis imaging on transgenic seeds

RT PCR was used to validate each transgene line's transcription level. The difference in the degree of induction of each mRNA gene and the synthetic mRNA identifies whether or not the plant transformations worked. Both transgenic plants were inducible from the oestrogen system, so oestrogen was used for treatment and ethanol was used as a control. The level of expression of the target gene, whether overexpressed or downregulated after estradiol use, was calculated by estradiol therapy. PCR operates at a specific annealing temperature and then undergoes gel electrophoresis.

The PCR products were loaded into 1% agarose gel with a TBE buffer solution after the PCR procedure. The container was connected to the electrical current and the voltage was set up to separate the DNA pieces, from the negative charge to the positive charge. The segments of DNA and mRNA were split according to their size. A dye was added to allow the DNA to be seen on the gel after it is separated. The gels were imaged under UV light, based on the gel that runs with TBE, and the DNA fragments appeared on the gel as bands. The level of expression of transgene seeds' mRNA was shown: whether overexpressed or downregulated, the expression of mRNA can be confirmed according to the band appearing on the gel image.

The DNA fragments were isolated by gel electrophoresis. The DNA fragment has a negative charge so that the electric current will be drawn to the positive charge. The staining agent, ethidium bromide, was applied to the gel and buffer solution to visualize the DNA fragments under UV light. To compare estradiol's induction level, positive and negative controls (*Actin 7* (-/+) estradiol) were loaded next to the target gene.

Glucose is transformed into starch by the *PGM* gene. However, *PGMi* destroys mRNA, so no starch is produced. *PGM* synthetic mRNA primers were designed to run the PCR. The artificial mRNA was caused by it, then the *PGM* mRNA degraded. It has an induction level dependent on *PGMi* without estradiol treatment. There should be no degradation level for the PGMi gene with the presence of estradiol treatment because the *PGM* mRNA has degraded. The line was *PGMi,* but *PGM* was the PCR amplicon. According to Figure 4.10, it can be established that *PGMi* had different levels of induction during treatment, and was degraded after treatment with 30 mM estradiol because the transgenic seeds were wild before oestrogen was added to them. A degraded level of mRNA was seen in *PGMi* 1-7, *PGMi* 2-4, *PGMi* 3-3 and *PGMi* 4-2.

The *SEX1* gene can make starch glucose-degradable; however, *SEX1i* kills mRNA, so starch cannot be degraded. *SEX1* artificial mRNA primers were developed for PCR. The artificial mRNA was induced, and the *SEX1* mRNA was degraded. Based on *SEX1i* without the presence of estradiol treatment (wild type), there should be no degree of expression for the *SEX1i* gene with the presence of estradiol treatment as the mRNA degraded it. *SEX1i* was the line, but the PCR amplicon was *SEX1*. According to the results (Figure 4.11), it can be verified that *SEX1i* has a different treatment induction level and has deteriorated after

treatment with 30 mM estradiol. Degraded mRNA levels were observed for *SEX1i* 3-6, *SEX1i* 7-5, *SEX1* 8-2 and *SEX1i* 11-1.

The *BAM* gene works to make the starch degrade. The depleted starch has been turned into glucose. To induce the gene expression of the *BAM* gene, 30mM estradiol was applied. Due to the PCR inducing the *BAM* cDNA, the induction levels increased. In Figure 4.12, *BAM 1-3*, *BAM 2-3* and *BAM 6-8* showed increased mRNA induction levels after estradiol treatment.

The *AAM* gene works to make the starch degrade. The genes from the transformation of the plant were amplified during the PCR reaction. The primers are designed explicitly for cDNA induction. To generate AAM's gene expression, 30 mM of estradiol was applied to the treatment because the transgenic seed used the oestrogen-inducible systems. Figure 4.13 shows that expression levels of *AAM* 2-4, *AAM* 3-1 and *AAM* 5-3 increased after estradiol treatment, confirmed by the bands.

It can be inferred that *PGMi* and *SEX1i* have little to no gene expression on gel PCR imaging, whereas AAM and BAM have gel PCR imaging expression. This means they have no changes in gene expression based on estradiol treatment (PGMi and SEXli), whereas AAM and BAM are affected by estradiol treatment. The PCR is merely a way of visualising the gene expression

4.7.4 Phenotyping with 20% PEG

The 20% phenotyping by PEG-6000 was the best germination seen after pre-testing on several PEG percentages. This correlated with other studies that selected PEG 6000 because it is considered better for plant function than lower molecular weight (Kaufmann and

Eckard, 1971; Michel, 1971). In this situation, the greater the PEG concentration, the lower the seed's germination is because the seed could not uptake water: PEG restricts water uptake. According to the phenotyping, PEG restricted seed germination. In line with previous findings (Ahmadi and Baker, 2000), PEG solution controlled the germination of wheat seeds by monitoring sucrose and starch accumulation in the embryo. This resulted in water-stress by reducing the sucrose synthase activity and causing a reduction in germination.

4.7.4.1 Germination for WT Col

The germination for WT Col on 20% PEG showed different effects between both treatments of PEG, with and without estradiol.

4.7.4.2 Germination for transgene seeds

During seed germination, the effect of PEG induced molecular drought stress on Arabidopsis. The transpiration and water potential caused by delaying germination were reduced by the PEG treatment used during the studies. In plants, PEG mainly accumulates in the leaves, then is dispersed in mesophyll environments. Higher molecular PEG will cause the plant's water movement to be blocked, causing desiccation to minimize water absorption. (Lawlor, 1970).

The gene *PGM* acts as glucose to starch conversion. Phenotyping of *PGMi* could, based on this hypothesis, increase germination with estradiol therapy. PGM's AmirRNA can prevent glucose conversion into starch; therefore, there was no conversion of starch at all. There was a lot of glucose in the cell as a result of glucose not being converted into starch. A high concentration of glucose in the cell will decrease the water potential and allow water to be absorbed.

As a consequence, the cell will grow, turgor will increase and lead to germination. The *PGMi* lines showed the highest germination based on the accumulation of glucose inside the cell. Based on the result (Figure 4.8), the lines *PGMi 1-7* and *PGMi 3.3* showed the highest germination among *PGMi* lines germination, and different effects on PEG were observed with and without estradiol treatment. These findings followed the hypothesis.

The *SEX1* gene acts in reverting the starch into glucose. AmirRNA of *SEX1i* functions to prevent the degradation of starch into glucose. Thus, no glucose will be produced because the amirRNA of *SEX1i* avoids starch conversion into glucose. The accumulation of starch caused the water potential in the cell to increase and no water was taken up inside the cells. As a result, there was no effect on turgor, and it showed less germination. According to the results (Figure 4.9), the lines *SEX1i 3-6*, *SEX1i 7-5* and *SEX1i 11-1* showed the lowest germination rates and have a different effect on PEG with and without estradiol treatment. These findings followed the hypothesis.

As starch degrades, *Beta amylase*, and *Alpha-amylase* act, and starch is degraded into glucose molecules. Consequently, there will be a lot of glucose in the cell from the starch converting into glucose. The cells then have a lower water potential with high glucose in the cell, which affects the solution outside the cell entering. Water absorption causes cells to turgor and increase in size, and then germination is induced to occur. As a result, higher germination comes from *Alpha-amylases AAM 4-1* and *AAM 5-5*.

In contrast, the best phenotypes among *Beta-amylase* were *BAM1-3, BAM 2-3 and BAM 6-8.* They also showed a different effect on PEG with and without estradiol treatment. These findings followed the hypothesis.

This confirms the hypothesis for *PGMi* 1-7, *PGMi* 3-3, *SEX1i* 3-6, *SEX1i* 7-5, *BAM 3-2, BAM 3-2, BAM 6-8, AAM 4-1* and *AAM 5-3,* based on the phenotyping of transgenic seeds.

4.7.5 Phenotyping with 200 mM NaCl

The phenotyping by 200 mM demonstrated a different result for the 200 mM NaCl experiment. After pre-testing with other concentrations, the concentration of 200 mM NaCl was selected. For germination, 200 mM is the highest. Concentrations of NaCl over 200 mM showed poor germination due to salinity stress.

4.7.5.1 Germination of WT Col

The germination of WT Col at different NaCl concentrations (0, 100, 150, 200, 250 and 300 mM) showed different effects with and without estradiol in both treatments. The best germination for this analysis was at 200 mM NaCl.

4.7.5.2 Germination of transgene seeds

Based on the hypothesis, PGM's phenotyping showed higher germination with estradiol therapy. However, PGM's amirRNA can destroy the conversion of glucose to starch. A lot of glucose in the cell will decrease the water potential and allow water to be absorbed. As a consequence, it induces cell expansion and rapid germination.

*SEX1i'*s amirRNA works to block the degradation of starch so that no glucose is released. It raised the water potential of the cell and caused the cell to not uptake water. It was expected to show less germination as a result.

As starch degrades, *Beta-amylase* and *Alpha-amylase* act and starch is degraded to glucose. At a lower water potential, a lot of glucose in the cell allows the solvent to enter the cell. Water uptakes allows cell turgor and spread, causing germination to occur.

