

MANIPULATION OF AGRONOMIC TRAITS IN BARLEY AND LILY FOR CROP IMPROVMENT

Jamal Mahmood Faraj

BSc (Horticulture Department)

MSc (Plant Breeding)

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Division of Plant and Crop Sciences

School of Biosciences, University of Nottingham

Sutton Bonington Campus, LE12 5RD

Leicestershire United Kingdom

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ABSTRACT

Genetic manipulation offers opportunities to improve ornamental and agricultural crops, which can be used to increase their economic value, either by targeting specific traits or via general improvements to yield. Such improvements could have important impacts on the world economy, either by increased food production or via general economic development. This research has focused on the manipulation of genes aimed at improving agronomic traits in both Lily (*Lilium)* and barley (*Hordeum vulgare L*.) The traits targeted have been pollen development, with the aim of controlling fertility for hybrid breeding, and delayed senescence.

These transgenic approaches were adopted in *Lilium* to try to generate plants with altered fertility and senescence pathways. These approaches used promoters targeting expression to specific organs and developmental stages, which were tested by the use of GUS reporter genes. A range of constructs were therefore prepared for their future application to control gene expression in *Lilium* and *Arabidopsis*

The *Arabidopsis DYSFUNCTIONAL TAPETUM* 1 *(DYT1)* transcription factor is expressed in the tapetum and is vital for regulation of tapetum development and pollen viability. Extending this information into cereal crops, such as barley, offers a potential mechanism for understanding the process of pollen development, and to control fertility to facilitate hybrid seed production. In this work, barley male sterile lines were obtained by over-expression of the barley *HvDYT1* gene, which is proposed as the ortholog of the *Arabidopsis DYT1* gene. These lines showed impaired pollen formation without affecting the development of the whole plant. This work demonstrates the role of *HvDYT1* in barley pollen development and offers the potential for future control of fertility for hybrid seed production and to produce genetically engineered plants with controlled fertility.

Agricultural production of many economically important crops, fruit and vegetables are impacted by significant economical losses associated with early senescence. Delayed senescence is a key target for manipulation since it offers the potential for prolonged growth for increased yield, or via prolonged quality, as in the cut flower industry. Molecular studies of the genes involved in cytokinin metabolism and signalling, particularly altered expression of the *ISOPENTENYLTRANSFERASE (IPT)* genes, have been used as approaches to modify senescence. In this work, transgenic barley plants were produced that contained the *Arabidopsis IPT4* gene under the control of the Ubi1 promoter. These were assessed for the impact on growth and senescence progression.

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DEDICATED TO:

To soul my father, he was pass away when I was 6 years old.

My Lover Roshna, My beloved daughter Lily.

My lovely families, particularly my mother that who spent all her life for us.

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LIST OF ABBREVIATIONS

µg	microgram
µg	microgram per milliliter
µl	microliter
bp	base pair
cDNA	complementary Deoxyribonucleic acid
cm	centimeter
CTAB	cetyl-trimethylammonium bromide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
Fig	figure
GFP	green fluorescent protein
GUS	β-glucuronidase
HL	hipper lader1
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT-PCR	reverse transcription -polymerase chain reaction
TBE	tris, borate EDTA
v/v	volume to volume ratio
w/v	weight to volume radi

CHPTER 1: GENERAL INTRODUCTION

1.1. APPLICATION OF BIOTECHNOLOGY FOR CROP AND HORTICULTURAL IMPROVEMENT.

Agriculture biotechnology can be defined as collection of scientific techniques that has been used to improve plant or animals, based on DNA molecular techniques (Herdt, 2006). Plant tissue culture, genetic engineering and plant molecular markers are three approaches in plant biotechnology that have been utilized extensively for crop improvement (Herdt, 2006).

Plant tissue culture is the first application that involves specific techniques for regeneration of plants in the laboratory from disease-free plant parts and mass propagation for plant transformation. The second application of plant biotechnological tools is genetic engineering (GE) that uses gene isolation and cloning to produce transgenic plants and to transfer genes from one organism into another. Finally, molecular markers can improve the efficiency of selection and breeding of varieties (Herdt, 2006).

Recently, genetic manipulation has been developed for economically important crops, to improve traits related to food and horticulture production and other human necessities (Subburaj *et al.*, 2016). Economically important plants such as rice, wheat, barley and maize, vegetables, fruits and ornamental plants have been cultivated since the end of 18th Century (Xu, 2010). The Food and Agriculture Organization of the United Nations (FAO) estimates that the global population will grow from 7.4 to 9 billion by 2050, which is expected to increase the demand for food by 70% (Riaz and Gul, 2015). On the other hand, alongside the elevated

demands, increased urbanisation due to the rise of the human population also directly affects land availability for crops and has a direct effect on the global climate and environmental changes (Chandrasekaran *et al.,* 2010; Tester and Langridge, 2010). Therefore, crop improvement through biotechnology, to improve yield/production and resistance to environmental conditions is a primary target for plant breeders and biotechnologists around the world, and it is an important approach for sustainable agriculture to produce high-quality production for human consumption (Subburaj *et al.,* 2016). Plant biotechnology has a direct impact on the economic sector by increasing crop yield through various mechanisms, and by producing novel crops. Agronomically important crops and horticultural plants are critical for economic development in the agriculture sector and will have important impacts upon life and future generations around the world (Chandrasekaran *et al.,* 2010; Tester and Langridge, 2010).

Agronomically important traits, including altered fertility (flower, anther and pollen development), longevity (delayed senescence), increased yield and yield quality, resistance to pathogens and environmental stress, are being targeted by biotechnology and selective breeding (Tester and Langridge, 2010). The role of reproduction in global food security is being addressed through control of male reproduction that can provide breeding tools to generate hybrids with increased yield (hybrid vigour). For instance, hybrid crops can produce 15-50% higher yields than inbred varieties (Tester and Langridge, 2010) and therefore the use of hybrid crops particularly in maize and rice is economic beneficial for global crop production. Thus, hybrid breeding contributes significantly to food supply in the

world (Tester and Langridge, 2010). Barley is an important temperate cereal crop, but also serves as a good cereal model due to its simple genome characteristics; it is diploid with a relatively small genome that can be transformed. As such it can be used to help understating and extend knowledge to control fertility to facilitated hybrid seed production.

A key horticultural and ornamental plant that has been used to investigate manipulation of agronomically important traits is lily, which is one of the most important ornamental cut flowers in the word (Núñez de Cáceres González, 2013). Lily pollen can trigger allergic in pets and humans, and is undesirable in the cut flower industry due to the mess and staining caused by the pollen (Stokes and Forrester, 2004; Bright *et al.*, 2009 and Thompson, 2012). Therefore, prevention of pollen release is a key target for lily improvement. Altering these features in Lily is difficult and genetic manipulation can play a significant role to help such developments, for example in the control of pollen release without affecting flower morphology. Additional key targets for commercial development in horticulture crops are increasing flower longevity and delaying senescence, alongside altered plant morphology. These are also major goals for many ornamental plants, fruit and vegetables that have significant economic losses due to senescence, which occurs during many developmental stages and growth in the plant (Nooden *et al.*, 1997 and Buchanan-Wollaston, 2003). Therefore, to reduce damage and losses in agricultural production, genetic manipulation is being increasingly used as a possible option. For example, many of the desired ornamental traits and marketable qualities in lily plants can potentially be developed by genetic improvement such as

altering the progression of flower senescence, developing new flower colours and increasing resistance to pathogens by genetic engineering (Chandler and Lu 2005; Nooden *et al.*, 1997 and Grassoti and Gimelli, 2011).

1.2 CEREALS AND BARLEY CULTIVATION

Barley (*Hordeum vulgare L.)* is an annual crop from the grass family and is the fourth most agronomical important feed crop worldwide, after rice, wheat and maize (Schulte *et al.*, 2009; Mrizova *et al.*, 2014). Archaeological findings have shown that barley was being cultivated in the Fertile Crescent about 10000 years ago. Reports indicate that barley was first domesticated around 8000 BC in the Himalayas (Badr *et al.*, 2000). However, the precise origin of barley is debatable, but Hockett, (2000) suggests that the originators may have come from around the area of Egypt, the Near East or Tibet and Ethiopia.

Barley is among the earliest domesticated crops used for beer production, animal feed and human consumption (Mrizova *et al.*, 2014). Barley can be differentiated into two kinds, two-row and six-row barley; two-row barley consists of three spikelets per side where only the central one is fertile, whilst in the six row type the three spikelets per side are fertile. Two-row barley is normally used for malting and human feeding, whereas the six-row varieties are used for animal feed due to its higher protein contain (Bowden *et al.*, 2010). Barely can be divided into winter and spring types. Winter barley planted in the late autumn, tends to be higher yielding and poorer quality than spring, and it is used for animal feed and is normally harvested in July, whereas the spring type is planted in spring and then harvested in summer (Bowden *et al.*, 2010).

Barley is a diploid (2n = 14) with a relatively large genome of 5.1 GB and small number of chromosomes, which are relatively large (6-8µm) (Mayer *et al.*, 2012). It has short life cycle making barley a genetic model for much research in the area of small grain cereals. In addition, the barley genome has been sequenced (The International Barley Genome Sequencing Consortium., 2010; Matsumoto *et al.*, 2011; Christiansen *et al.*, 2011and Mayer *et al.*, 2012).

Cereal production has increased dramatically because of the Green Revolution (Pingali, 2012), which resulted in an unprecedented improvement in agricultural production from the 1960s. This represents a total increment in cereal production from 1 to 2.6 billion tonnes in 2011 (FAO, 2013). However, The Food and Agriculture Organization of the United Nations (FAO) estimates that worldwide population will grow from 7.4 to 9 billion by 2050, which is expected to increase the demand for food by 70% (Riaz and Gul, 2015), therefore it has been estimated that an increase of 70% more food, (44 million metric tons per year) is required by 2050 (FAO, 2009; Tester and Langridge, 2010). Alston *et al.*, (2009) reported that only a liner average rate of increase (32 million metric tons per year) was achieved in global food production, by agronomic improvement using molecular breeding. Tester and Langridge, (2010) showed that cereal production had risen from 877 million metric tons in 1961 to 2351 million metric tons in 2007 (Fig 1.1), but indicated that production will need to rise to over 4000 million metric tons by 2050 (FAO, 2006; Tester and Langridge, 2010), an increase of 37% that is sustained for about 40 years, will be needed to meet the predicted targets of increasing populations.

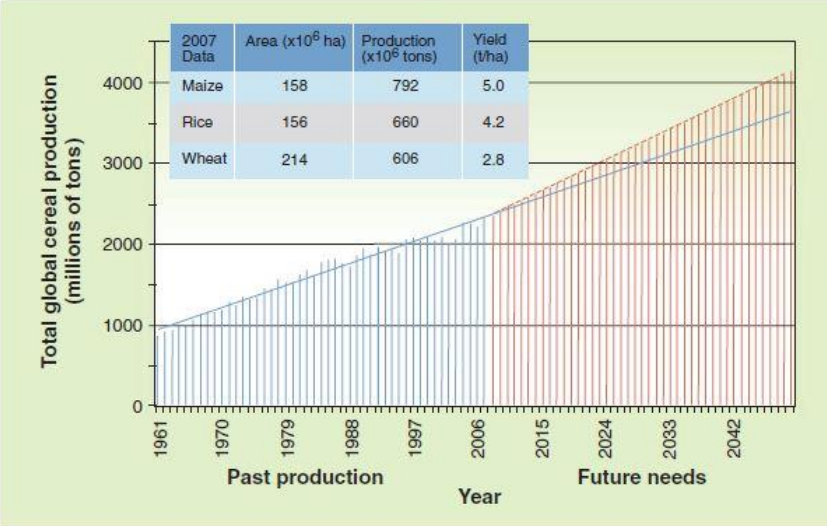


Figure 1. 1: Cereal production.

Production has risen from 877 million metric tons in 1961 to 2351 million metric tons in 2007 (Blue). However, to meet predicted demands, production will need to rise to over 4000 million metric tons by 2050 (red). The inset table shows the 2007 data for the three major cereals. (taken from FAO, 2006; Tester and Langridge, 2010).

However, achieving this scale of increase in global food production is unprecedented, therefore altered methods for agronomic process and improvement of crops are required (Tester and Langridge, 2010). In addition, to increasing crop production and animal productivity, research needs to be applied to new technological development and knowledge, but also to reducing environmental impact such as reducing greenhouse gas emissions, improving water efficiency and energy (Qaim, 2016). Therefore, new technologies can be developed via breeding and genetic manipulation to introduce a range of desirable traits for increase production and improving food security (Tester and Langridge, 2010). (Fig 1.2) shows the increase yields of some major cereal crops between 1960 to 2015 that plant breeding has been significantly affected (Qaim, 2016).

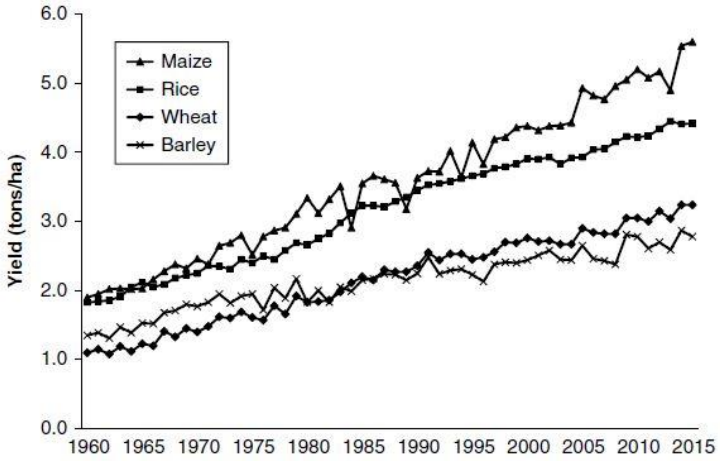


Figure 1. 2: Increasing and development of major cereals yield worldwide from 1960 to 2015 (taken from Qaim, 2016).

As mention previously, production of high-quality of food must be increased to feed the increasing human population, but this will be particularly challenging in face of climate change. It is also evident that developing countries are likely to experience the greatest increase in demand. (FAO, 2006; James, 2010 and Tester and Langridge, 2010) (Fig 1.3). Recently, genetic engineering of plants is starting to provide solutions to agricultural problems and this is likely to increase over the coming years, particularly given the gene editing approaches that are being developed.

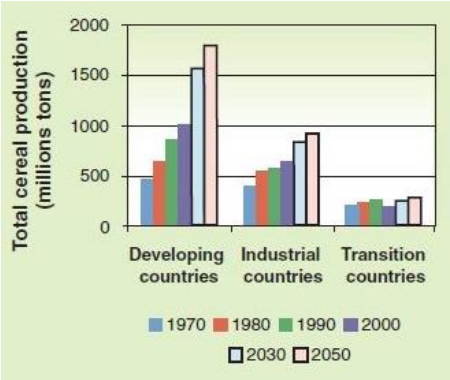


Figure 1. 3: The greatest demand for yield increases will be from countries in the developing world (taken from Tester and Langridge, 2010).

The FAO defines Food Security as “the situation that exists when all people, at all time, have physical, social, and economic access to sufficient, safe and nutritious food that meets their dietary needs and food preference for an active and healthy life” (FAO, 2002). Food Security was split into four topics by the World Food Summit in 1996, which are availability, access, stability and utilization. The first is availability that relates to quality and quantity, alongside the diversity of food. Second, is Access that includes physical access and infrastructure. Third, is Stability that divided into two types: (1) factors linked to food security risks, (2) focuses on the incidence of shocks. Finally, utilization is determined by the ability to utilize food as well as the outcomes of poor utilization (thus defines safe and healthy utilization of food) (FAO, 2014).

Gregory *et al.,* (2005) demonstrated that Food Security is diminished when food systems are affected by a range of factors: such as climate change and other agents of environmental change for instance conflict, disease (e.g. HIV/AIDS) and it may be particularly severe when these factors overlap with food production systems alongside the impact of urbanization and globalization.