The hypothesis is confirmed by a line for *PGMi 1-7, PGMi 3-3, and SEX1i 3-6, SEX1i 7-5, BAM 3-2, BAM 6-8, AAM 4-1 and AAM 5*-3 based on the phenotyping of transgene seeds.

4.8 Conclusions

Selection of the transgenic line, from heterozygous to homozygous, is required before other experiments are completed. Treating kanamycin seedling will kill the recessive seedling, and the dominant seedling will survive. To ensure that the trait carries two copies of the same allele in each gene, it was essential to select for homozygosity. When homozygous choices were made, an extensive collection of seeds were obtained by planting homozygous plants. For several weeks, the seed collection was retained to lose its dormancy for further experiments.

Next, to show that the transgenic seed plants used in this study were present and down-regulated and that the reaction was up-regulated, RT PCR validation was carried out. The RT PCR was done to ensure that the transgene gene was present in each gene's seed. The RT PCR measured the level and quantity of genes in the seeds of transgenes. When oestrogen was applied, the induction levels for the induction stage of gel electrophoresis were shown. In this research, the presence of genes in transgene seeds was shown by RT

PCR, as an example in PGMi, indicating that the induction level decreased with estradiol treatment.

In contrast, the level of induction decreased with estradiol treatment in SEX1i. For further phenotyping of the PEG and NaCl therapy, both PGMi and SEX1i lines showed degraded induction levels have been selected. The induction level has been shown to increase following treatment with estradiol for both AAM and BAM. For the next NaCl and PEG phenotyping tests, both the AAM and BAM lines, which showed increased induction levels, were chosen. The RT PCR first confirmed that the gene was present in the transgene seeds to verify it.

The drought and osmotic stress induced by PEG and NaCl were compared in this analysis. However, no related mechanism correlated with the findings shown. Some of the seeds may germinate in both NaCl and PEG treatments for both therapies. Some seeds, however, delay germination more than is typical for germination. The therapy slows natural germination. However, before the seedling, the germinated seed sprouted from NaCl and PEG treatment will not survive. The greater the germination rate, the better adaptability to the environment will be achieved. At the beginning of the germination stage, treatments were developed for NaCl and PEG to determine whether the seeds could germinate and respond to stress. PEG treatment was for drought stress, while NaCl treatment was for salt stress.

In general, the worst problem facing all plants is drought and salinity, which decreases crop growth and reduces crop yields. NaCl and PEG therapy can create a real stress situation. The stress produced in this experiment was used to simulate drought, salinity and osmotic stress, to test the seed's ability to adjust to a real situation. Seed treatment, such as osmopriming, as a guideline, can improve seed germination and the treatment of plants.

CHAPTER 5

MULTI GENE FAMILIES FOR *ARABIDOPSIS THALIANA* AND *CAPSICUM ANNUM*

5.1 Introduction

Capsicum annuum is an essential global crop for horticulture. Chilli peppers are used in many parts of the globe. *C. annuum* is commonly grown in many countries, and it is the largest crop produced in Asia. China is the world's largest chilli producer, with 18 million tons per year, followed by Mexico, with 3.5 million tons per year (FAOSTAT, 2017). Whereas in Malaysia, chillies are cultivated for local consumption. Malaysians mostly prefer to add chillies to their food to enhance the taste. Chillies are also exported outside the Malaysian market. Chillies are used daily by consumers in cooking and serve as a herb to improve the taste. There are lots of benefits of *C. annuum,* from plant biotechnology to health.

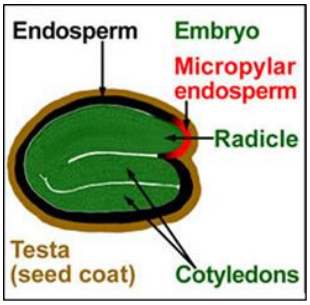
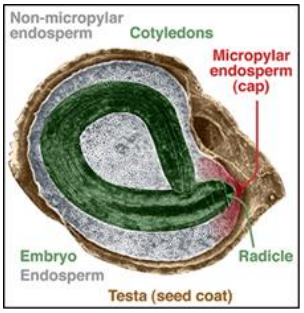
Chilli peppers offer many advantages, such as weight control *(*Whiting *et al.*, 2012). The capsaicinoids found in the chilli have a weight control relationship that helps prevent weight loss. Antioxidants, bioactive compounds, enable DNA damage and tissue in the body to be preserved and repaired. To help antioxidant phytochemicals circulate through the body, vasodilation increases. An analysis conducted by (Nadeem *et al.*, 2011) showed that the chillies' antioxidant can protect food and tissues from oxidative damage caused by free radicals and reactive oxygen. *C. annuum* is primarily used as a food additive in cooking, based on the many advantages of chillies. The capsaicin content found in chillies and capsicum species secretes a pungent fruit material that gives the fruits a warm taste. These have a distinct flavour that can improve the taste.

The biotechnology of *C. annuum* science is widely studied. Several scientists have now discovered genomic experiments. Most of the studies on *C. annuum*, such as insect control in the field, can decrease yield planting. There is also research on how biotechnology can improve the productivity of chilli crops. Intensive research and extensive studies on starch germination in *A. thaliana* have been performed. In this research, I tried to identify the gene present in Arabidopsis that affects seed starch and germination, to determine whether they exist within the chilli, and describe how it works.

5.1.1 General introduction for *Capsicum annuum*

In Southeast Asia, *C. annuum* is known as chilli and pepper, and it is well known. It comes from the family Solanaceae and the genus Capsicum. During the Columbus discovery of America in the 16th century, the pepper's roots originated from North America and Northern South America. In 1942, while looking for the pepper, Columbus took it to Spain (Chamchalow, 2001). Chilli peppers have been distributed all over the world. However, because of warm temperate temperatures, the chilli pepper is better suited to tropical climates. Then in the early 16th century, it expanded into Asia.

Table 5.1: Comparison of *Arabidopsis Thaliana* and Capsicum *Annuum.*

Crop	***Arabidopsis thaliana***	***Capsicum annuum***
Family	Brassicaceae	Solanaceae
Genus	Arabidopsis	Capsicum
Seed cross-section	 Muller, 2006	 Finch-Savage and Leubner-Metzger (2006).
Size	135 megabase pairs in size	3.5 Gigabase (Gb) in size
Time to germinate	1 to 2 days	7 to 10 days
Life cycle	6-8 weeks	Over than one year

5.1.2 Objectives

The objectives of this study are summarised below:

i. To observe the germination phase of *C. annuum* during imbibition with water.

ii. To observe the different morphological physical changes seed transition to germination of *C. annuum.*

iii. To define whether the genes present in *A. thaliana* for starch and germination are present or not in *C. annuum* before and after the genes have been expressed during germination.

5.2 Chili plantations in Malaysia

Malaysia's chilli planting gives farmers immense profits, because it is welcomed by the local and foreign markets. Approximately 2,900 hectares of red chillies are cultivated locally to meet local and export demand for consumption (Anim, 2017). The chillies are produced throughout the year in Malaysia, every year. However, due to flooding or the monsoon season, the cultivation of chillies decreases at the end of the year. To maximise income during the monsoon season, techniques are introduced under a glasshouse to solve the flooding problem.

Chillies are a significant crop, used as vegetables and spices in Malaysia. Malaysia's chilli plantations are grown every year, but the yield decreases at the end of the year during monsoon season. Instead of planting chillies directly in the field, they are also grown under shelter to avoid flooding and monsoon damage. The wet condition can decrease crop yield during the flooding and tend to increase fungal and disease infection.

Anthracnose is a disease caused by a species of *Colletotrichum*. *Colletotrichum* is the eighth most crucial plant pathogenic fungi (Dean *et al.*, 2012). *Colletotrichum* causes anthracnose disease. The most reliable and eco-friendly method of controlling the disease is by biological regulation, that is, the safest, simplest and cheapest way to apply crop disease control is by using a resistant cultivar. However, compared to *A. thaliana*, there are limited studies on chilli crops. After an anthracnose outbreak, post-harvest decay also occurs. Among the many other crops, anthracnose strikes chillies. As recorded by the crop yield of chillies based on anthracnose disease, up to 50% yield loss can occur (Pardeevaraporn *et al.*, 2005).

Following the rise in demand for chillies in Malaysia, MARDI takes the initiative to boost the available types of chilies. There are several local varieties, but there is not too much yield production, and the demand from the market cannot be fulfilled. There is also an absence of high quality chilli seeds available. The outside use of hybrid seeds is often used to solve this situation. Hybrid seed applications cause the local chillies to not be well established. The local source of chillies is not adequately qualified. The chillies that come from seeds of low quality appear to have late maturity. The seed's poor quality offers low yield productivity, and then they show no resistance to the disease. In addition, productivity and uniformity are also affected during the fruit production process. MARDI has come out with the latest hybrid seed variety based on this scenario, a superior variety called *MC 11*, to resolve the hybrid seed situation.