1.3 HORTICULTURE AND THE ORNAMENTAL FLOWER INDUSTRY

One of the most traditional and popular ornamental flowers worldwide is Lily *(Lilium spp*.) (Benedito, 2004). Lilies have a high commercial value in the cut flower, pot plants and garden design industries. Numerous ornamental traits and marketable qualities can be targeted for genetic improvement in lilies, such as flower longevity, senescence and flower development and anther and pollen development (for example control pollen release without affecting flower structure). In addition, other important traits are flower colour and fragrance, because they are desirable for consumers (Chandler and Lu, 2005).

Lilium species are diploid (2n=2x=24), with a few exceptions of triploids (2n=3x=36) lilies such as *L. tigrinum* and *L. bulbiferum* (Noda, 1978; Kim *et al.,* 2006; Shahin *et al.,* 2011). The genome of *Lilium* comprises ~34 billion base pairs (approximately 36Gbp), which is a very large genome size compared to the model plant *Arabidopsis* which comprises of 157 million base pairs of DNA (Bennett and Smith, 1976; Bennett *et al.,* 2003; Shahin *et al.,* 2011). Lily plants have been frequently used for physiological, cytological, and molecular investigation of pollen development, because lily pollen grains are relatively large (Vidali *et al.,* 2009) and the *Lilium* genus has a very big flower compared to the model plants. Nevertheless, the manipulation of this species is currently very limited.

One of the major industries in many countries is the flower industry, which has developed significantly over recent years. Chandler and Tanaka, (2007) reported that the production value of ornamental plants was estimated in 2006 to be around

50 billion Euros, and the consumption value was 100 - 150 billion Euro world-wide. In addition, since 2007, the floriculture industry has been estimated as ~80 billion Euros. The area used for the production of bulbs, flowers and pot plants is estimated to be 740,600 hectares worldwide, with 79,033 ha in Europe (Schubach, 2010). The Netherlands is one of the most important countries for floriculture production and marketing and was estimated at 31,121 ha in 2009 (Grassotti and Gimelli, 2011).

Three important sections in ornamental plant production are cut flowers, pot plants and garden plant design (Benschop *et al.,* 2010), with *Lilium* ranked as the fourth most important cut flower. In addition, ornamental horticulture industries have been important for commercial bulb and flower production including fresh-cut flowers, potted crops and garden landscaping design. These production areas can be represented and developed about 90% and these are increasing rapidly in several countries such as Latin America, Asia and Africa (Benschop *et al.,* 2010).

In the past, classical breeding has had an important role in developing the novelty of flowers for industry. Recently, genetic engineering has significant roles in de-veloping of novelty of cut flower (Tanaka *et al.,* 2005). The first commercially successful group of hybrid lilies were produced by Jan de Graaff, a well-known breeder from Oregon in the USA, in 1950. Asiatic hybrid lilies like Enchantment and Connecticut King were derived from species native to Korea, Japan, China and Europe and were the first commercial cultivars of hybrid lilies (Grassotti *et al.,* 2011).

1.3.1 Description of the genus of Lilium.

The genus *Lilium* L. belongs to the Liliaceae family and consists of approximately one hundred species of monocotyledonous and perennial crops. It is distributed between 10° to 90° latitudes of the Northern Hemisphere (McRae, 1998). During the 18th century, *Lilium* started as ornamental plants (Nunez de Caceres *et al.,* 2011). In 1836 Endlicher, first classified this genus, dividing *Lilium* into five sections; Amblirion, Martagon, Pseudolirium, Eulirion and Cardiocrinum. In 1871 Baker manipulated Endlichers' taxonomy and created a new section, *Archelirion*. After that, in 1925, Wilson modified and divided the genus into four subgenera. Subsequently this genus was classified depending on two physiological germination times and thirteen morphological descriptions, for nine subsections and seven sections by Comber at 1949, Relationships have also been defined using Phylogenetic trees constructed from internal transcribed spacer (ITS) sequences of 55 *Lilium* species (Nishikawa *et al.,* 1999) (Fig 1.4).

McRae, (1990) and Van Tuyle *et al.,* (2002) demonstrated that generally, *Lilium* can be crossed easily between each wild type species within these sections and that the hybrids are fertile. Interspecific hybrids of Leucolirion, Archelieion and Sinomartago, are the most important cultivated groups, which include the following:

1) Longiflorum hybrid (L-genome) which has trumpet-shaped, mostly outward-facing white flowers, with a strong scent and year-round forcing ability. This hybrid derivation is originated from interspecific hybridization in the Leucolirion section (McRae, 1990).

2) The second important group is Asiatic hybrids (A-genome), which have enormous colour variation in their tepales (white, yellow, orange, purple, red, salmon and pink) and early flowering. These hybrids are the consequence of interspecific crosses amongst at least 12 species from the Sinomartagon section; the international lily register (http://www.lilyregister.com). Many Asiatic hybrids have pathogen resistance, particularly to *Fusarium* and viruses (McRae, 1998).

3) The final group, Oriental hybrids (O-genome), comprises of very important lily hybrids that are derived from hybridization amongst the five species of the Archelirion section. Generally, they have big flowers, pleasant fragrance and are late-flowering (McRae, 1998). In addition, most Oriental hybrids have a significant resistance to pathogens, especially *Botrytis elliptica* (Barba-Gonzalez *et al.*, 2005).

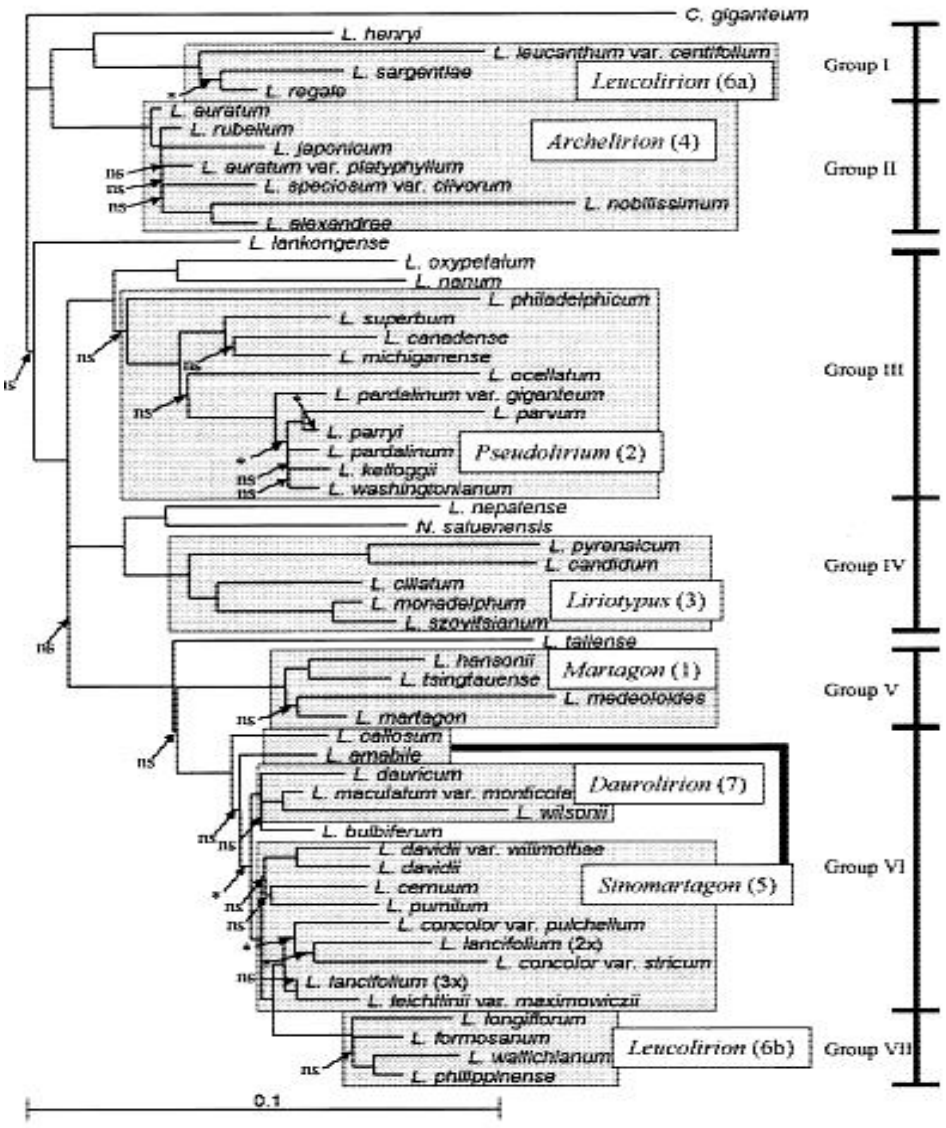


Figure 1. 4: Phylogenetic tree constructed from internal transcribed spacer (ITS) sequences of 55 Lilium species, Cardiocrinum gaiganteum and Nomocharis saluenensis by the maximum-likelihood method (taken from Nishikawa et al., 1999)

1.4. PLANT LONGEVITY AND SENESCECE.

Plant longevity is defined as the length of plant life cycle, which is a consequence of natural selection and environmental fluctuation (Woolhouse, 1974). Longevity of flowers is the most crucial traits for ornamental plants to increase productivity of crops in the agronomic sector (Gregeren *et al.*, 2013).

Plant senescence is a developmentally regulated and genetically programmed process that cause plant death (Tripathi and Tuteja, 2007; Zhou and Gan, 2010). Senescence is the last phase of development and associated with degeneration causing flower death. It is a highly controlled process, which can result from endogenous ontogenetic processes or from external factors, and involved in structure, biochemical and molecular change, which also can be one example for (PCD) programed cell death (Tripathi and Tuteja, 2007).

Plants exhibit two types of senescence, mitotic and post-mitotic. Mitotic senescence is called also (proliferative senescence) refers to the loss of capability of cells to mitotically divide. Post-mitotic senescence is an active degenerative process that occurs after cellular maturation leading to cell death in the plant organs including leaves and floral petals (Gan, 2003). Post-mitotic senescence (particularly leaf senescence) appears to be a form of programmed cell death (PCD), which is regulated by internal and external factors (Lime *et al.,* 2007 and Gan., 2003) Regulation of plant senescence is important, because of it is relevance to productivity and development, it is observed in many different tissues including germinating seeds, root cap, cortex, leaves, petals and reproductive organs, however it is of particular importance for leaves and flowers (Lim *et al.,* 2007; Tripathi and Tuteja, 2007).

1.4.1 Genetic regulation and manipulation of leaf and flower senescence.

Regulation of senescence mechanisms and manipulated of this process is a major objective for agricultural improvement particularly delaying leaf and flower senescence (Zhou and Gan, 2010). Currently, senescence has a negative impact on crop yield and biomass accumulation for the production of many ornamental plants, fruit and vegetables as well as other crops (Nooden *et al.*, 1997 and Buchanan-Wollaston., 2003). Genetic manipulation is a possible option to reduce such losses in agricultural production, which can significantly improve agronomic traits, such as quality, quantity of crops and increased post-harvest storage, stress tolerance as well as delaying leaf and flower senescence in floriculture sector (Nooden *et al.*, 1997).

1.4.1.1 Genetic manipulation of leaf senescence.

Leaf senescence is the final stage of leaf development that is highly regulated and complex process, controlled by multiple developmental and environmental factors, and involves changes in metabolic and cellular physiology, biochemistry and gene expression, leading to cell death (Fig1.5) (Nooden, 1988; Lim and Nam, 2005). Genetics and molecular approach can be used to develop understanding of the regulation and manipulation of senescence (Lim *et al.*, 2007). For example, molecular approach has been used to identify and characterize genes increased or decreased expression level during leaf senescence. (Lim *et al.*, 2007).

Arabidopsis has been used as a good model plant for investigate and identifying senescence regulators, and for elucidating the regulatory mechanisms of leaf senescence (Hensel *et al.*, 1993; Lim and Nam, 2005).

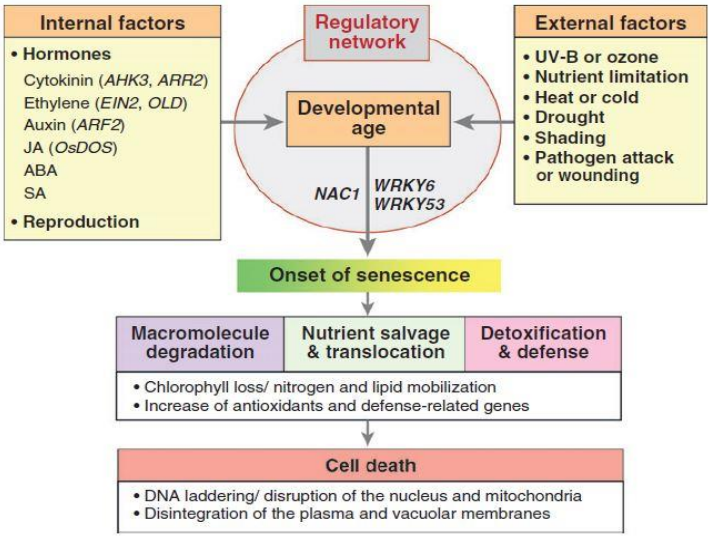


Figure 1. 5: A model for regulatory pathways in leaf senescence.

Internal and external signals are integrated into developmental, age-dependent senescence pathways for leaf senescence. Multiple pathways that respond to various factors are interconnected to form regulatory networks. These regulatory pathways activate distinct sets of senescence-associated genes, which are responsible for executing the degeneration process and ultimately lead to cell death (taken from Lime *et al.*, 2007).

Leaf senescence is a highly coordinated process that is regulated by many senescence associated gene (SAGs) (Guo and Gan, 2005; Lim *et al.*, 2007) (Fig 1.6).

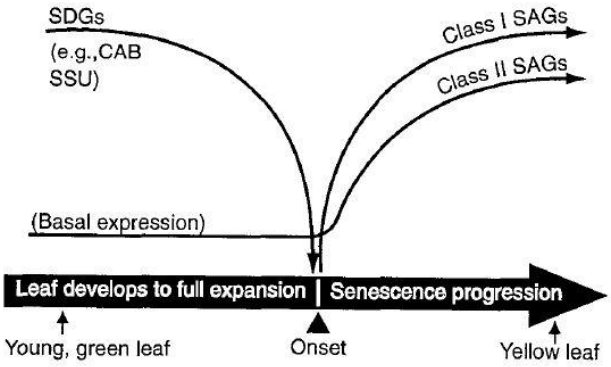


Figure 1. 6: Differential gene expression during leaf senescence.

Senescence down-regulated genes (SDCs) include genes involved in photosynthesis such as the chlorophyll a/b-binding protein gene **(CAB)** and the Rubisco small subunit gene (SSU). **SACs** are those genes with expression up-regulated during leaf senescence. Class I **SACs** are expressed only during senescence (senescence-specific). Class II **SAGs** have a basal level of expression during early leaf development, but this level increases during senescence (taken from Gan and Amasino, 1997).