5.2.1 *Capsicum annuum* variety (MC11)

The *C. annuum* seed materials for this analysis were obtained from the MC 11 variety from the Malaysia Agricultural Research Development Institute (MARDI). Many commercial chilli varieties are commonly grown in Malaysia, but popular forms include *MC 11*, *MC 12*, *MC 4*, *Ipoh Chili* and *Kulai chilli*. Chillies are mono-crop plants, cultivated on an annual basis. The red chili, *MC 11*, has been popular since the 1980s. To solve the problem of low-quality seeds from a local crop, MARDI produces the *MC 11* variety. This variety originates from a tiny tree. Ten weeks after planting, this variety of chilli begins to yield and continue to deliver yield until the end of the season. The fruit's reliable content is high, around 20%, and is suitable for other food processes. This variety also indicates disease tolerance.

5.2.2 *Capsicum annuum* economic and worldwide use

The transformation of genes to increase the quality and quantity of chillies is part of the research into chillies. This study also aims to enhance its results. A system of Agrobacterium has been introduced for economic reasons in the processing of chilli. Hence, there have been many experiments. Chilli peppers are now commonly cultivated in subtropical and tropical areas. The global production of chillies varies depending on the country's use. The chilli pod may be used fresh or dry. Chillies are dried for preservation for long-term use. As stated by (Mejia, 1988; Sun *et al.*, 2007), the benefits of chilli are that they're used as a vegetable condiment, spice, medicine, colouring agent and vitamin source. Research has recently discovered other uses of chilli in the products because of the beneficial composition.

In *C. annuum*, the chemical compound capsaicin has a high degree of biological activity that affects the nervous, cardiovascular and digestive systems. This has been confirmed and defined by (Virus and Gebhart, 1979; Surh and Lee, 1995). Other studies also note that capsicum tissues have potential value in the treatment of medical conditions, such as the possible antimicrobial effect (Gottshall *et al.*, 1949; Bushnell *et al*,., 1950) .In addition, based on a study by Brederson *et al*. (2013), capsaicin compound used as a medication to treat disease addressed the challenges of creating TRP channel antagonists as a new generation of pain therapeutics.

Capsaicin compound is often used in pepper spray. As the capsaicin compound gives the hot effect, pepper spray can be used as an insect control in agriculture. Naturally, it is organic and does not harm the environment. When compared with other commercial chemical pesticides, it is better. The pepper part of *C. annuum* is used as a biochemical pesticide. It

serves to repel and destroy the insect. It causes damage to the insect membrane and metabolic disruption due to the capsaicin "hot and heat" taste. Previous research (Antonious *et al.*, 2007) investigated the use of hot pepper fruit extract to control cabbage looper and spider mite as an insect repellent. It showed 94% insect/pest mortality during the investigation. A further analysis (Antonious *et al*., 2009) found that the pungency level of *Capsicum chinensis* was taken from the various pepper regions grown in silty loamy soil with different capsaicin concentrations. In addition, candidates with a higher concentration of capsaicin could have health-promoting properties in agriculture, as a source of pest control agents.

5.2.3 Chemical composition of *Capsicum annuum*

Chilli's active ingredient is an alkaloid compound called capsaicin. The pungency of the Capsicum genus is caused by capsaicin. C18H27NO3 is the molecular formula of capsaicin, and its molecular weight is 305.40 g/mol. Capsaicin (8 methyl-N-vanillyl-6 no amide) is a fat-, alcohol- and oil-soluble crystalline, lipophilic, colourless and odourless alkaloid (Cao *et al.*, 2015). The latest research shows that the Capsicum capsaicin compound has an anti-cancer effect in cancer models and the mechanisms behind it (Cao *et al.*, 2015).

When it comes into contact, capsaicin can create the sensation of burning in any tissue, resulting in a sensory neuron interaction. Research conducted by Yaldiz *et al*. (2010) found that capsaicin levels vary according to the Capsicum species. The capsaicin content is influenced by the harvesting time and various forms of drying methods. In the chilli placenta tissue and internal membranes, capsaicin is abundant. The seed itself produces no capsaicin. Capsaicin stimulates a pain receptor when swallowed, and the usual evolutionary function is to alert the body to any harmful physical heat. TRPV1 (transient receptor of potential cation channel subfamily V member 1) is the temperature sensation receptor, and

is also known as the capsaicin receptor and the vanilloid receptor 1. TRPV1's essential functions are to sense body temperature and provide a feeling of heat and scalding discomfort.

5.3 The development of the *Capsicum annuum* plant

There are several stages in which seeds can become a crop and grow as a new plant. For each stage, starting from seed until becoming a fruiting plant, distinct times are taken.

5.3.1 Germination of *Capsicum annuum*

Seed germination is not the same for every crop. Some seeds germinate more quickly, and some seeds germinate more slowly, depending on the crop itself. The normal practice for *C. annuum* seeds is to treat the seeds with anti-fungus to kill the fungus infection that may hinder the germination of the seed. In initiating seed germination, warm temperature plays a critical role. Farmers usually use black plastics or mulches to keep the soil wet. It takes about ten days for normal *C. annuum* seeds to germinate, five times slower than *A. thaliana*. The emergence of the radicles from the seed coat is the end of germination, and the seedling starts to develop.

5.3.1.1 *Capsicum annuum* requirements for germination

There are some requirements for chilli to germinate. The main thing to consider is the temperature. Chilli peppers need a warm state, and require 25–27°C to grow maximum chilli seeds. In the stage of seed germination, the weather plays a crucial role. They need light to start germination, like many other seeds, but for chilli, once sprouting, the seedlings need light for further growth.

The chilli seed has been developed by transplanting seedlings before moving to the field site for better survival. Compared to seeds sown directly in the field, the probability for seedling survival is high for the transplanted seedlings. Seeds are planted in the media tray with the compost for the transplant, then kept indoors. To keep warm, the top of the media is covered with mulch or black plastic.

5.3.2 Flowering

The stem elongates when germinated, and new leaves grow. When the mature stage is reached, the leaves then turn dark green. Depending on the species and variety, capsicum plants grows to a height of 2–4 ft. It begins flowering when the plant reaches maturity. The *C. annuum* produces many white flowers as a result of the self-pollinated flower. However, not all the flowers retained in the plant become the fruit because the flowers are quick to drop down and do not stay on the plant well.

5.3.3 Fruiting

Two weeks after flowering, and about 90–120 days after sowing, the plant will bear fruits. The pod is green when the chilli first begins to grow, and then the most ripened chilli will turn red, yellow, orange, brown and purple. When the pod fruit grows in size and develops, it is ready for harvesting.

5.4 Multigene family

The multigene family is identified as a group of genes from the same organism that encodes a protein with similar sequences. It is depicted in different copies and can be replicated by DNA replication.

5.4.1 Orthologues

Gene orthologues are used to find parallels and links with *A. thaliana,* within *C. annuum*. Orthologues are homologous genes; they are focused on the same genes in different organisms. Since they come from the same ancestor, different species' orthologues will typically have the same gene functions. Many plants have orthologous genes and share the same mechanism with other distinct plants. It was expected that *C. annum* and *A. thaliana* would have similar orthologues. In this analysis, genes such as phosphoglucomutase (*PGM),* starch excess 1 (*SEX1*), starch synthase (*SSI*), and *phosphoglucisomerase (PGI)* were used.

5.5 Results

The germination of *C. annuum* was present until day 18, based on the day before imbibition. The time taken for the seed to germinate is seen in the graph (Figure 5.1). Compared to *A. thaliana,* the different time taken can be seen. The cross-section of the chilli seeds was also observed. Following this analysis, the pre-and post-gene DNA was extracted from the chilli seed embryo induced germination for further PCR research. The PCR test is listed below. Also attached are the ID genes from the BLAST website.

5.5.1 Germination curve of chilli seed

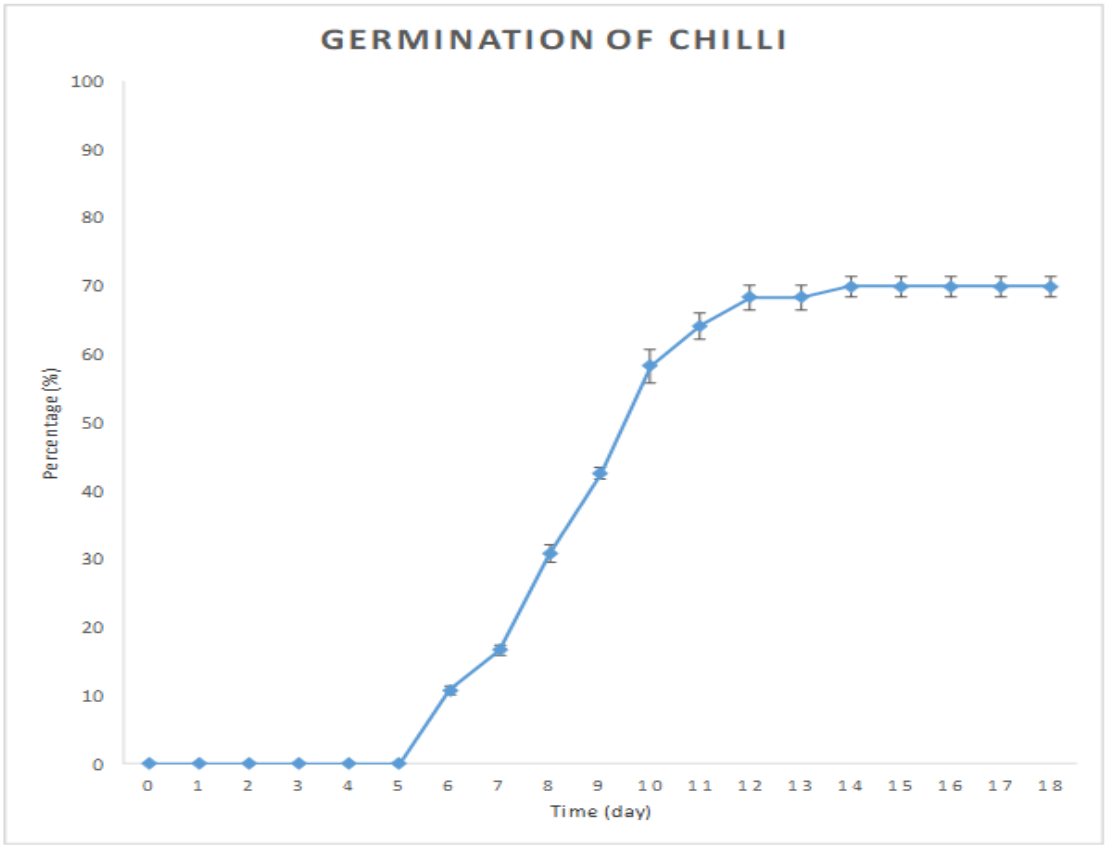


Figure 5.1: Germination curve for *Capsicum annuum* during imbibition with water.

Generally, germination will take about seven to ten days for chilli peppers. In these tests, the seed was treated with 30% bleach before soaking with water. Bleach works to destroy the seed fungus. The previous work shows that if the seed is soaked directly in water, the seed begins to germinate after ten days, so it was bleached. The germination, after imbibition, starts on day six. Based on the germination curve, the *C. annuum* seed begins to sprout on day six after imbibition. Following the curve (Figure 5.1) shows a plateau between day six and day fourteen. This means that on day fourteen, no more seeds germinate. The sprouted

seed will not last longer than two weeks in the petri dish since there were no nutrients. Only water to initiate germination was supplied.

However, it will take four to six weeks for seeds planted directly in the soil to germinate normally, if the temperature is at 4°C (Anim, 2017). The seed would germinate around the first two weeks. The chilli seeds are soaked in the water at least overnight before planting in the field. This softens the seed coat's outer layer for the easy penetration of seed germination radicles. In general, to germinate well in the field, the chilli seed needs a temperature of 23–30°C.

5.5.1.1 Morphological changes of *Capsicum annuum* from seed to seedling

Compared to *A. thaliana*, the morphological changes of *C. annum* seeds are very distinct. Compared to *A. thaliana*, the chilli seed takes a long time to germinate and takes one to two days to sprout, while for *C. annuum*, it takes about six days to germinate.

5.5.1.2 Seed dissection

The seed is soaked with water and then dissected. The two conditions were 24 h for imbibition and six days for imbibition at the time of imbibition. The 24 h time was selected as this is before the gene was induced, while the gene was induced for six days, based on the *C. annuum* germination curve. The seeds were then pictured under a microscope. After 24 h of imbibition and six days after imbibition, the chilli seed's cross-section was finished. From the seed cover, the chilli seed was cut horizontally. Figure 5.2 shows the un-dissected seed, the seed cross-section and the embryo removed from the endosperm.

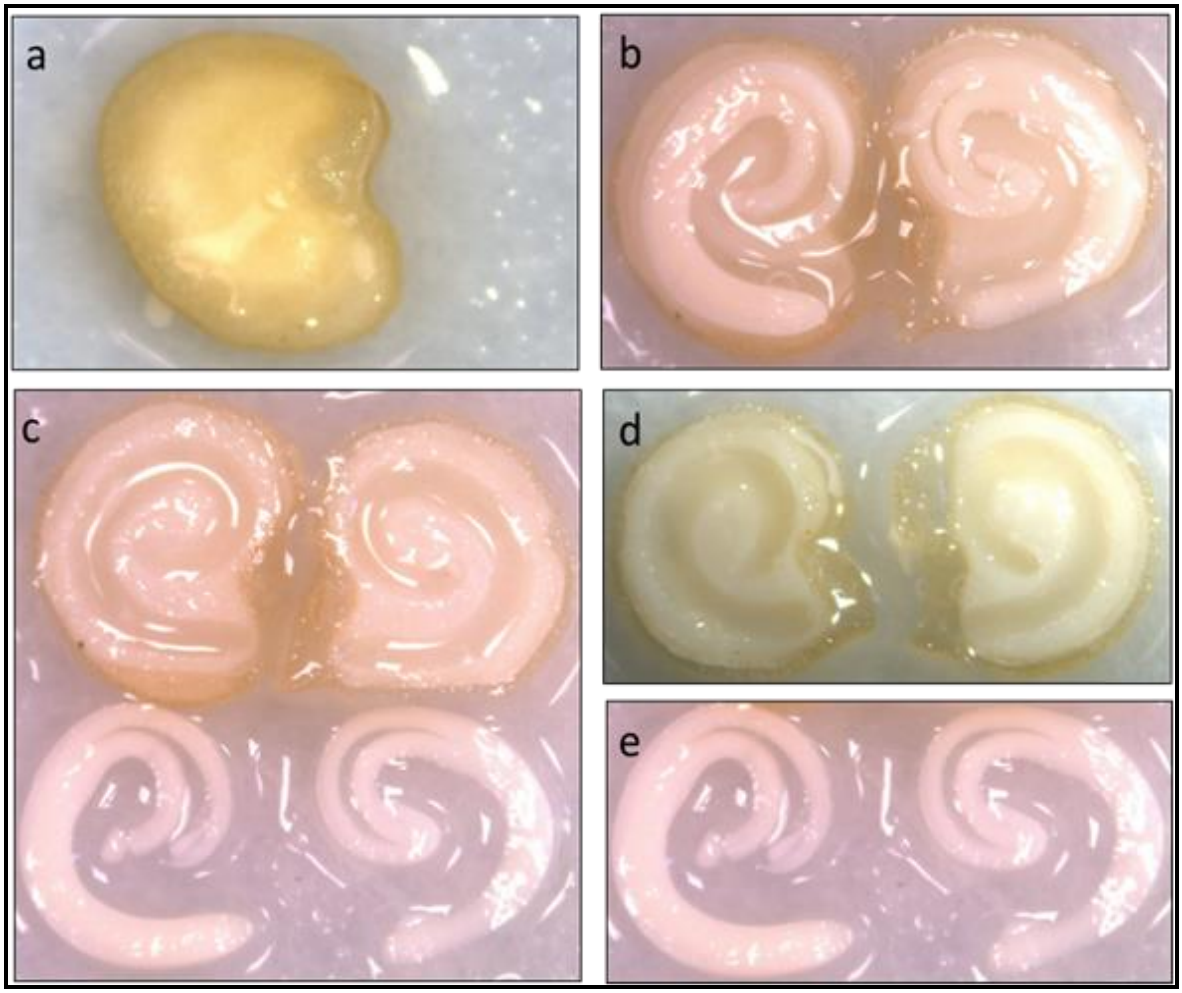


Figure 5.2:The cross-section of *Capsicum annuum* showing a: the un-dissected seed; b: cross-section of the seed; c: the embryo removed from the endosperm; d: the embryo.