Gan, (2007) demonstrated that the SAG12 gene could be used as a promoter for production and regulation of cytokinin to delay senescence in many crops; such as tobacco (Gan and Amasino, 1995; Ori *et al*., 1999), lettuce (McCabe *et al*., 2001), petunia (Chang *et al*., 2003) and *Arabidopsis* (Huynh *et al*., 2005). In addition, Gan and Guo, (2014) reported that a large range of plant species have been successfully manipulated to delay senescence using *PSAG12-IPT* regulatory senescence inhibition system (Fig 1.7)

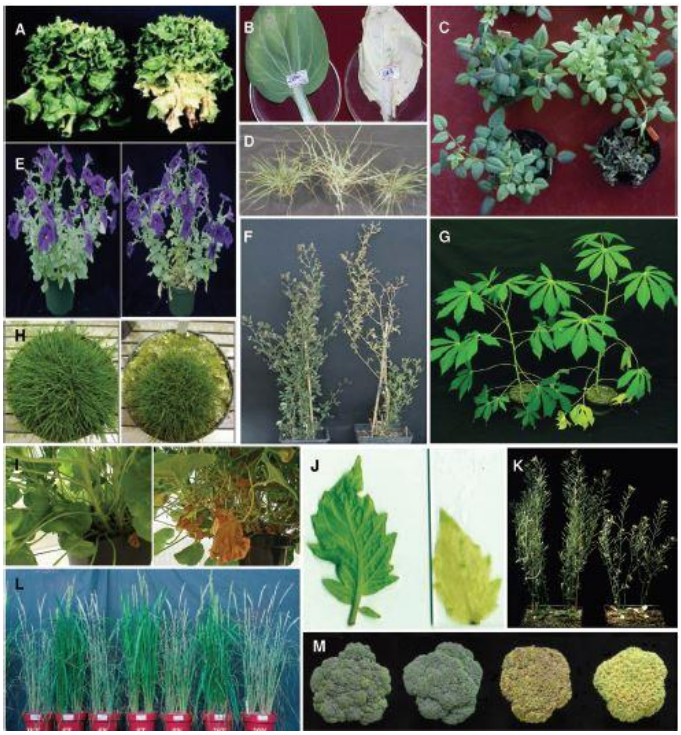


Figure 1. 7: Examples of PSAG12-IPT transgenic plants with delayed leaf senescence phenotypes.

(A) WT and transgenic (left) lettuce plants (McCabe *et al.*, 2001). (B) WT and transgenic (left) bok choy leaves (Yuan *et al.*, 2002). (C) WT and transgenic (left) potted rose (Zakizadeh *et al.*, 2013). (D) WT (middle) and transgenic *Zoysia sinica* plants (Zhang *et al.*, 2005*a*). (E) WT and transgenic (left) petunia (Clark *et al.*, 2004). (F) WT and transgenic (left) alfalfa plants (Calderini *et al.*, 2007). (G) WT and transgenic (left) cassava plants (Zhang *et al.*, 2010). (H) WT and transgenic (left) creeping bentgrass plants (Xu *et al.*, 2010*b*). (I) WT and transgenic (left) *Pelargonium* plants (García-Sogo *et al*., 2012). (J) Detached leaves of WT and transgenic (left) tomato (Luo *et al.*, 2005). (K) WT and transgenic (left) *Arabidopsis* plants (Gan, 2005). (L) WT (Pot 1 from left), transgenic rice plants (Pots 2, 4, and 6), and their nulls (Pots 3, 5, and 7) (Peleg *et al.*, 2011). (M) WT and transgenic (left 2) broccoli (Liu *et al.*, 2011). (taken from Gan, and Guo, 2014).

Transcriptional networks in leaf senescence have been analysed and many senescence genes were shown to be regulated by many transcription factor including WRKY, NAC, BZIP, C2H2, EREBP and MYB (Gan, 2007; Schippers, 2015). Transcriptome gene-expression during leaf senescing has been analysed in many crops including, wheat (Gregersen and Holm, 2007), barley (Parrott *et al.*, 2007; Christiansen *et al.*, 2011), rice (Liu *et al.*, 2008), *Medicago trucatula* (De Michele *et al.*, 2009), Arabidopsis (van der Graff *et al.*, 2006; Breeze *et al.*, 2011).

Leaves are critical organs in plants, where solar energy is absorbed and utilized to fix CO2 and produce carbohydrate and release O2 via photosynthesis, and nutrient and amino acids and antioxidants are synthesized (Gan and Guo, 2014). Photosynthetic capability is reduced significantly during leaf senescence with associated decreases in crop yields and biomass accumulation (Gan, 2007; Gan and Guo, 2014). Chloroplasts are one of the first organelles targeted for breakdown during senescence, which contain up to 70% of the proteins that are associated with chlorophyll degradation and loss of the green colour during leaf senescence, (Lim *et al.*, 2007; Zhou and Gan, 2010). Cell death occurring during leaf senescence can be defined as a type of programmed cell death, during which chlorophyll and macromolecules including proteins, membrane lipids and RNA, are catabolism and degraded, alongside a loss of photosynthetic activity, and minerals, nitrogen, phosphorus and metal ions are transferred out of the leaf (Wollaston-Buchanan *et al.*, 2003; Lim *et al.*, 2007; Fernandez-Lopes *et al.*, 2015). The ability to delay leaf senescence is therefore a significant target in agriculture aspects (Wollaston, 2003; Lim and Nam, 2005).

In cereal crops, quality and quantity of crop are affected by limiting the growth staged during leaf senescence and may cause postharvest spoilage including nutrient loss in vegetable plant and leaf yellowing. Therefore, studying leaf senescence not only enhances our understanding of fundamental biological processes, but also provides a potential mechanism for controlling leaf aging to improve the agricultural traits of crops (Lim *et al.,* 2007). This is because during delayed senescence, leaves will remain photosynthetically active for longer and the flowering period will be extended (Gan and Amasino, 1995; Wingler *et al.*, 1998; Jordi *et al.*, 2000; Cao, 2001; Zhang *et al*., 2005*a*, 2010; Bu *et al.*, 2007; Fu *et al.*, 2009*b*; Xu *et al.*, 2010*a*; Gan and Amasino, 1996; Zhang *et al.*, 2000). This can result in more seeds and a greater accumulation of biomass (Table 1.1), for example transgenic tobacco plants carrying the *PSAG12-IPT* transgene showed an increase of yield /biomass (Gan and Guo, 2014).

Table 1. 1: Increase of yield/biomass in transgenic plants with outoregulated IPT expression.

Plant species	Increase in biomass/yield (%)	Harvested organ type	Growth conditions	Reference
Arabidopsis	61	Shoot	Controlled	Zhang *et al.* (2000)
Arabidopsis	85	Shoot	Flooded	Zhang *et al.* (2000)
Arabidopsis	35	Shoot	Flooded	Huynh *et al.* (2005)
Brassica napus	6.4–32.9	Grain	Controlled	Luo *et al.* (2002)
Cotton	34.2	Lint	Field	Liu *et al.* (2012)
Cotton	96.43*	Whole plant	Salt stress	Liu *et al.* (2012)
Creeping bentgrass	160*	Root	Heat stress	Xu *et al.* (2010*b*)
Creeping bentgrass	30.5*	Root/shoot ratio	Drought stress	Merewitz *et al.* (2011*b*)
Peanut	58	Fruit	Field	Qin *et al.* (2011)
Rice	7.99	Grain	Field	Lin *et al.* (2002)
Rice	16.18	Grain weight	Controlled	Wang and Liang (2005)
Rice	144	Grain	Water stress	Peleg *et al.* (2011)
Tobacco	40.26	Shoot	Controlled	Gan and Amasino (1995)
Tobacco	83.33*	Leaf 7–9	Controlled	Jordi *et al.* (2000)
Tobacco	14.29	Shoot	Controlled	Boonman *et al.* (2006)
Tomato	22	Fruit	Field	Feng *et al.* (2003)

Three approaches have been used for manipulation leaf senescence for agricultural improvement, including manipulation of i) plant hormone biology-based leaf senescence, ii) leaf senescence-specific transcription factors and iii) translation initiation factor-based technology (Guo and Gan, 2014).

1.4.1.2 Genetic manipulation of flower senescence.

Flower senescence is the terminal stage of developmental processes that leads to the death of the floral organs, which are responsible for sexual reproduction and seed set for continuation of the generations. It has been used a good model for both senescence and abscission for improving post-harvest flower quality and longevity (Tripathi and Tuteja, 2007). This is a highly regulated process and Fig 1.8 shows a model of the regulatory pathways in flower senescence.

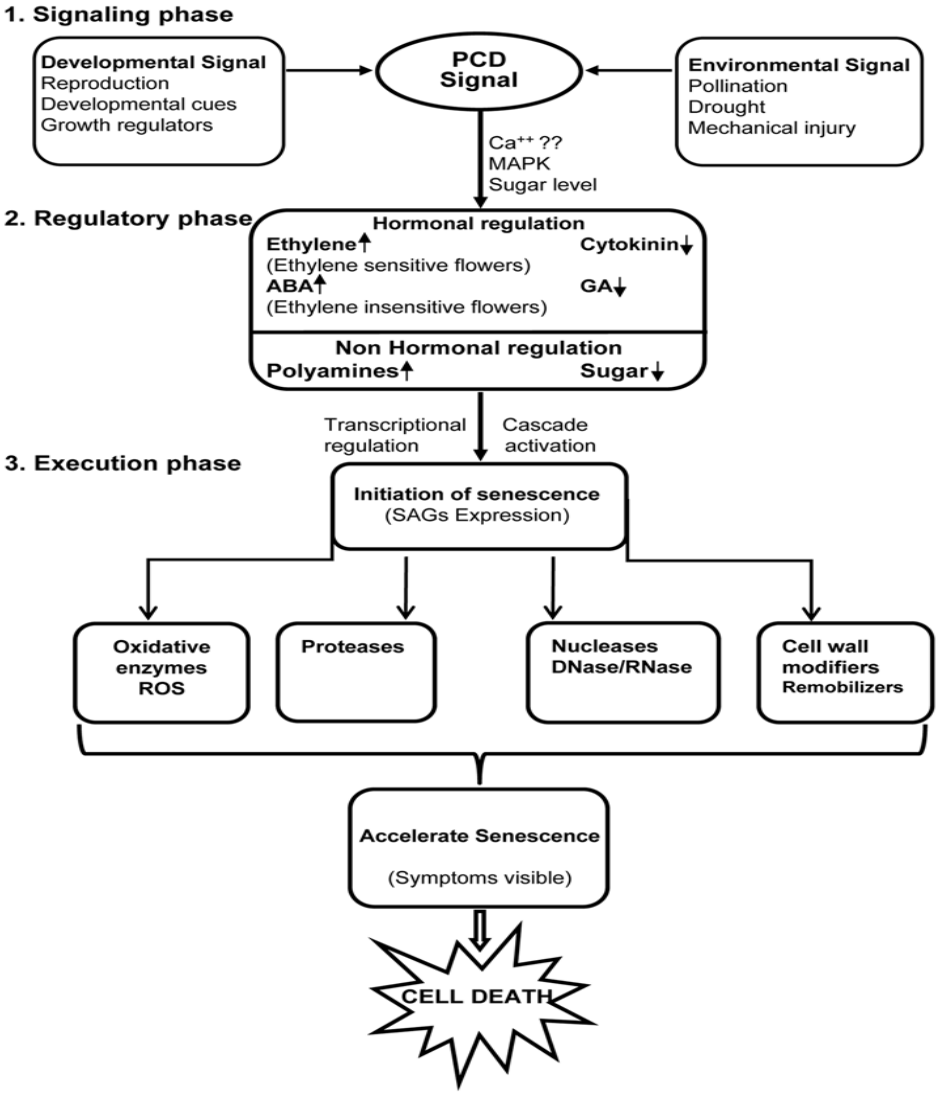


Figure 1. 8: A model for regulatory pathways in flower senescence.

The PCD signal is generated by both external and internal stimuli and transduced by signals resulting at least in part from hormonal imbalances in the cell. This altered level of hormones further activates several cascade and transcriptional regulation. The initiation of senescence starts with expression of several SAGs like proteases, nucleases, wall degrading and oxidative enzymes. The collective actions of all these enzymes accelerate the senescence process and it became irreversible. At the later stages of senescence, symptoms become visible and ultimately lead to cell death of flowers (taken from Tripathi and Tuteja, 2007).

There are many examples of manipulation of leaf and flower senescence; many genes have been isolated from numerous flowers using different tools that are associated with floral senescence (Senescence Associated Genes (SAGs). (MCM1 AGAMOUS DEFICIENS AND SRF) MADS-box genes like *AGAMOUS like 15 (AGL15)* and *AGL18* have played a significant role in flower senescence (Tripathi and Tuteja, 2007). Fernandez *et al.,* (2000) reported that plants can delay flowering time, floral organ abscission and senescence via constitutively overexpression of AGL15 gene.

In addition, retention of perianth organs and prolonged longevity in plants can be achieved by over-expressing MADS box gene AGL18 (Tripathi and Tuteja, 2007; Adamczyk *et al.,* 2007). Genetics and molecular analysis have also been used to delay leaf and flower senescence base on plant hormone biology by supression of ethylene production or enhancing cytokine production (Lim *et al.,* 2007 and Zhou and Gan, 2010).

In conclusion, multiple experimental examples have indicated that delayed abscission and flower senescence, which is a most important target in the flower industry, can be obtained in ornamental plant by genetic manipulation.

1.4.2. Effect plant hormones on senescence

Plant hormones play an important role in the senescence process in all stages of the plant life cycle. There are many hormones that affect longevity and senescence, such as ethylene, cytokinins, auxins, gibberellic acid, jasmonic acid and abscisic acid (Shahri and Tahir, 2011). Among them, cytokinins, gibberellins and auxin are known to up regulate senescence, which can be involved and play significant role in delay of senescence process, while ethylene, jasmonates and abscisic acid down regulate this process (Nooden, 2004 and Smart, 1994).

However, the effect of pant hormones on senescence and longevity depends on the condition, concentration and plant species (Gan and Amasino, 1996) For example, in petunia cytokinins can delay petal senescence by reducing sensitivity to ethylene (Chang *et al.,* 2003). Arrom and Munne-Bosch., (2012) concluded that hormonal alteration in different floral tissues occurs throughout flower development; for example, the levels of cytokinin and auxin-mainly decrease later during senescence, and increase in the tepals prior to anthesis, also the hormone ABA can accelerate flower senescence by reducing longevity and increases during the final stages of female development.

Changes in hormonal balance can influence whole parts of cut flowers. Sepals and petals are thought to be the most important parts of the flower for longevity and senescence because they affect vase life, which is a crucial for the quality of cut flowers (Arrom and Munne-Bosch, 2012). Pollination plays a significant role in alteration of the flower particularly anther and petal abscission and senescence, because endogenous ethylene production is increased during pollination. Therefore, ethylene can promote the pollination-regulated perianth senescence that is

seen in many flowers such as Orchid, *Petunia* and carnations (O'Neill, 1997). It is not clear which flower parts trigger flower bud abscission and perianth senescence in *Lilium* cut flowers. However, anthers in cut flowers, particularly lily, are trigger organs that may induce flower bud abscission, therefore the opening of anthers may have possible effects on bud abscission in cut flowers (Hwang *et al.,* 2012). Overall, cytokinin has a significant role in delaying leaf and flower senescence.

1.4.2.1 Cytokinins.

Cytokinin is plant growth regulator, which is derived from adenine and can significantly affect the morphology and physiological processes in higher plants. This phytohormone has been implicated in many aspects of plant growth and development such as cell division, cell differentiation, shoot initiation and response to biotic and abiotic factors, as well as leaf expansion, chlorophyll synthesis and delayed leaf senescence (Kieber and Schaller, 2014). Mutant and transgenic tissues with altered cytokinin activity have shown altered properties associated with agriculturally important traits including senescence, productivity, sink activity and tolerance (Werner *et al.,* 2001; Balibrea- Lara *et al.,* 2004; Zalabak *et al.,* 2013; Mrizova *et al.,* 2014).

Akiryoushi *et al.,* (1984) reported manipulation of the genes involved in cytokinin synthesis, in *Agrobacterium tumefaciens*. After infection of plants by this bacteria plant crown-gall disease results, due to the transfer of the Ti-plasmid to the plant cell, containing the *tms1* and *tms2, ipt* or *tmr* genes that are responsible for production of auxin and cytokinin (Gelvin, 2003 and Slater *et al.,* 2003). This gene, which encodes for isopentenyl transferase (IPT) enzyme, which is the first step in

the cytokinin biosynthesis pathway in both bacteria and plants. This enzyme transfers the isopentenyl group from DMAPP to ADP, ATP in plants and AMP in bacteria (Akiryoushi *et al.,* 1984; Takei *et al.,* 2001**;** Kakimoto, 2003; Kieber and Schaller, 2014 and Linder *et al.,* 2014); Fig 1.9 shows the metabolic and biosynthetic pathway of cytokinins in the plant.