5.5.2 ID similarities of *Capsicum annuum* and *Arabidopsis thaliana*

Both *Capsicum annuum* and *Arabidopsis thaliana* have a similar ID. The ID similarities are shown in the screenshot below (Figures 5.3 – 5.6):

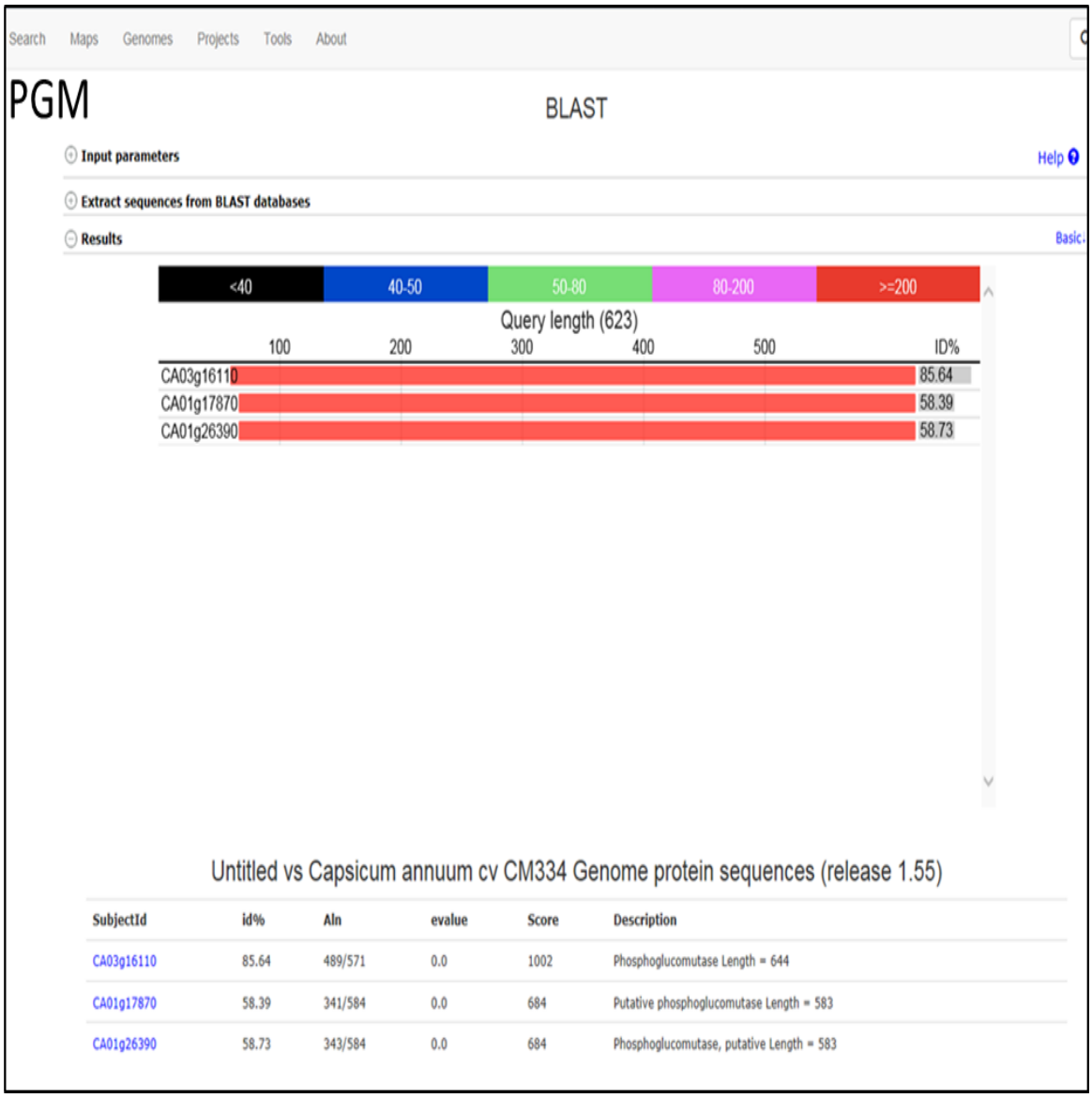


Figure 5.3: Screenshot of the similarity matches founded for gene *PGM*.

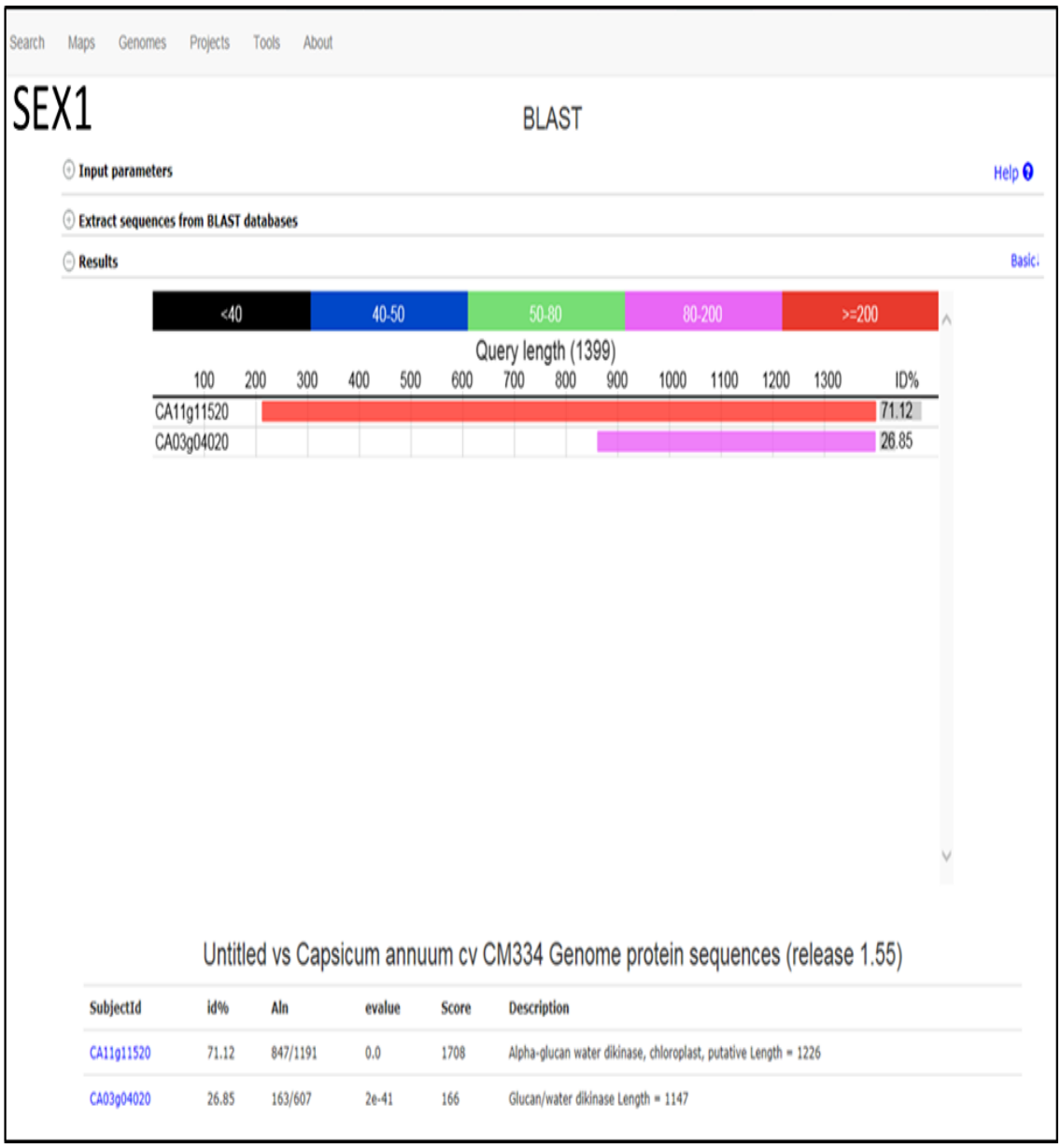


Figure 5.4: Screenshot of the similarity matches found for gene *SEX1*.

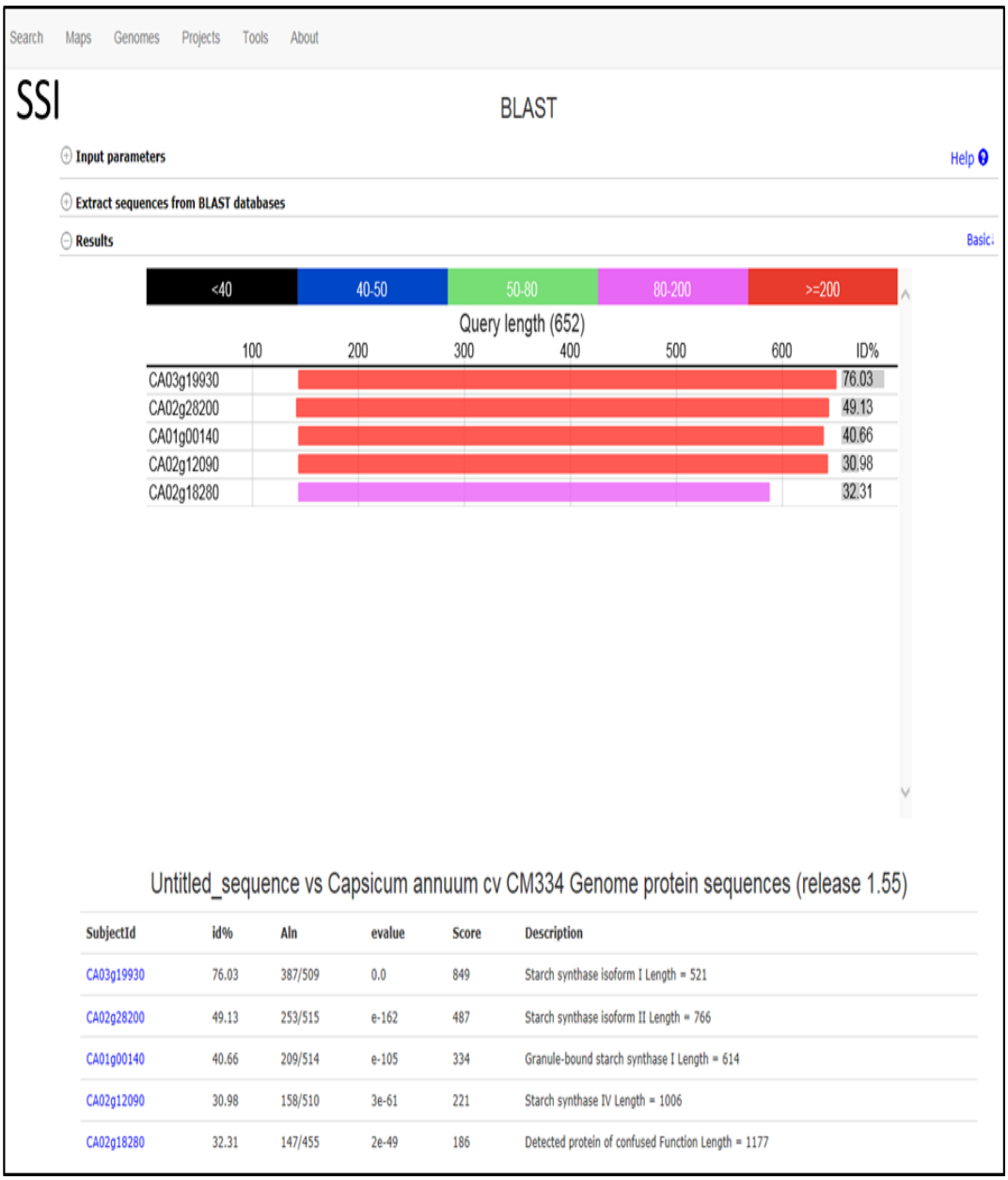


Figure 5.5: Screenshot of the similarity matches found for gene *SSI*.

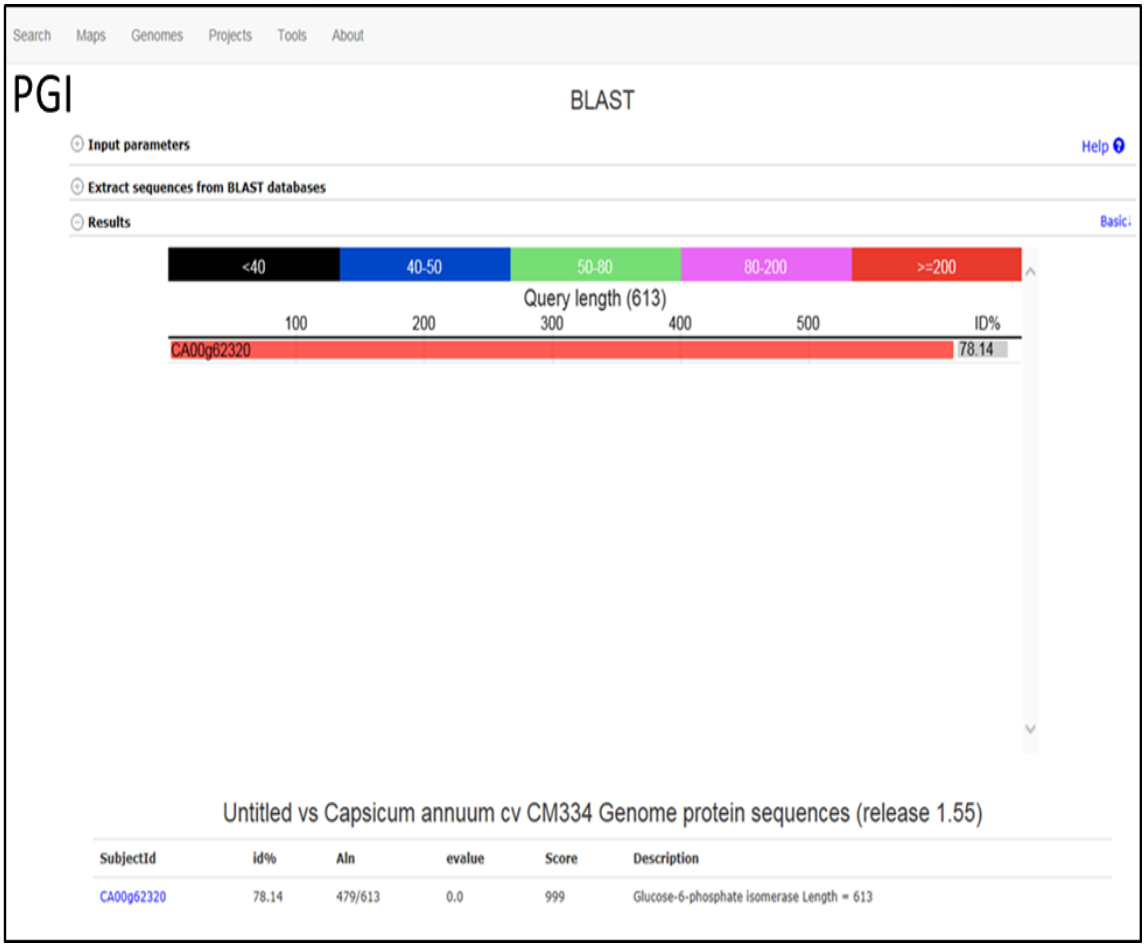


Figure 5.6: Screenshot of the similarity matches found for gene *PGI*

The similarity ID of these genes was from the BLAST tool. Then the primers were created for each gene, as shown in Table 5.2.

Table 5.2: The primers created from the BLAST tool using the ID name.

Primers	ID Name	Size	Sequences
PGM 1	CA03g16110	200	Forward: TGACTACGAGGCAAGCACATT Reverse: AAACAAACCGAACCCCCTGT
PGM 2	CA01g17870	223	Forward: ATCATTCGCCAACACTGGGT Reverse: AGACCGAACCATCAATGGGG
PGM 3	CA01g26390	633	Forward: TGGTTCAGTCTCGCAGCATC Reverse: TAGTAGCACCCTCTGAGCCA
SEX1	CA11g11520	312	Forward: TGGGAAGGTAGTTTGCTTTGC Reverse: GGAATTCCCACCCAGGAAGG
SSI 1	CA03g19930	421	Forward: GGGCTATGGTCCACTGCTTT Reverse: CTCCATACCATTCCGGAGGC
SSI 2	CA02g28200	293	Forward: TTTTGGTGGCTGCAGAATGC Reverse: CACACGGTTCCCTCCGTAAA
SSI 3	CA01g00140	726	Forward: TAAACGTGGGGTTGATCGCA Reverse: CTGTCAACAGGCAAGCCAAC
SSI 4	CA02g12090	624	Forward: TGCCATGATTGGCAAACAGC Reverse: ATGTGGCACTGGGCTAGAAC
PGI	CA02g00010	155	Forward: TTTCCGGTTGCGTAAAGGCT Reverse: TTCCCCGAGTCTGCTGATTG
ACTIN7 1	CA03g11540	235	Forward: AACGGAGGCACCCCTTAATC Reverse: ACGACCAGCAAGGTCCAAAC
ACTIN7 2	CA04g06670	885	Forward: AAGCTCAGTCGAAGAGAGGTATTCT Reverse: ATGTGCTGAGGGATGCAAGG

5.5.2.1 PCR imaging

The PCR product imaging showed two distinct conditions of the seed which is before and after the germination. The conditions are 24 HAI and 6 DAY. Compared to having the gene present or not, the imaging before and after the gene caused it. Separately, different genes were present. *Actin 7* was selected as a control.

5.5.2.1.1 *PGMi*

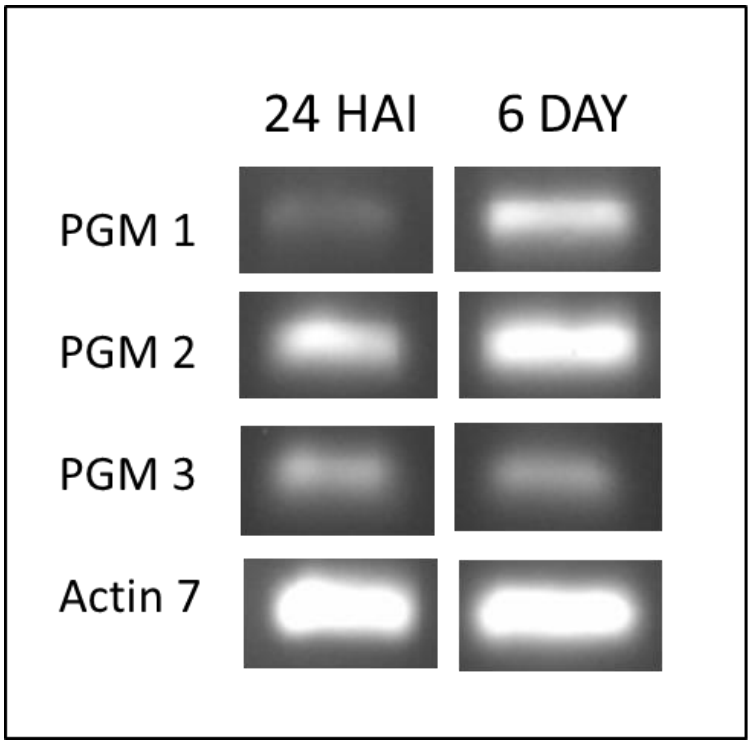


Figure 5.7: *PGM* product imaging at 24 HAI (before the gene was induced) and at 6 DAY (after the gene was induced).