The molecular mechanism of cytokinin action in plants is still unclear, however nine IPT coding genes have been identified in the *Arabidopsis* genome (Lim *et al.,* 2003; Lime *et al.,* 2007; Kieber and Schaller, 2014); Table 1.2 describes the genes involved in cytokinin biosynthesis in *Arabidopsis*.

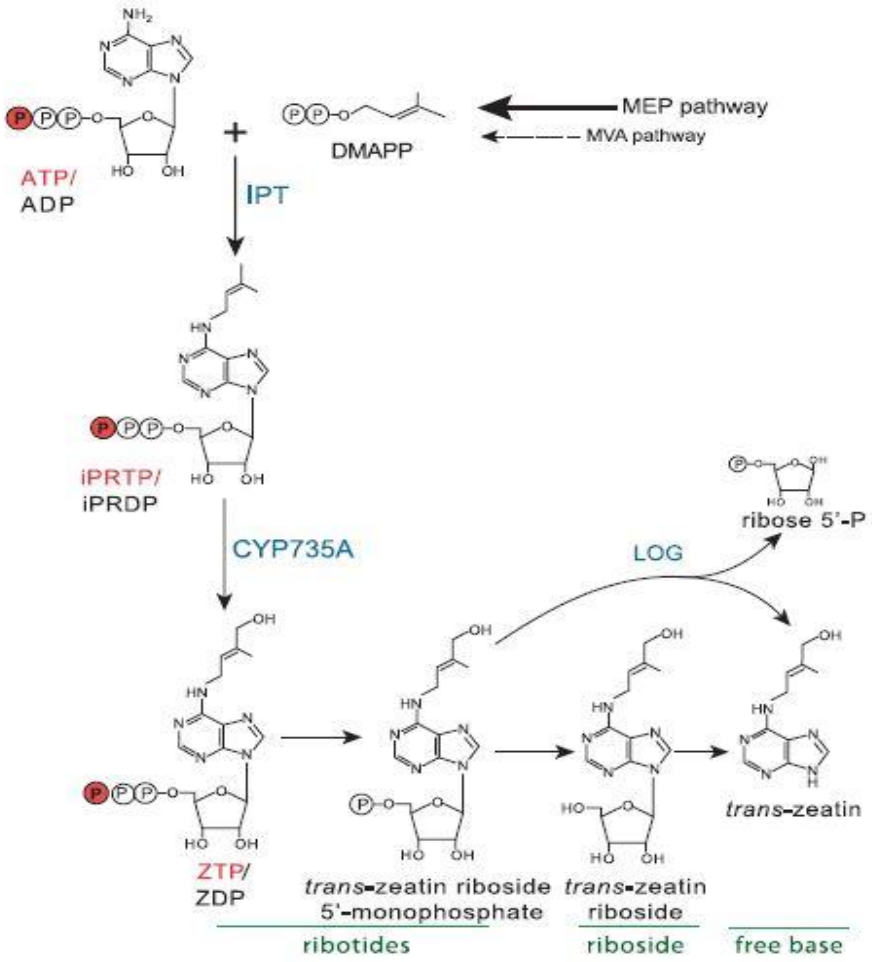


Figure 1. 9: Metabolic and biosynthetic pathway of cytokinins in plants (taken from Kieber and Schaller, 2014).

Table 1. 2: Genes involved in cytokinin biosynthesis in Arabidopsis (taken from Kieber and Schaller, 2014).

IPT genes	AGI Codes	Location	Notes
IPT1	At1g68460	chloroplast	
IPT2	At2g27760	cystol	tRNA isopentyl transferase
IPT3	At3g63110	chloroplast	upregulated by nitrate; expressed primarily in phloem
IPT4	At4g24650	cystol	
IPT5	At5g19040	chloroplast	
IPT6	At1g25410	not yet determined	Pseudogene in some accessions
IPT7	At3g23630	mitochondria	Induced by STM in SAM
IPT8	At3g19160	chloroplast	
IPT9	At5g20040	not yet determined	tRNA isopentyl transferase
Cytokinin trans-hydroxylase genes			
CYP735A1	At5g38450	not yet determined	
CYP735A2	At1g67110	not yet determined	
Cytokinin nucleoside 5' monophosphate phosphoribohydrolase genes			
LOG1	At2g28305	Cystol and nucleus	
LOG2	At2g35990	Cystol and nucleus	
LOG3	At2g37210	Cystol and nucleus	
LOG4	At3g53450	Cystol and nucleus	
LOG5	At4g3519	Cystol and nucleus	
LOG7	At5g06300	Cystol and nucleus	
LOG8	At5g11950	Cystol and nucleus	

Over-expression of the *IPT* gene in transgenic plants can cause an increase in the level of cytokinins and reduce leaf senescence (Can and Amsino, 1996). Therefore, this gene has been used under the control of different promoters, such as CaMV35S and SAG12, for expression modification in commercial ornamental plants (Can and Amsino, 1996; MaCabe *et al.,* 2001; Cai *et al.,* 2012; Zakizadeh *et al.,* 2013). Chang *et al*. (2003) reported that (Petunia x hybrida cv V2) transformed with the *PSAG12-IPT* gene had significantly extended flower longevity, from 6 days to 10, compared to wild type. In addition, transformation of miniature potted rose (*Rosa hybrida* cv.Linda) with the *PSAG12-IPT* gene delayed leaf senescence up to 10 days, compared to non-transgenic, under dark stress (Zakizadeh *et al.,* 2013).

Recently researcher have been attempt alter the regulation of CKs by molecular processes in plants such as barley to improve the quality and quantity of crop and delay leaf senescence (Zalabak *et al.,* 2013). In higher plants, several multigene families are affected on levels of concentration of cytokinin including: Isopentenyl transferases (IPT) for cytokinin biosynthesis; Cytokinin oxidases (CKX) for degradation; Zeatin O-glucosyltransfeases (ZOG) for reversible inactivation; B-glucosidases (GLU) for reactivation; Lonely Guy (LOG) for activation and UDP glycosyltransferases(UGTs) for irreversible N-glycosylation; cytokinin concentration levels are regulated by a balance of these (Song *et al.,* 2012; Song and Jameson, 2015).

In Arabidopsis plant nine IPT genes, seven cytokinin dehydrogenase enzymes (CKK genes), three zeatin O-glucosyltransferases (ZOG genes) and three β-glucosidases (GLU) genes have been characterised and functionally verified (Bilyer *et al.,* 2001; Hou *et al.,* 2004) in barley, eleven Hv CKX family gene have been identified (Zalewski *et al.,* 2014).

Increasing endogenous cytokinins have been shown to significantly effect quality and yield by enhancing of IPT expression and activation depending on the specific promoter and developmental stage and environmental conditions (Song *et al.,* 2012). The level of active cytokinins are decreased during senescence and lead to decreased quality and quantity of yield (Jameson and Song, 2015), whereas decreased expression of CKK, enhanced expression of IPT gene via manipulation of cytokinin levels lead to increase of production yield. However, plant is a sink limited or source -limiting environments during cytokinin modified through IPTor CKx targeted (Jameson and Song, 2015 Fig 1.10).

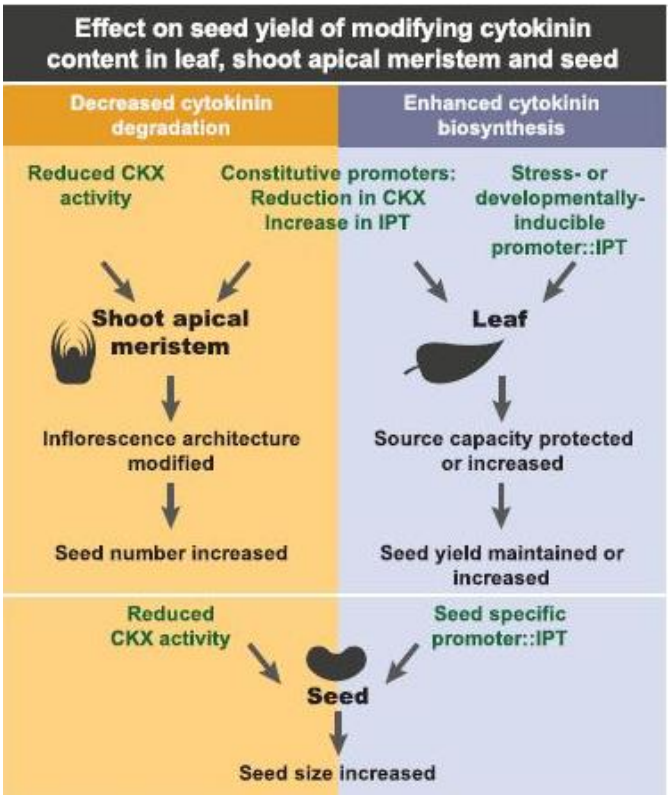


Figure 1. 10: Effect on seed yield of modifying cytokinin content in leaf, shoot apical meristem, and seed. IPT: isopentenyl transferase is the key cytokinin biosynthetic enzyme. CKX: cytokinin oxidase/dehydrogenase causes cytokinin degradation (taken from Jameson and Song, 2015).

1.4.2.2. Ethylene

Ethylene is a gas produced by plants, which plays a significant role in plant growth and development, and in the regulation of leaf and floral senescence, organ abscission and fruit ripening. Ethylene synthesis can affect the post-harvest quality of cut flowers and therefore has implications for the commercial market of ornamental plants (Paliyath *et al.,* 2008). Cut flowers, like fruit, are divided into climacteric and non-climacteric groups, climacteric is described by ripening associated with increased respiration, in contrast in non-climacteric is considered by lack of ethylene associated in respiration. For example, carnation, Gypsophila and orchids are climacteric flowers, and respiration and increased ethylene production can induce petal senescence. Whereas in non-climacteric flowers, including Tulip, Gladiolus and Iris, production of the plant hormone ethylene and respiration are not increased during flower senescence and petal senescence is not affected by exogenous ethylene (Serek *et al.,* 2006).

Many researchers have demonstrated that senescence and abscission of flowers might be trigged by endogenous ethylene (ethylene receptors). Consequently, study of ethylene receptors with linked of signal transduction pathway is fundamental for understanding of ethylene perception in flower (Paliyath *et al.,* 2008; Patterson and Bleecker, 2004) Genetic manipulation has a significant potential for regulation of this process and altering senescence. Therefore, genes associated with ethylene biosynthesis, perception and signal transduction have been isolated in important floricultural crops. Genetic manipulation of the ethylene pathway has successfully improved flower longevity in many ornamental plant species, including *Petunia*, Carnation, *Nemesia* and *Campanula* (Shahri and Tahir, 2011 and

Shibuya, 2012). Several studies have demonstrated the role of the ethylene pathway in senescence, for example plant leaf longevity was increased in ethylene-insensitive mutants *etr1-1* and *ein2*/ *ore3* (Gebic and Bleecker, 1995; Oh *et al.*, 1997).Transgenic petunia flowers expressing the *Ethylene receptors 1* (*Etr1-1)* gene under control of the CaMV35s promoter, resulted in an extended life cycle, two or four times longer, and delayed abscission, compared to non-transgenic plants (Tanaka *et al.*, 2005). In addition, Shibuya *et al.*, (2004) demonstrated that the *EIN2* gene, which is involved in the ethylene signalling pathway, plays a role in petal senescence in petunia since suppression of the *phEIN2* gene resulted in significant delays in petal senescence after pollination (Fig 1.11).In conclusion, manipulation of the ethylene pathway may have a significant role for decreasing postharvest loss of ornamental cut flowers and increasing the market sector.



Figure 1. 11: Delayed petal senescence in transgenic petunia with reduced PhEIN2 expression.

A wild-type flower 2 d after pollination (left) and a *PhEIN2*-RNAi transgenic flower 8 d after pollination (right). The petals of transgenic plants are still turgid even after the ovaries have been fully expanded (taken from Shibuya *et al.*, 2004)

1.5 CONTROLLING FERTILITY- HYBRID BREEDING SYSTEMS

1.5.1 Flower development in *Arabidopsis*.

The morphology of organs of typical flowers consists of petals (corolla) sepals (calyx), stamen (anther and filament) and carpels (stigma, style, ovary) (Fig 1.12); many genes are associated with the development of these organs and tissues (Blazquez, 2000), with complex genetic systems associated with the control of these mechanisms of flower development (Benedito *et al.,* 2004). Over the past three decades, molecular and genetics analysis have made major progress towards understanding the key mechanisms involved in floral regulation and how to control of flower morphogenesis (O'Maoileidigh *et al*., 2014 and Stewart *et al.,* 2016)

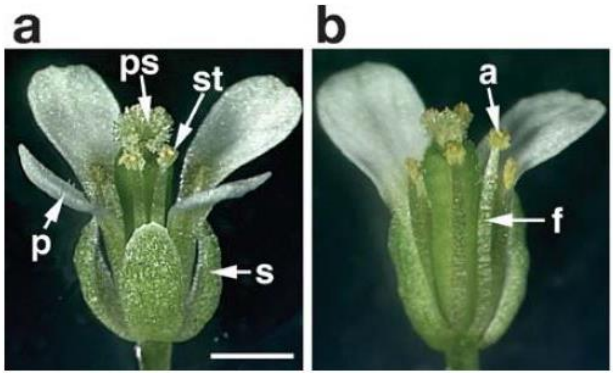


Figure 1. 12: Arabidopsis flower.

(A) An intact mature (stage 13) Arabidopsis flower with four types of organs, (S) sepals, (P) petals, (St) stamens, and (Ps) the pistil. (B) A mature flower with one sepal and two petals removed to reveal some of the stamens, which have (a) an anther, (f) a filament. A and B are of the same magnification bar=1.0mm, (taken from Ma, 2005).

1.5.1.1 Anther and pollen development

Anther and pollen development is crucial for final crop yield, and therefore understanding its development is vital. Recently, this understanding has been expanded using molecular genetics of pollen and anther development in several model systems. Many mutants have been isolated, which have identified genes linked to anther and pollen development, anther dehiscence and pollen delivery (Yang *et al.,* 2001). In flowering plants, male gamete formation occurs in the stamen (Ma, 2005). Stamens are the male reproductive organs consisting of a filament and an anther; the filament is a tube of vascular tissue, attached to the anther by connective tissues, which supplies water and nutrients to the anther (Scott *et al.,* 2004).

The anther contains the reproductive and non-reproductive tissues that are responsible for producing and releasing pollen grains so that pollination and fertilization processes can occur (Goldberg *et al.,* 1993). Anther development in *Arabidopsis* has been classified into 14 stages (Fig1.13 and 1.14); anther development initiates from a single archesporial cell with the emergence of the stamen primordia in the third whorl of the floral meristem (Goldberg *et al.,* 1993; Scott *et al.,* 2004). A key stage of this is the establishment of the adxial-abaxil polarity and the differentiation of the microsporangial cell layer (Scott *et al.,* 2004) (Fig 1.15 and 1.16).

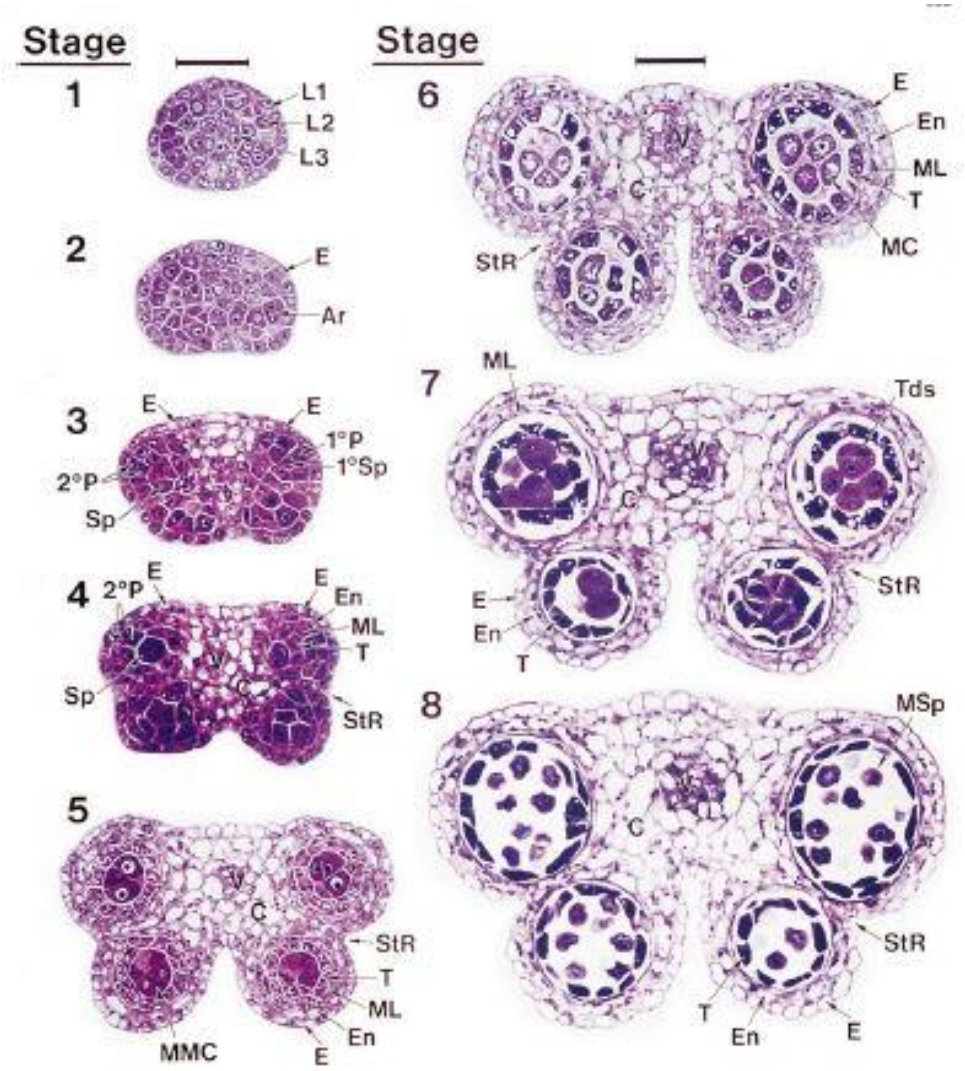


Figure 1. 13: ***Arabidopsis thaliana*** **anther development phases.**

1. *Ar*, archesporial cell; *C*, connective; *E*, epidermis; *En*, endothecium; *L1*, *L2*, and *L3*, the three cell-layers in stamen primordia; *MC*, meiotic cell; *ML*, middle layer; *MMC*, microspore mother cells; *MSp*, microspores; *1°P*, primary parietal layer; *2°P*, secondary parietal cell layers; *1°Sp*, primary sporogenous layer; *Sp*, sporogenous cells; *StR*, stomium region; *T*, tapetum; *Tds*, tetrads; *V*, vascular region. Stages 1to 4 *Bar* over stage 1=25µm but for stages 5 to 8 *Bar* over stage 6=25 µm (taken from Sanders *et al.*, 1999).

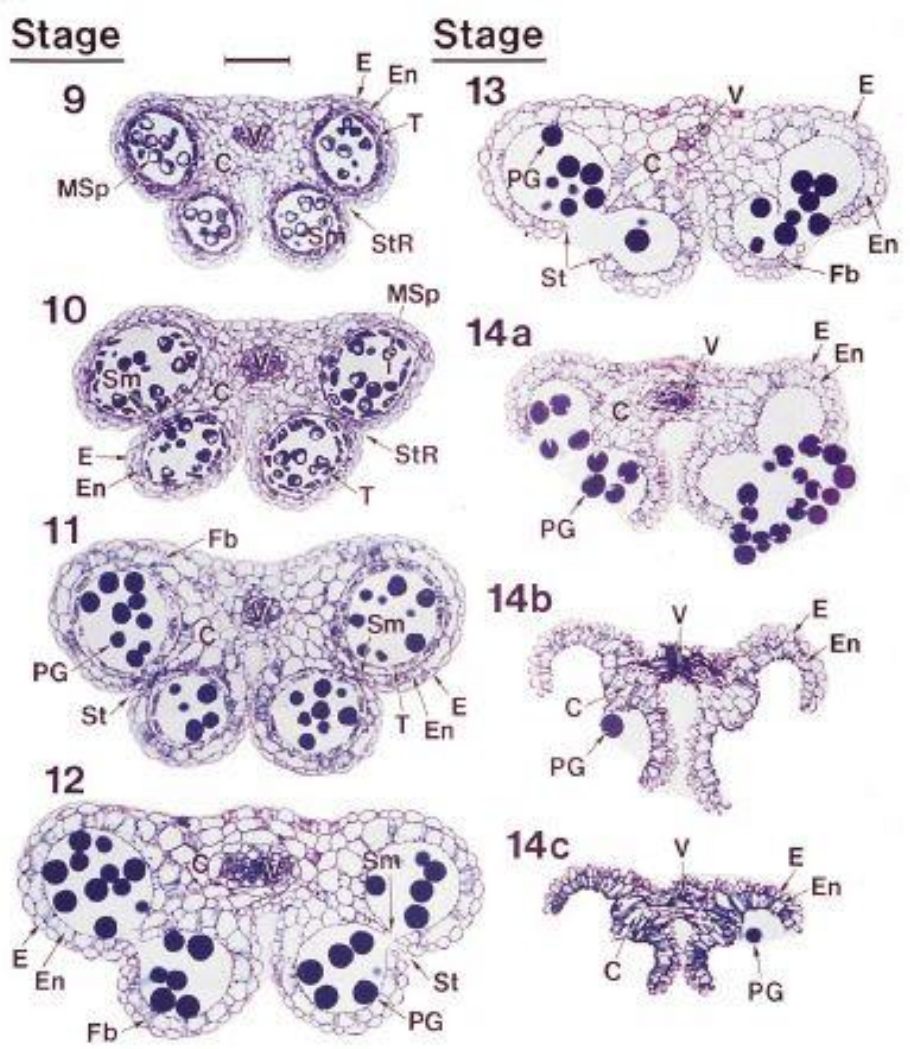


Figure 1. 14: ***Arabidopsis thaliana*** **anther development phase.**

Stages 9 to 11, 12 to 13, and 14a to 14c represent anther late development, dehiscence, and senescence, respectively. *C*, connective; *E*, epidermis; *En*, endothecium; *Fb*, fibrous bands; *MSp*, microspores; *PG*, pollen grains; *Sm*, septum; *St*, stomium; *StR*, stomium region; *T*, tapetum; *V*, vascular region. *Bar*=50 µm (taken from Sanders *et al.*, 1999).

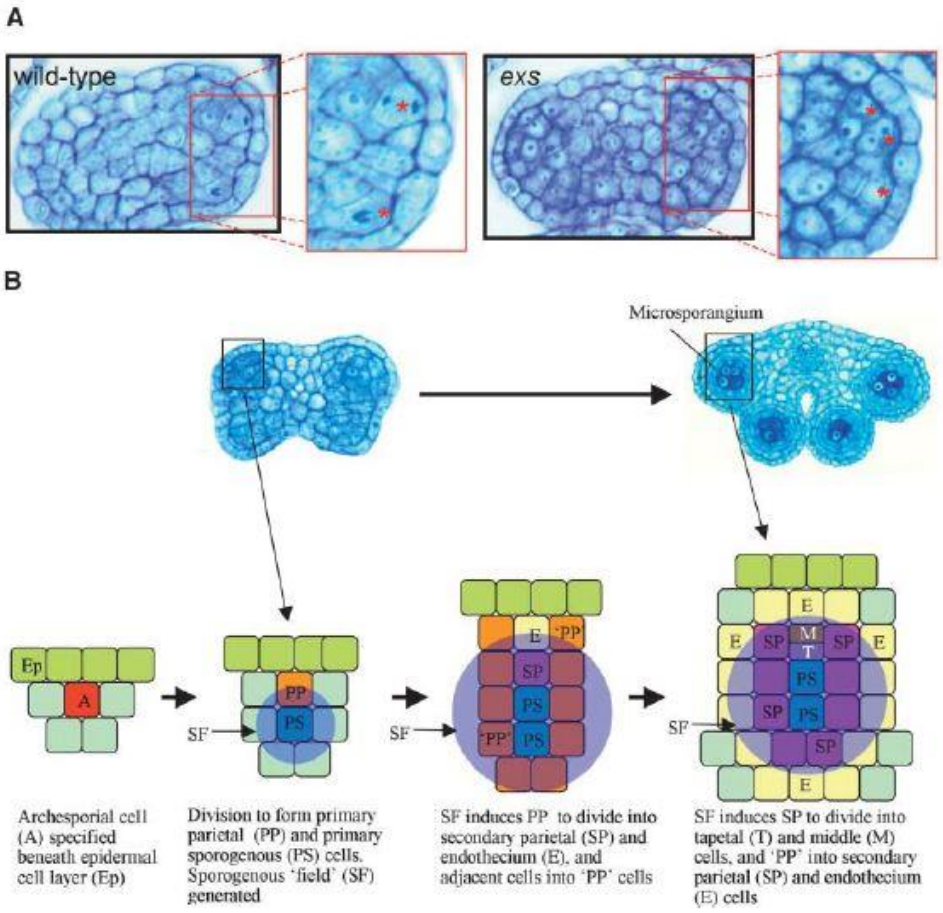


Figure 1. 15: Model of the differentiation of the microsporangial cell layers in Arabidopsis. (Taken from Canals et al., 2002)

(A) Light micrographs of wild-type and *exs* anther primordia with archesporial cell marked by asterisks; extra archesporial cells arise in *exs* mutants.

(B) Model for the differentiation of the microsporangial cell layers in *Arabidopsis*.

(C)

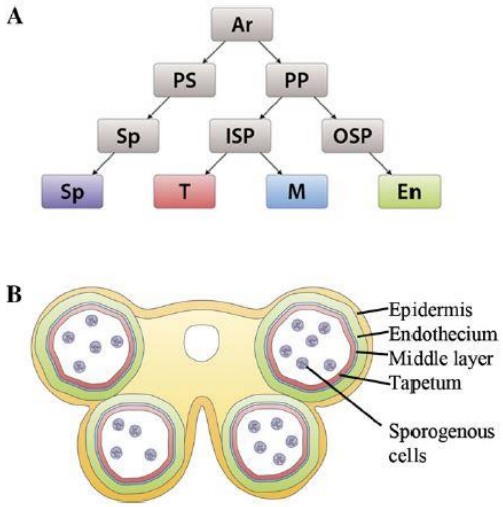


Figure 1. 16: Anther structure and cell lineage of the anther (taken from Wilson et al., 2011).

(A) Cell lineage model that shows archesporial cell division to form the final cell layers of anther (Scott *el al.*, 2004; Feng and Dickinson, 2007).

(Ar) Archesporial cells; (PP) Primary parietal layer; (SP) Primary sporogenous layer; (SP) sporogenous layer; (ISP) Inner secondary parietal layer; (OSP) outer secondary parietal layer; (En) Endothecium layer; (T) tapetum; (M) Middle cell layer.

(B) Schematic diagram of the cells layers of an *Arabidopsis anther*.

Development of male gametophyte consists of two stages, microsporogenesis and microgametogenesis that is takes place within specialized male reproductive organs called the stamens. Tetrads of haploid microspores are produced from a diploid pollen mother cell by meiotic division during microsporogenesis (Scott *et al.*, 2004; Borg *et al.*, 2009; Twell, 2010). The released microspores enlarge and a single large vacuole is produced during microgametogenesis (Owen and Makaroff, 1995; Yamamoto *et al.*, 2003). (Fig 1.17).

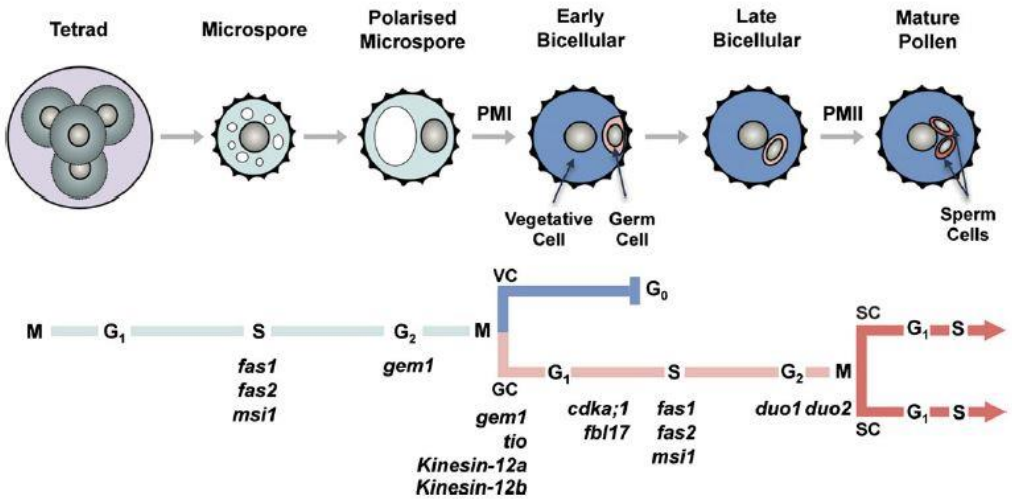


Figure 1. 17: Schematic diagram illustrating the distinct morphological stages of male gametophyte development in Arabidopsis (Taken from Borg et al., 2009).

Pollen development comprises of three major development stages; firstly, the differentiation of the sporogenous cell and meiosis (sporogenesis), secondly the post-meiotic development of free microspores and finally, microspore mitosis (Chaudhury, 1993). Pollen mother cells (meiocytes) are enclosed in a tapetum-derived callose wall and undergo meiosis to form tetrads of haploid microspores (Owen and Makaroff, 1995). This callose layer appears essential for development and formation of the scaffold for the pollen wall, it then breaks down and the microspores are released into the anther locule to begin male gametophyte development (post-meiotic development). The free microspores then go through one/ two rounds of mitotic divisions and pollen wall formation continues. The final mature pollen may be tricellular, or as seen in some species e,g, Arabidopsis, or it can be bicellular with a second mitotic division of the sperm cells (sc) occurring inside the pollen tube after pollination for instance in Lily (Singh and Bhalla, 2007).

The tapetum cell layer plays an important role both in the regulation and coordination of pollen development, but also in providing materials for pollen wall formation. The single microspores are developed to produce a large vegetative cell (VC) and a smaller generative cell (GC) by asymmetric division during pollen mitosis I (McCormick, 2004). Following the first mitotic division, a second mitosis produces two sperm cells enabling double fertilization to produce the embryo and endosperm (Eady *et al.,* 1995).

Arabidopsis pollen development is similar to other species such as barley and lily. In barley anther, transverse sections showed high similarities when compared to Arabidopsis and rice (Fernandez Gomez *et al.,* 2015.) (Fig 1.17). However, differences were observed in the arrangement of tetrads within the anther locule, whereas tetrads can be observed filling the whole locule in Arabidopsis, in barley they aligned closely to the tapetum layer leaving a central empty locule (Fernandez Gomez and Wilson, 2012.) (Fig 1.18).