Figure 5.7 refers to the agarose gel electrophoresis (1%) of the PCR product for the orthologous genes from Chilli and Arabidopsis showing amplification of 24 HAI (before gene induction) and 6 DAY after gene induction. *Actin7* was used in the experiment to monitor the size (235 bp). The amplified items showed a scale of 200 bp, 223 bp and 98 bp for *PGM 1*, *PGM 2* and *PGM 3*, respectively.

5.5.2.1.2 *SEX1i*

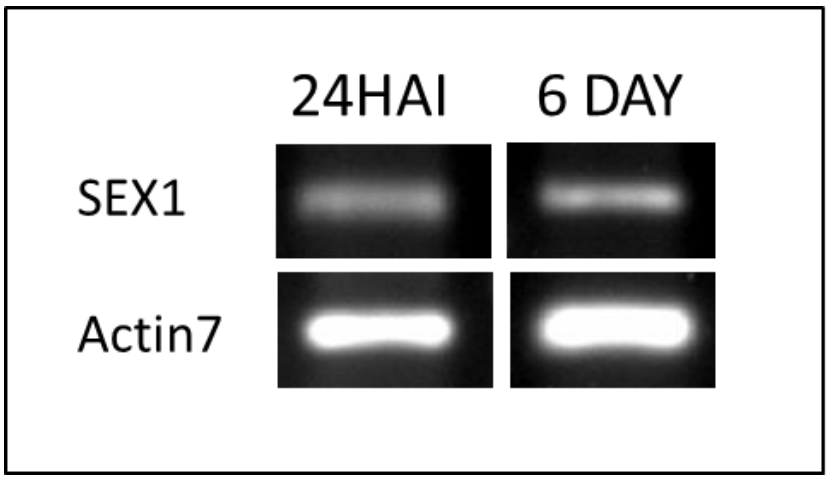


Figure 5.8: *SEX1* product imaging at 24 HAI (before the gene was induced) and 6 DAY (after the gene was induced).

Figure 5.8 shows the PCR product imaged with *Actin7* used as a control at 235 bp after gel electrophoresis, whereas the amplified gene, *SEX1*, was 312 bp.

5.5.2.1.3 *SSI*

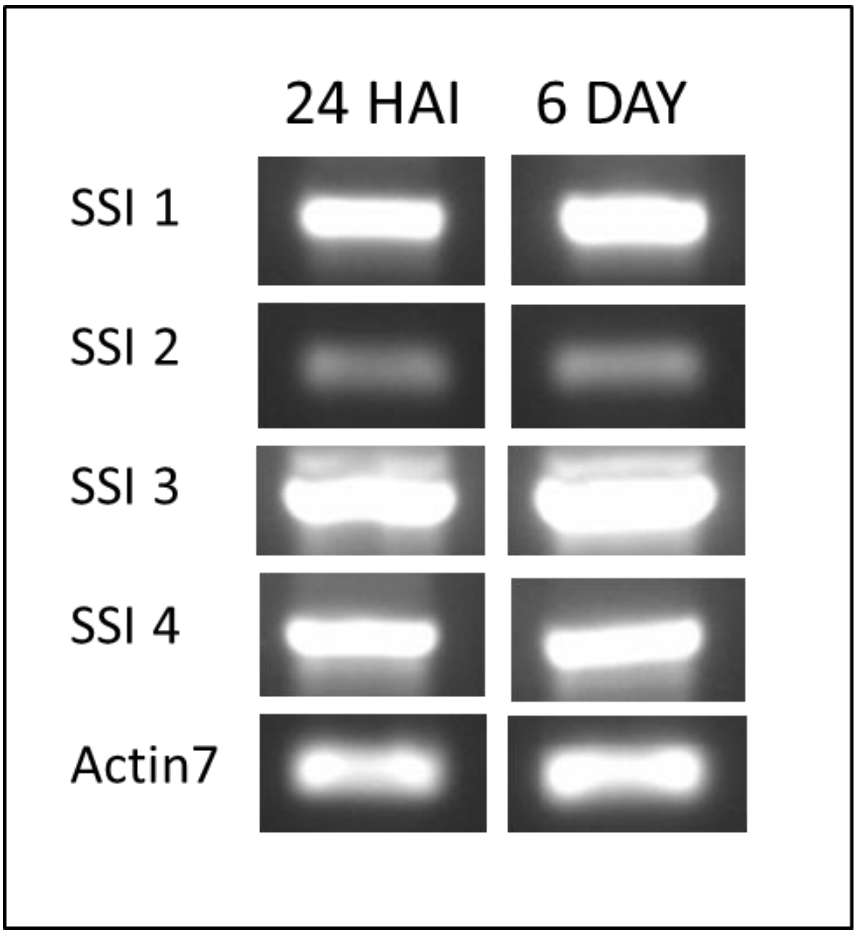


Figure 5.9: *SSI* product imaging at 24 HAI (before the gene was induced), and 6 DAY (after the gene was induced).

Figures 5.9. The *SSI* gene PCR product has been multiplied to a different scale. *Actin 7* was the control for this gel electrophoresis, and it was amplified at 235 bp. The amplified *SSI 1*, *SSI 2*, *SSI 3* and *SSI 4* gene products range in size from 420 bp, 293 bp, 726 bp and 624 bp, respectively.

5.5.2.1.4 *PGI*

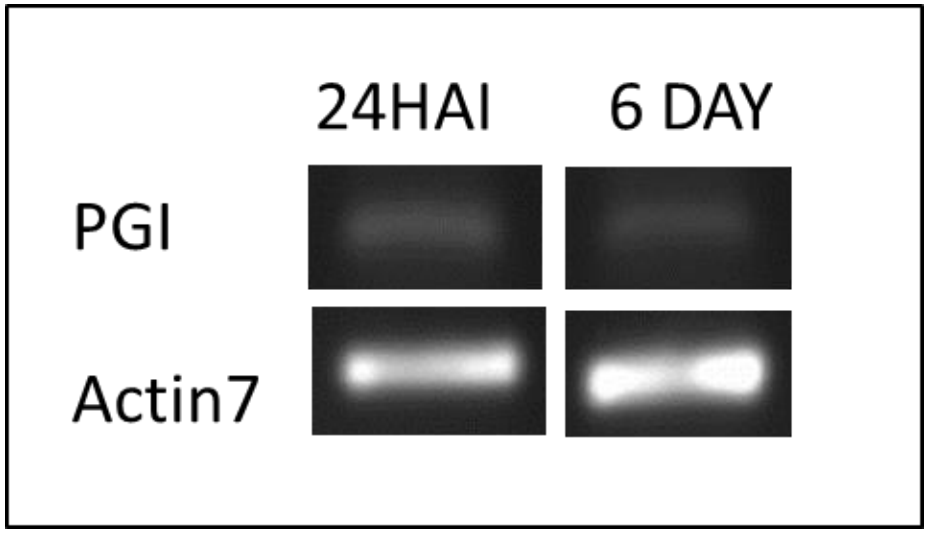


Figure 5.10*: PGI* product imaging at 24 HAI (before the gene was induced), and 6 DAY (after the gene was induced)

Figure 5.10 shows the PCR product imaged with *Actin 7* as a control at 235 bp after gel electrophoresis, whereas the amplified gene, *PGI*, was 155 bp for amplified gene.

5.6 Discussion

Some observations for the germination of *C. annum*, which differed from the seed germination of *A. thaliana*, are accompanied by the data. On the other hand, the PCR reaction of the gene showed some gene similarities with *A. thaliana* present in *C. annuum.*

5.6.1 Germination curve of chilli seeds

C. annuum seeds were treated with 10% bleach to stop fungi during the seeds' germination. The presence of fungi will disrupt the germination of the seeds and then reduce the rate of germination. The treated seeds were then imbibed with water, with 30 seeds per plate on three filter sheets, and four replications. The imbibed seeds were put in the room for light

growth. Germination ranking was monitored every day. The seeds did not show any evidence of germination until day five. Then on day six, the seeds began to germinate, and was ~10.83% grown. When the radicle protrusion from the seed starts, germination begins. The seed began to germinate on day six. On day 13, the graph showed deflation until day 18. From day 13 onwards, no further germination occurred. The time caused by genes can be estimated based on the germination curve (Figure 5.1). Following the germination curve, it can be inferred that the early period before affecting the genes was 24 h after imbibition. Whereas the late duration after induction of the gene was five to six days after imbibition. It is approximately four times slower than the germination of *A. thaliana* following the *C. annuum* germination graph.

5.6.2 PCR

The unique primers were designed to amplify the target genes in the chilli seeds during the PCR reaction. Orthologues are used to discover the similarity to another organism; the gene from *A. thaliana* contrasted with *C. annum*. To acquire the embryos, RNA samples were obtained from the dissected seed. The two other points were 24 HAI (before induction of the gene) and 6 DAY (after the gene has been induced). To eradicate genomic DNA contamination, both distinct RNA conditions were treated with DNase 1 to ensure that pure DNA is ultimately obtained. The cDNA primer size was obtained after running gel electrophoresis with 1% agarose gel. Following gel electrophoresis, the gels were imaged under UV light to see the PCR product's band size using Image J software. It showed that the expression of the DNA band appeared under both conditions.

This means that both the *A. thaliana* and *C. annuum* genes *PGM, SEX1, SSI* and *PGI* were present and have similarities during the seeds' germination.

The *PGM, SEX1, SSI* and *PGI* genes were used in this research to continue project two (Starch and Germination). Following the previous article, this is a detailed study of the use of the *C. annuum* crop species widely grown in Malaysia. The genes were selected based on project two, and the similarities between *A. thaliana* and *C. annum* continue to be identified. Four, three, one and one were created according to the BLAST and SOL Genomics Networks for *SSI, PGM, SEX* and *PGI* gene similarities.

During the germination of *A.thaliana*, genes associated with starch metabolism are turned on. This chapter investigates whether genes related to starch metabolism were also induced in the chilli seed embryo during germination. The genes are present, and chilli has expressed them. The way genes in Arabidopsis and Chilli are regulated is not the same.

5.7 Conclusions

The seed germination of *C. annum* takes ~6 days, whereas *A. thaliana* take just 1.5–2 days to germinate. The germination time for capsicum is three times lower than Arabidopsis. As a conclusion of this project, I may infer that the multigene family of *A. thaliana* and *C. annuum* resemble the genes associated with starch metabolism. The genes were induced during the germination of seeds for both conditions. After water imbibition, the germination of *C. annuum* seeds began on day six. This study showed that the early gene induced HAI at 24, while the late gene induced HAI at day 6 after imbibition. For both conditions, the most apparent band was present. They showed that the gene was present in *C. annuum* in *A. thaliana*, but the way the gene is regulated is not the same.

CHAPTER 6

CONCLUDING REMARKS

6.1 Overall conclusions

As a consequence of the spatial-temporal distribution of gene expression in Arabidopsis, gene expression variations at different times and locations for other genes studied were involved. During the analysis, gene behaviour showed a different pattern depending on the time and location. The gene expression pathway patterns are distinct, starting from 1 h of imbibition to the seedling stage. Plant hormones also lead to the transfer of seeds to seedlings. In promoting seed dormancy, the ABA hormones work. During the seed process, the ABA level was high, preventing germination until a seed was given a suitable germination condition. When the seed begins to germinate, the GA hormones are involved, and the seed's germination is initiated. The radicle extends, and the germination results in the protrusion of the radicle into the seed coat. In completing the germination of seeds, ABA and GA communicate with one another. 1 HAI, 3 HAI, 6 HAI, 18 HAI, 24 HAI, JTR, STR, JG and seedling were included in the research analysis at various points, offering a different gene expression pattern.

The key structure in the plant is the cell wall. The cell wall defines the cell form and becomes the shield since it consists of pectin and cellulose fibre (Cosgrove, 1997). It works by manipulating the size of the elements. Owing to the matrix polymer's rearrangement, the cell wall can be extended and increased in dimension. Recent studies have shown how the cell wall develops and expands in response to the CWRE (cell wall remodelling enzyme). The gene expression is different, and for various points, the activities within the seed are different. In addition, the plot of the 2D embryo model shows the gene expression of the

ABA, GA and Expansin groups. Various colours were used to plot the gene activity where the genes were expressed.

In addition, further starch and germination studies have been carried out on *A. thaliana* as a continuation of the seed transformation to seedling project. During the seed's transformation to seedling, the starch GUS reporter of the seed was used to examine the gene expression. The seed lines of the starch GUS reporter were soaked with water and dissected at that particular time, then the dissected seed was imbibed at 20% PEG for five days. For each gene, the GUS expression starch reporter lines displayed a different GUS staining pattern that was depicted in the 2D embryo model. The different colours represented the expression of the gene induced in the model. Then, for other purposes of the report, the analysis continued with the additional seed stock.

At the molecular level, transgenic seeds of the oestrogen-inducible system from the various *PGMi, SEX1i, BAM* and *AAM* constructs were tested to confirm the level of induction of amirNA cDNA present in the gene. The RNAs were extracted from the seedling to undergo RT PCR. As a result, it was inferred that *PGMi 2-4, PGMi 3-3, SEX1i 3-6, SEX1i 7-5, BAM 1-3, BAM 2-3* and *AAM 2-4* were the best level of induction for RT PCR. Phenotyping was based on the selected line from the RT PCR production after these findings. The phenotyping test of the transgenic seed of oestrogen-inducible seeds from different constructs was used to induce gene expression selected by PEG and NaCl treatment during germination.

A. thaliana is an oilseed. Oil is reserved for use by plants as energy storage. Oil has been mobilised and converted into sugar during the germination of Arabidopsis seeds. The conversion into sugar is achieved via the glyoxylate cycle and gluconeogenesis. Therefore,

by converting glucose (simple sugar) into starch, starch synthesis regulates cell turgor (complex carbohydrates) osmotically. The sugar content will determine the osmotic effect of water absorption on the PEG and NaCl treatment during seed germination. The drought-tolerance of the transgenic seeds was researched, and PEG solution were used to establish the drought condition. In this analysis, NaCl was used to generate the salinity state for seeds during germination. The stress circumstances produced a real environment to know the transgenic seeds capacity to survive in the field. According to the pre-tested PEG experiment, 20.5% PEG was ideal for seed germination during drought stress and 200 mM NaCl was better for seed germination under salinity stress.

This research was continued using the same genes applied to the crop plant, *C. annuum*, to expand the starch and germination experiment results. The *A. thaliana* gene locus is used for the *PGM* (phosphoglucomutase), *SEX1* (starch excess 1), *SSI* (starch synthase) and *PGI* (phosphoglucoisomerase) genes. It also used the SOL Genomic Network's genes to find similarities. Using BLAST, identical proteins for each gene were used to find the homologues in *C. annuum*. With the gene similarity to the cDNA sequence, the gene ID names were present; therefore, the ID names and cDNA sequences were used to build the primers to run the RT PCR. According to the BLAST and SOL Genomic Networks, many similarities were identified and used for the analysis. The gene similarities were present and can be inferred based on the outcome of the PCR. The DNA band expression exists for both conditions, dependent on the RT PCR outcome; at 24 HAI and six days of imbibition. Therefore, it can be concluded that both *A. thaliana* and *C. annuum* expression of *PGM, SEX1, SSI* and *PGI* were present and showed similarities during seed germination. However, in *A. thaliana,* the genes' methods were regulated, and *C. annuum* was different.

6.2 Contribution to this research

Based on this analysis, some contributions during the transition from seed to seedling for these research studies are summarised, such as finding the time series of gene expression for the GUS staining for the various related genes. In the beginning, before germination, specific genes were expressed differently and then behaved differently after germination. The embryo of the 2D model illustrates the different time-based gene expression behaviour. The new knowledge gained from this research is that as the seed germination and seedling transition physically changes, various morphological changes occur for development. Then the gene activity during germination can be understood for any shift that shows distinct gene expression.

The imbibition of the seed for five days in 19% PEG is the same as for two days of the seed imbibing water. The PEG slowed down the seed germination and gene expression activities. Starch and germination studies have shown the effect of salt and drought on germination. Seed germination can be hindered by excessive dryness and salinity, thereby leading to loss due to un-germination, or the seedling may not survive in the stress state. According to these tests, the transgenic plant seeds of *PGMi, SEX1, AAM* and *BAM* show a different effect on PEG and NaCl due to the phenotyping effect.

6.3 Recommendations for further work

As for my next suggestion, given additional time for the extent of the analysis, I would like to divide the seed germination time into three stages: the pre-germination phase, the germination phase and the post-germination phase. Then, for each time point, the distinct gene expression activity will be observed. I would also like to use software to represent the gene expression model and its operation. I can discern the time phase and their actions via these methods.

I would like to observe and assess the starch abundance in the embryo after the starch and germination analysis using confocal imaging before and after stress treatment to see the differentiation of starch present in the cell. Therefore, before and after the stress therapy, I can assume an impact or no change in the starch component. I want to generate more tension for the phenotyping effect to see the seed's ability to germinate and to know the germinated seeds' ability to become seedlings, and then grow into a plant under stress.

Of the different crops commonly grown in Malaysia, brassica crops with a short life cycle are fitting for the analysis. Some of the plants, e.g. *Brassica juncea Ipomoea aquatica* (water spinach) and many other kinds of leafy vegetables, have a short germination period. There may be the same gene and functional similarities in the various crops.

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