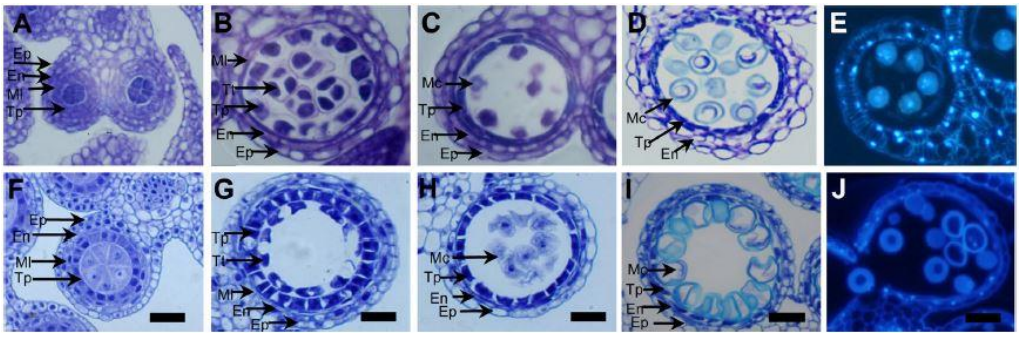


Figure 1. 18: Anther and pollen development sections of (A–E) Arabidopsis and (F–J) barley

Anther and pollen development follows a similar pathway in Arabidopsis and barley (A and F): Secondary sporogenous cells to pollen mother cells. Four cell layers surrounding the anther. (B and G): Microspore release from the tetrad; the tapetum becomes vacuolated. (C and H): Free microspores; middle layer becomes crushed and the prominent tapetum starts to degenerate. (D and I): Microspores become vacuolated and tapetum degenerates. (E and J): Trinuclear pollen; septum breakage and pollen release. Ep, epidermis; En, endothecium; Mc, microspores; Ml, middle layer; Tp, tapetum; Tt, tetrad. Bars= 50µm (taken from Fernandez Gomez *et al.,* 2015.)

It can be also be seen that in lily, the second mitotic division of the sperm cells (sc) occurs inside the pollen tube after pollination, however in *Arabidopsis* sperm cells (sc) this occurs in the anther prior to pollen release (Singh and Bhalla, 2007) (Fig1.19).

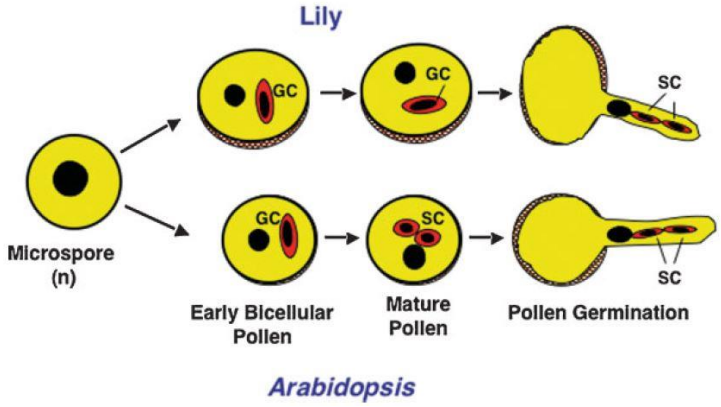


Figure 1. 19: Overview of sperm cell formation representing the stages of mature pollen in lily and Arabidopsis plants.

In lily the second mitotic division and sperm cells (sc) develop inside the pollen tube after pollination. In Arabidopsis sperm cells (sc) are produced from generative cell (GC) during pollen maturation in the anther (Singh and Bhalla, 2007)

1.5.1.2 Regulation of anther and pollen development

The regulation of anther and pollen development has been extensively studied in *Arabidopsis*, this information is being utilized for other crop species including Rice, (Zhang and Wilson, 2009), maize, barley and lily. Many genes involved in regulation of anther cell differentiation and development of pollen formation have been identified in Arabidopsis (Wilson *et al.,* 2011) (Fig 1.20).

Schiefthaler *et al.,* (1999) and Yang *et al., (*1999) demonstrated that the SPOROCYTELESS/ NOZZLE (SPL/NZZ) is one of the earliest gene required for the initiation of the cell division and differentiation in both anther and carpels of *Arabidopsis*. It is induced by the MADS box transcription factor AGAMOUS (AG) (Ito *et al.,* 2004) and during late development via induction of (DAD1) DEFECTIVE IN ANTHER DEHISCENCE1 (Ito *et al.,* 2007b). NZZ/SPL is thought to act in the anther L2 cell layer during archesporial division. Archesporial cells form normally in the *nzz/spl* mutant, however the formation of pollen mother cells (PMCs) and the surrounding cell layers fails (Schiefthaler *et al.,* 1999; Yang *et al.,* 1999). EXTRA SPOROGENOUS CELLS/ EXS MICROSPOROCYTES1 (EXS/EMS1) genes are also linked to the archesporial cell division in the anther and control of the division number of cells in L2 layer that sporogenous cell initials are regulated (Canales *et al.,* 2002; Zhao *et al.,* 2002). Fig (1.21) shows genes regulating cell differentiation in early stages (1-5) of anther development (Sharma and Nayyar, 2016).

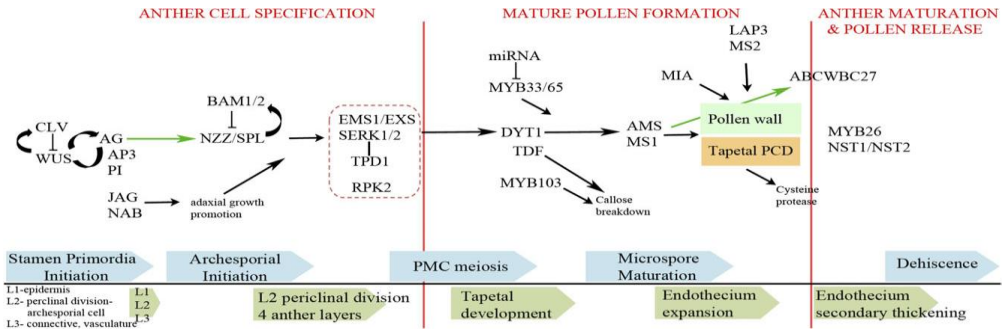


Figure 1. 20: Anther and pollen development pathway (taken from Wilson et al., 2011).

Anther and pollen development pathway. WUSCHEL (WUS) activates AGAMOUS (AG) at the centre of the floral apex, the number of pluripotent cells increases, then AG represses WUS to promote differentiation. Stamen initiation is controlled by the homeotic genes APETALA3 (AP3), PISTILLATA (PI), and AG, with the primordia forming as a tetrad of archesporial cells. AG induces microsporogenesis via activation of NOZZLE/SPOROCYTELESS (NZZ/SPL) (Ito *et al.*, 2004). The transcription factors JAGGED (JAG) and NUBBIN (NAB) are also involved in the process of defining stamen structure (Dinneny *et al.*, 2006). Two CLAVATA 1-related leucine-rich repeat receptor-like protein kinases, BARELY ANY MERISTEM1 (BAM1) and BAM2, act in a regulatory loop with NLZ/SPL to promote somatic cell types and to restrict NZL/SPL expression to the inner region of the locule (Hord *et al.*, 2006; Feng and Dickinson, 2010). Archesporial cell number and tapetal cell fate is controlled by a leucine-rich repeat receptor kinase EXTRA SPOROGENOUS CELLS/EXCESS MICROSPOROCYTES1 (EXS/EMS1) (Canales et al., 2002; Zhao *et al.*, 2002) and its ligand TAPETAL DETERMINANT1 (TPD1) (Jia *et al.*, 2008). The SERK1 and SERK2 complex is thought to form a receptor complex with EMS1 in the tapetal plasma membrane (Albrecht *et al.*, 2005; Colcombet *et al.*, 2005), which then binds TPD1 (Yang *et al.*, 2003, 2005) (indicated by the red dotted line). Tapetal development is initiated by DYSFUNCTIONAL TAPETUM1 (DYT1) (Zhang *et al.*, 2006) and DEFECTIVE IN TAPETAL DEVELOPMENT AND FUNCTION1 (TDF1) (Zhu *et al.*, 2008), with tapetal maturation, pollen wall formation, and tapetal programmed cell death (PCD) involving ABORTED MICROSPORES (AMS), MALE STERILITY1 (MS1) (Wilson *et al.*, 2001), and ABCWBC27 (Sorensen *et al.*, 2003; C. Yang *et al.*, 2007; Xu *et al.*, 2010) with the MALE GAMETOGENESIS IMPAIRED ANTHERS (MIA) gene, encoding a type V P-type ATPase, MALE STERILITY2 (MS2), and LEUCINE AMINOPEPTIDASE (LAP3). The final stage of dehiscence involves jasmonic acid (JA)-induced gene expression and transcription factors associated with endothecium secondary thickening, MYB26 (Steiner-Lange *et al.*, 2003; Yang *et al.*, 2007) and the NAC SECONDARY WALL THICKENING PROMOTING FACTOR1 (NST1) and NST2 double mutant (Mitsuda *et al.*, 2007). Regulation is indicated by arrows; green arrows indicate proven direct regulation; inhibition is shown by a T-bar.

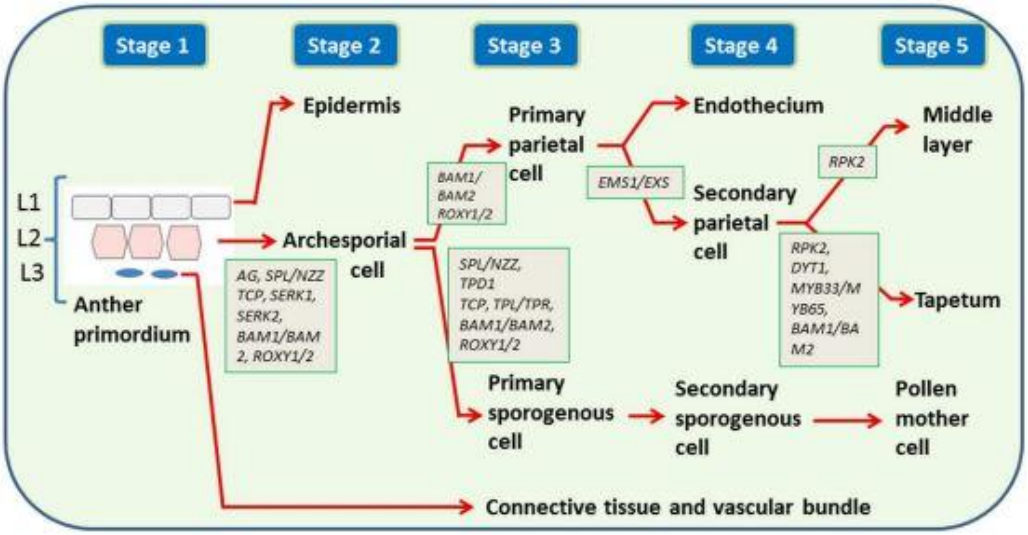


Figure 1. 21: Genes regulating cell differentiation in early stages (1-5) of anther development.

Three cell layers (L1, L2, L3) present in anther primordium divide to form: L1: epidermis (E), L2: endothecium (En), middle layer (ML), tapetum (T), and pollen mother cell (PMC), L3: connective tissue and vascular bundle (C). The development of L2 to En, ML, T, and PMC proceed through several intermediates, e.g., primary parietal cell, archesporial cell, secondary parietal cell, primary sporogenous cell, secondary sporogenous cell. The genes are shown in italics. AG: AGAMOUS, SEP: SEPTELLA, BAM: BARELY ANY MERISTEM, ROXY1/2: Arabidopsis CC-TYPE GLUTAREDOXINS, SPL/NZZ: SPOROCYTELESS/NOZZLE, TCP: TEOSINTE BRANCHED1/CYCLOIDEA/PCF transcription factors, SERK: SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE, EMS1/EXS: EXCESS MICROSPOROCYTES1/EXTRA SPOROGENOUS CELLS, RPK2: RECEPTOR-LIKE PROTEIN KINASE2, DYT1: DYSFUNCTIONAL TAPETUM1, TPD1: TAPETAL DETERMINANT 1, TPL/TPR: TOPLESS/TOPLESS-RELATED (taken from Sharma and Nayyar, 2016

In *Arabidopsis thaliana* the mutant gene *EXTRA SPOROGENOUS CELLS 1/EXCESS MICROSPOROCYTES1* (*exs1/ems1*), produces excess microsporocytes whilst the tapetum and middle cell layer are absent (Canales *et al.,* 2002 and Zhao *et al.,* 2002). TAPETAL DETERMINANT1 (TPD1) is required for the specialization of tapetal cell for microspore formation in the *Arabidopsis* anther. The absence of TPD1 causes the precursors of the tapetum cells to fail to differentiate into the tapetum cell layer, but develop into additional microsporocytes. Nevertheless, both *ems1* and *tpd1* mutants are male sterile with degeneration of the microsporocytes during meiosis II, suggesting that a functional tapetum is required for development of pollen (Yang *et al.,* 2003)

DYT1 (*DYSFUNCTIONL TAPETUM 1*) acts downstream of SPL/NZZ and EMS1/EXS, and is required for tapetum differentiation (Zhang *et al.,* 2006). It has been shown to be involved directly and indirectly in the regulation of many tapetum genes such as *ABORTED MICROSPORES* (*AMS)* and *MALE STERILITY1 (MS1)* (Zhang *et al.,* 2006 and Feng *et al.,* 2012). TAPETAL DEVELOPMENT AND FUNCTION (TDF1/MYB35) acts downstream of DYT1 and upstream of *ABORTED MICROSPORES* (AMS) (Zhu *et al.,* 2008; Zhu *et al.,* 2011). DYT1 directly regulates TDF1 in the tapetum, the expression of downstream gene including *AMS, MS188/MYB80, TEK, MS1* and other sporopollenin genes can be restored by expression of TDF1 in the DYT1 mutant (Gu *et al.,* 2014). AMS acts as a regulator of tapetal development as well as in the synthesis of pollen wall materials including lipidic and phenolic components (Xu *et al.,* 2010). The *ams* mutant showed premature microspore degeneration and absence

of sporopollenin deposition (Xu *et al.*, 2014); the tapetum also becomes abnormally enlarged and vacuolated (Sorensen *et al.*, 2003).

Generally, Arabidopsis pollen development is similar in other species such as rice, barley, wheat and lily, and therefore information on the regulation of these networks can be translated into these different crop species (Wilson and Zhang, 2009 and Gomez *et al.*, 2015) (Fig 1.22). Such knowledge provides opportunities to understand and manipulate these processes for agricultural improvement.

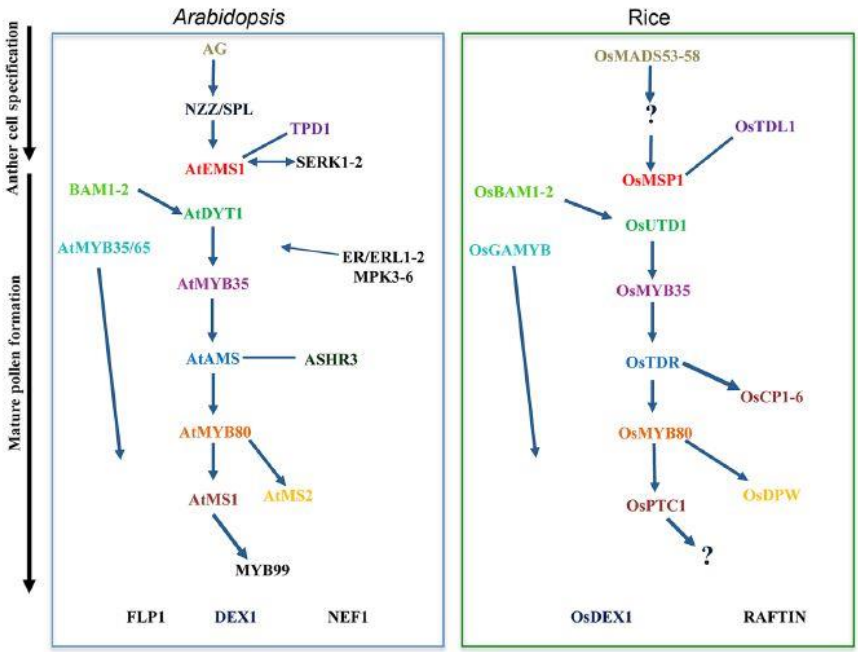


Figure 1. 22: Diagram comparison gene regulation network for Arabidopsis and rice during anther and pollen development from anther cell specification to mature pollen formation. (Gomez et al., 2015).

1.5.2 Conservation of pollen development pathway in another species

1.5.2.1 Barley pollen development

Barley flowers form as an inflorescence, referred to as a spike (ear, or head) which are composed of a number of individual spikelets or floret. A barley floret is perfect, consisting of 3 stamens with female organ (ovary) that has two feathery stigmas to collect the pollen, which is partially enclosed by two sheathes (barley empty glume or lemma) that are prolonged at the tip into a small awn, the palea sheath encloses the reproductive parts of the flower. (Kirby and Appleyard 1987; Komatsuda *et al.* 2007). Figure 1.23 shows schematic representation of barley inflorescence architecture.

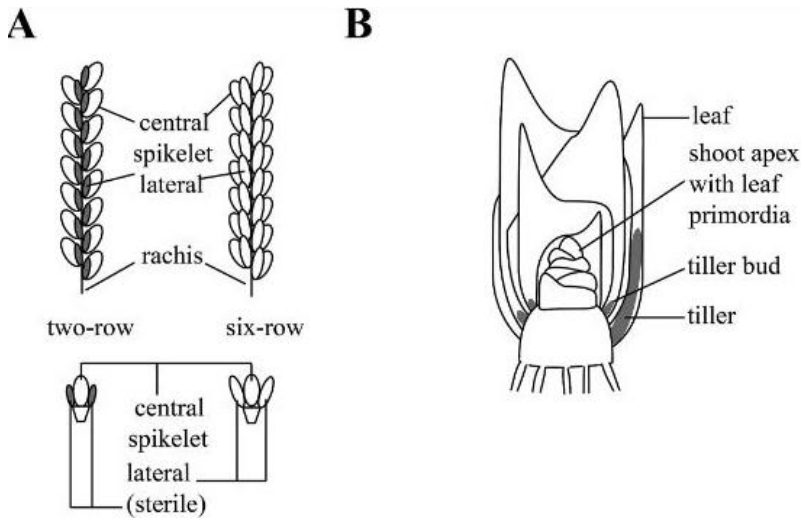


Figure 1. 23: Schematic representation of barley inflorescence architecture.

(A) Branched grass inflorescence architectures of; two-rowed and six-rowed barley *(H. vulgare*) spike. (A bottom) Single spike of two- and six-rowed barley. (B) Representation of the shoot architecture found in grass species (taken from Brit-Liller *et al.,* 2015).

Flower development in barley occurs mainly within the pseudostem shoot, making it difficult to study reproductive stages without dissection (Fernandez Gomez and Wilson, 2012). However, a staging system was developed that enabled the morphological characterization of anther and pollen development associated with spike size and the last flag elongation stages (Fernandez Gomez and Wilson, 2012). The key developmental stages relative to bud size and flag leave development are shown in Fig 1.24.

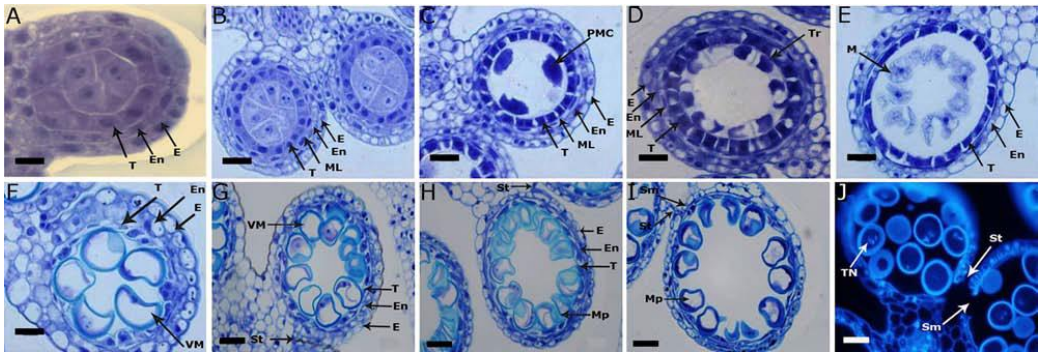


Figure 1. 24: Barley anther and pollen development relative to spike size: transverse sections of barley anthers from spike sized 0.6–5.7 cm.

(A–F) and the last flag elongation stages (G–J). These samples were collected from within the spike ranges specified in Table 1.1. (A) Primary sporogenous cells; three layers surrounding the anther locule (spike 0.6 cm). (B) Secondary sporogenous cells to pollen mother cells; four cell layers surround the anther (spike 1.7 cm). (C) Pollen mother cells enter into meiosis; tapetum layer is prominent (spike 2.8 cm). (D) Microspores released from the tetrad; tapetum becomes vacuolated (spike 3.5 cm). (E) Free microspores; middle cell layer undergoes crushing. The prominent tapetum layer starts to degenerate (spike 4.9 cm). (F) Microspores become vacuolated; tapetum is degenerating (spike 5.7 cm). (G–H) Mitosis I; tapetum is degenerating, but still present. (I) Binuclear pollen; mitosis II occurs. (J) Trinuclear pollen; septum breakage. Sections represented are examples of the mentioned ranges; A–I, toluidine blue; J, DAPI). BN, binuclear cells; E, epidermis; En, endothecium; ML, middle layer; Mp, microspores; N, nucleus; PMC, pollen mother cells; Sm, septum; St, stomium; T, tapetum; TN, trinuclear cells; Tr, tetrads; VM, vacuolated microspores. Bars, 40 lm. (Taken from Fernandez Gomez and Wilson, 2012)

Anther and pollen developmental in barley were characterised by Murray *et al.*, (2003) and anther development was divided into 11 development stages from early development (archesporial initials present) to dehiscence (Table 1.3). A detailed study of anther and pollen development was subsequently published by Fernandez-Gomez and Wilson, (2012). Transverse sections of barley anthers showed high similarities to Arabidopsis and rice (Fernandez Gomez *et al.*, 2015.) However, differences were observed in the arrangement of tetrads within the anther locule, whereas tetrads can be observed filling the whole locule in Arabidopsis, in barley tetrads were aligned closely to the tapetum layer leaving a central empty locule (Fernandez Gomez and Wilson, 2012.) (Fig 1.17).

Table 1. 3: Anther development stages in barley (Murray et al., 2003)

Stages	Developmental stages
1	Archesporial initials present; beginning of the four lobe condition
2	Primary sporogenous cells surrounded by three- layered wall
3	Four outer cell layers (epidermis, endothecium, middle layer and tapetum present. Secondary sporogenous cells present.
4	Tapetum prominent, pollen mother cells undergo meiosis.
5	Non-vacuolate microspores newly released from tetrad. Middle layer compressed
6	Vacuolate microspores (1.8mm anther) Tapetum degenerating
7	Vacuolated microspores (2.3mm anther)
8	Bicellular vacuolated pollen
9	Pollen grains accumulating starch
10	Pollen grains completely starch filled
11	Dehiscence

Regulation anther and pollen development has been widely studied in Arabidopsis and this regulation network seems to be very conserved between dicots and monocots, including crops such as rice and barley (Wilson and Zhang, 2009; Gomez *et al.*, 2015; Table 1.4).

Table 1. 4: Anther and pollen development gene network conservation in higher plants.

Arabidopsis	Ref	Rice	Ref	Barley	Ref	Wheat	Ref
AG	(1)	OsMADS53-58	(2)				
EMS1/TPD1	(3,4,5)	MSP1/OsTDL1	(6, 7)				
DYT1	(8,9)	UDT1	(10)				
TDF	(11)						
AMS	(12,13,14)	TDR	(13,15,16)				
MYB103/80	(17)	OsMYB103/80	(18)				
MS1	(19,20,21)	PTC1	(16)	HvMS1	(22)		
MYB33-65	(23)	OsGAMYB	(24)	HvGAMYB	(25)		
MS2	(26)	OsDPW	(27)				
DEX1	(28)	OsDEX1	(28)	HvDEX1	(28)		
		RAFTIN	(29)			RAFTIN	(29)

(1) Ito *et al.*, 2004; (2) Yamaguchi *et al.*, 2006; (3) Canales *et al.*, 2002; (4) Canales *et al.*, 2002; (5) Yang *et al.*, 2003; (6) Nonomura *et al.*, 2003; (7) Zhao *et al.* ,2008; (8) Zhang *et al.*, 2006; (9) Feng *et al.*, 2012; (10) Wang *et al.*, 2006; (11) Zhu *et al.*, 2008; (12) Sorensen *et al.*, 2003; (13) Xu *et al.*, 2010; (14) Xu *et al.*, 2014; (15) Zhang *et al.*, 2011; (16) Li *et al.*, 2006; (17) Zhang *et al.*, 2007; (18) Zhang *et al.*, 2010; (19) Vizcay-Barrena and Wilson, 2006; (20) Yang *et al.*, 2007; (21) Ito *et al.*, 2007; (22) Gomez and Wilson, 2014; (23) Millar and Gubler, 2005; (24) Kaneko *et al.*, 2004; (25) Murray *et al.*, 2003; (26) Aarts *et al.*, 1997; (27) Shi *et al.*, 2011; (28) Ma *et al.*, 2013; (29) Wang *et al.*, 2003(taken from Gomez *et al.*, 2015).

An important breeding objective is to control pollen release or viability, without affecting the other parts of the flower. One effective method to achieve this is to prevent self-fertilization by male sterility which is an important trait for hybrid breeding programmes (Whitford *et al.*, 2013 and Tien *et al.*, 2013). Cytoplasmic male Male Sterility (CMS) has been spontaneously indented in many plants and is

based on mitochondrial abnormalities that result in the inability to produce fertile pollen (Hanson and Bentolila, 2004 and Horn, 2006). This system has been used extensively for hybrid generation in rice, and maize (Longin *et al.*, 2012) and more recently the first commercial hybrid variety of barley (Colossus) was produced by CMS in the UK (Syngenta, July 2003). Hybrid rice is grown extensively across China using the CMS system (Yuan and Virmani 1988; Virmani 1994: Yuan *et al.*, 1994) and it is estimated that nearly 80-90% is derived from *Oryza rufipogon* (Longin *et al.,* 2012) Control of male reproduction helps facilitate plant breeding for generate stronger plant and high yielding, for instance F1 hybrid rice yields 15-20% more than most inbred varieties (Zhong *et al.,* 2004 and Cheng *et al.*, 2007). Therefore, the control of pollen development and release is of major commercial importance for development of hybrid lines, selective breeding and to reduce the spread of genetically modified pollen (Gomez and Wilson, 2014).

1.5.2.2 Lily pollen development

The lily flower comprises of three petals and three sepals (oblanceolate shapes), with six longer stamen and gynaecium, which have three carpels and a superior ovary (Judd *et al.,* 2008) **(**Fig 1.25 A and B**)**. Flower color in lilies depends on section and species, for instance, Asiatic hybrids have enormous colour variation in their tepals (white, yellow, orange, purple, red, salmon and pink) and are early flowering. In contrast, Longiflorum hybrids have white flowers, which are trumpet, flat or bowl-shaped. Lilies have non-truncated bulbs, which are white-yellow, consist of many scales, and food storage materials compressed into the stem and basal plate.

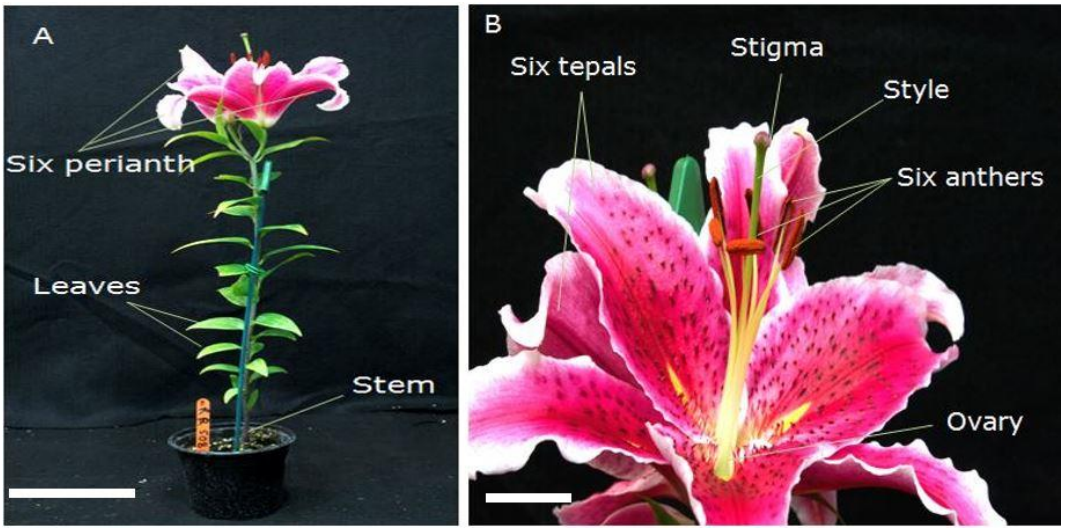


Figure 1. 25: (A) Lilium cv.Star Gazer showing all parts. Bar =9cm

(B) Detailed parts of the *Lilium* flower. Bar = 3 cm

Development of male germ-line in lily is similar to Arabidopsis except that mature pollen grain (two sperm cells) was produced from the haploid microspore by asymmetric division in Arabidopsis (Fig 1.26). Single microspores develop by asymmetric division to produce a large vegetative cell (VC) and a smaller generative cell (GC) (McCormick, 2004). The second mitotic division of the sperm cells (sc) occurs inside the pollen tube after pollination in lily, whilst in *Arabidopsis* and rice, sperm cells (sc) are produced during pollen maturation in the anther (Singh and Bhalla, 2007). In addition, cross-section of lily anther (*Lilium* sp.) was shown and explained apart of lily pollen development (Fig 1.27).

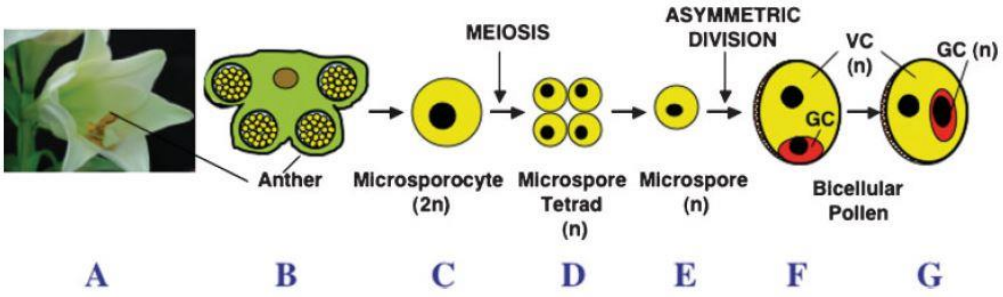


Figure 1. 26: Development of male germ-line in lily:

A; Male reproductive organs, stamen (yellow anther) in a Lily flower. B; Illustration of cross sections of an anther showing microsporocytes (2n). C: Microsporocytes (2n). D: Haploid microspores of tetrads (n). E: A microspore (n). F: Early bicellular pollen, vegetative cell (VC), Generative cell (GC). G: Mature bicellular pollen (take from Singh and Bhalla, 2007).

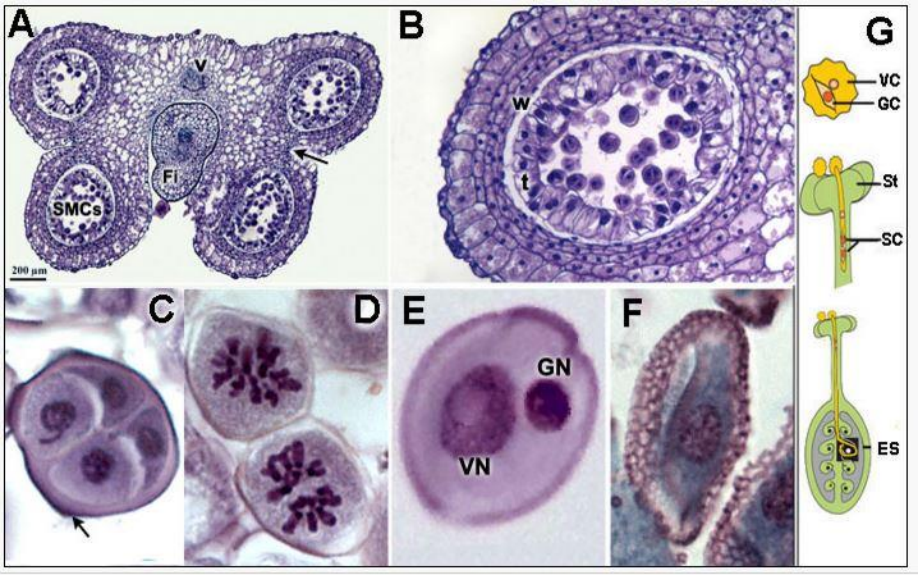


Figure 1. 27: Lily pollen development.

A: Cross-section through a lily anther (*Lilium* sp.) In the loculi sporemothercells (SMCs) can be seen from which the four spores develop through meiosis I and II. Inbetween the loculi of each pair a thin layer of cells (arrow) is visible along which the locules burst open at maturity to release the pollen grains. In the middle the cross-sectioned filament (Fi) to which the anther is attached is indicated.

B: Locule. The central lumen contains the developing pollen. On the inner wall (w) of the loculus a layer of block-shaped tapetum (t) cells can be seen.

C: Tetrad stage during pollen development. After the two meiotic divisions, the four daughter cells are still interconnected and form a tetrad. They are still surrounded by the wall (arrow) of the original microspore mother cell (MMC).

D: Mitotic division of the spore leading to the formation of a microgametophyte or pollen. Only the metaphase is shown here. The chromosomes lay in the equatorial plane of the cell.

E: Nearly mature pollen grains with a vegetative cell with nucleus (VN), which later will form the pollen tube, and a generative cell with its own nucleus (GN), which later will divide into two sperm cells.

F: Mature pollen grains in which the texture of the outer cell wall, the exine, can be recognized. The grainy dark purple structure in the middle of the pollen grain is the vegetative nucleus.

G: Schematic diagram showing (top) mature (middle) pollen grain consisting of the vegetative cell (VC) and therein the smaller generative cell (GC). After landing on the stigma (St) the pollen grain germinates and forms a pollen tube. In the pollen tube the generative cell divides into two sperm cells (SC). The pollen tube grows to the embryo sac (ES) and delivers the two sperm cells that are involved in double fertilization (bottom) (taken from http://www.vcbio.science.ru.nl/en/virtuallessons/pollendevelopment).

Lily plants can be used for physiological, cytological, and molecular investigation of pollen development, because lily pollen grains are relatively large (Vidali *et al.*, 2009) and the *Lilium* genus has a very big flower compared to the model plants. Nevertheless, the manipulation of this species is limited to date, particularly due to the difficulties of transformation.

Lily pollen can trigger allergies in pets particularly cats and humans. Many researchers have demonstrated that during plant reproduction Nitric oxide (NO) and nitrite are released from hydrating pollen, and can causes allergenic rhinitis (hay fever) in humans (Bright *et al.,* 2009). In addition, toxicity to cats and dogs has been identified (Stokes and Forrester, 2004; Thompson, 2012). Fitzgerald (2010) reported that the ingestion of lily pollen with water soluble flower parts can cause acute nephrotoxic syndrome, also after pollen ingestion in pets, vomiting and gastrointestinal problems can occur; both conditions can lead to death of the animals. The large amounts of pollen released in lilies also impairs the appearance of the flowers and can cause problems for commercial ornamental production, particularly as cut flowers; genetic manipulation may potentially provide a significant opportunity to resolve these problems. Thus, an important breeding objective is to control pollen release without affecting flower structure.

1.6 AIMS OF THE PROJECT

The aims of this project were to manipulate key traits of agronomic importance in Lily (*Lilium* Stargazer) and barley (*Hordeum vulgare L.*) by genetic manipulation using *Agrobacterium* transformation. Successful transformations were confirmed and transgene expression has determined using molecular techniques. The overall goal of this work was to develop novel traits of importance in commercially significant crops.

The specific objectives of this project were:

1- Tissue culture and transformation of *Lilium* to prepare constructs for targeted manipulation of traits in lily flowers. This work included firstly, analysis of pollen development pathways in lily, characterization and preparation of reporter constructs using a variety of promoters, both tissue-specific and constitutively expressed. An HvMYB26SRDX construct under control of the rice OsERFLP anther-specific promoter was also generated to target repression of the anther dehiscence associated MYB26 gene, to the anther. Secondly, to delay senescence in Lilium, particularly flower and petal longevity, by overexpression of the AtIPT4 gene (Chapter 3).

2- To manipulate and characterise pollen development, and thus male fertility, in barley for application in future breeding programmes, by overexpression of the putative barley orthologue of the *Arabidopsis DYSFUNCTIONAL TAPETUM* 1 (*DYT1*) gene (Chapter 4).

3- To manipulate senescence in barley by controlling the levels of cytokinin by manipulating the expression of *ISOPENTENYL TRANSFERASE* (*IPT*) genes. Molecular studies of the genes involved in cytokinin metabolism and signalling have demonstrated that over-expression of the *IPT* genes in transgenic plants can cause an increase in the levels of cytokinin and reduce leaf senescence. Overexpression of the *Arabidopsis AtIPT4* gene was conducted in barley under the control of the ubiquitin promoter to try to delay senescence in barley (Chapter 5).

CHAPTER 2: GENERAL MATERIALS AND METHODS

2.1 PLANT MATERALS AND GROWTH CONDITIONS

2.1.1 Barley plant

Spring barley c.v, Golden Promise seeds were obtained from University of Nottingham and grown in 36 well pots with John Innes N°3 compost, in a growth room under controlled conditions (16 h photoperiod; 15°C for day and 12°C for night; 80% relative humidity and 500µmol/m2/s light levels from metal halide lamps (HQI) complemented with tungsten bulbs at the mature plant canopy level). After, 2-3 weeks Zadok stage 11-12 (one shoot with 1- 2 unfolded leaves) plants were transplanted, four plants per 5 litres pot, (Levington C2 compost) according to Zadoks *et al.*, (1974).

2.1.2 Lily plants

Lily bulbs of variety Star Gazer were obtained from J. Parker Dutch Bulbs Company. These were transferred into standard compost, comprising Levington M3, John Innes No.3, perlite and vermiculite in the ratio (3:3:1) containing the systemic insecticide intercept 5 GR (21g in 75L Scott Company UK). Bulbs were grown in a controlled environment room (20˚C ± 2 ˚C for day and night temperatures), under light intensity 111µmol s^{-1}m $light^{-2}$ (warm fluorescent bulbs). Materials were individually grown in 13cm pots. After 40 days leaves from young plant were taken for genomic nucleic acid extraction.

2.1.3 Arabidopsis plants

Arabidopsis ecotype *Landsberg* seeds were obtained from Nottingham Arabidopsis Stock centre (NASC). Seeds were sown in 13 cm plastic pots containing Levington M3 compost (Scott's Company, UK). Intercept (21g in 75l Scott Company UK) was added. Plants were grown in a controlled growth room (20˚C ± 2 ˚C day/night, 22 hour photoperiod and 75% RH; 111µmol s^{-1}m $light^{-2}$; warm white fluorescent light. Transparent plastic sleeves were used to avoid cross-pollination.

2.2 TISSUE CULTURE AND PLANT MATERIALS MAINTENANCE.

2.2.1 Immature embryo isolation from barley

Golden Promise variety was grown as described at Section (2.1.1). Spikes were collected at grain filling stage; 1.5 to 2 mm immature embryos were needed which were found within seeds that present a milky texture. After spikes were collected, seeds were transferred to a sterile jar and sterilised by washing them with 70% (v/v) ethanol for 30 seconds and 4x 50% (v/v) sodium hypochlorite solution for 5 minutes. Finally, seeds were rinsed 4 times with sterile water. Immature embryos were extracted in a sterile laminar flow hood using sterile forceps and a sterile black tile. The embryogenic axis was carefully removed without damaging the embryo to avoid the regeneration of roots and shoots (Fig 2.1). The isolated embryos were placed on Callus Induction Medium without antibiotics (Appendix 4) scutellum side up. Then plates were kept in a dark room, 22-23°C for 24 hours after which embryos were ready for transformation.

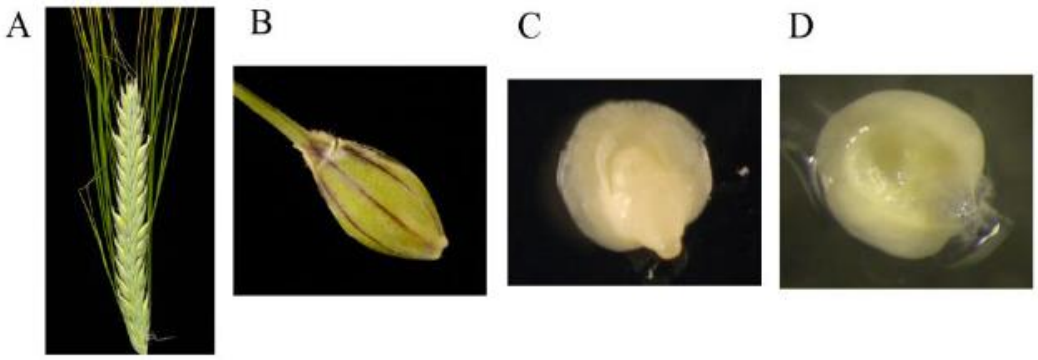


Figure 2. 1: Stages for embryo isolation for genetic transformation take from (Harwood et al., 2009)

A: A barley spike with immature embryos. B: Isolated immature seed from spike.

C: Immature embryo with embryonic axis. D: Immature embryo without axis.

2.2.2 Lily tissue culture

Star Gazer bulbs were used for *in vitro* bulb-let induction; bulb scales were washed under running tap water to eliminate the excess soil, any rotten or necrotic part were removed with a scalpel and then the upper 1cm apex of scales were selected. The cleaned tissues were then sterilized by immersion in a 2% (v/v) trigene solution for 10 minutes and then rinsed 3 times with sterile water. They were immersed in 15% (v/v) Domestos solution (Unilever) for 30 minutes with 2-3 drop of Tween 20 (0.1%) and then rinsed 3 times with sterile water. Finally, they were immersed in 50% (v/v) ethanol solution for 30 seconds and then rinsed 3 times with sterile water. The sterilised scales were placed onto sterilized filter paper (Whatman® qualitative filter paper grand 1 (110mm)) in a laminar airflow cabinet to dry. Scales were then sliced horizontally (1-2 mm thin depending the scales

size) and were placed horizontally onto MS-SI (MS-Shoot Induction) medium (Appendix 4) in Petri dishes. Each dish contained 25ml of 4.4g/L MS basal salts supplemented with 60g/L sucrose, 2.5g/L activated charcoal, 2.8 g/L phytagel, 2.0mg/L, picloram, 1.0mg/L BAP and lipocacid 100µM (Appendix 4). All Petri dishes were stored in the dark at 23±1°C. Petri dishes were checked every day for contamination, basal plates that were contaminated were removed and the rest was moved into new fresh medium and placed back into the growth room. After 5 to 7 weeks' bulblets developed from explants, these were then transferred individually into 50 ml glass jar containing 20 ml of MS-S1 medium (Appendix 4), and then incubated in the dark at 23±1°C.

2.2.3 *Arabidopsis* seed sterilization for transgenic screening

Mature seeds were sterilized in 1.5 ml Eppendorf tubes containing 1ml of 70% (v/v) ethanol and vortexed for 5 min. The tube was then centrifuged for 3 min at 12000 rpm, and the supernatant was removed. The previous step was repeated and 1000µl of 100% ethanol was added and then poured onto sterile filter paper and left to dry in a laminar airflow cabinet; dry seeds were screened on MS plate (Appendix 4) with selective antibiotics under sterile conditions. Plates was kept in 12 hours light, at 21±1°C for about 2 -3 weeks.

2.3 MOLECULAR BIOLOGY METHODS

2.3.1 Genomic DNA extraction from Barley.

Genomic DNA was extracted from barley tissues using Isolate II Plant DNA Kits according to the protocol supplied by the manufacturer, except that 30 µl of Sigma water was used to finally dissolve the DNA rather than buffer. DNA (1.5 µl) samples were checked for purity, quantity and quality using a Nano-drop (ND-1000 Spectrophotometer, Nano-Drop Technologies, Inc., and Wilmington, USA) and then stored at -20°C.

2.3.2 Genomic DNA extraction from *Lilium* plant by modified protocol of Doyle and Doyle, 1987.

The CTAB DNA extraction protocol (modification of Doyle and Doyle, 1987 protocol) was utilized to isolate genomic DNA. CTAB buffer was prepared as describe in (Appendix 4) Genomic DNA was isolated from young leaves of *Lilium cv.* Star Gazer. Pieces of leaf tissue (approximately 2-3cm) were collected from each plant and transferred into 1.5 ml microcentrifuge tubes and snap frozen using liquid nitrogen. A sterile Micro-pestle was then used to grind the frozen leaf to a fine powder; 500 µl of CTAB buffer was added into the tube and mixed by vortexing, and then incubated in water bath at 55°C for at least 1 hour and mixed once after 30 minutes. Then, samples were centrifuged at 13000rpm for 10 minutes. The liquid supernatant was carefully transferred in a new 1.5 ml microcentrifuge tubes; 2 µl of RNase A (10mg/ml) was added for each sample and then incubated at 37°C for 15 to 30 minutes. 500 µl of chloroform (99%) was then added with gentle mixing by pipetting and then centrifuged at 13000rpm for 10 minutes. The aqueous phase was carefully transferred into new 1.5 ml micro-centrifuge tubes. 0.08 volumes of cold

7.5M of ammonium acetate with 0.54 volumes of cold isopropanol was added for the precipitation of DNA and then mixed by inverting tubes 20 to 30 times by hand. This was stored in freezer -20 for 30-40 minutes, before samples were centrifuged at 13000 rpm for 3 minutes. The supernatant was discarded and then 700 µl of 70% (v/v) EtOH was added and tubes inverted 5-10 times to mix, and then centrifuged 13000rpm for 1 minutes. The supernatant was carefully discarded. This 70% EtOH step was then repeated. The supernatant was very carefully discarded without dislodging the pellet. To dry samples, tubes with pellet was left at room temperature for 1 hour. Once dry, 50 µl of TE buffer (Appendix 4) was added to suspend the DNA at room temperature for 1 hours, samples were then stored at -20˚C.

2.3.3 Genomic DNA extraction from *Arabidopsis* tissues.

Young leaves (2-3mm diameter) were placed in directly into 100µl of sucrose buffer on ice (Table 2.1) and then crushed using a yellow pipette tip which was then used to label the sampled plant. The samples were then heated to 100°C for 10 min and stored at -20°C; 1 µl of supernatant was used per 10 µl PCR reaction.

Table 2. 1: Sucrose solution for Arabidopsis DNA isolation.

Component	Stock conc. mM	Working conc. mM	For 10 ml
Tris- Cl (pH 7.5)	1000	50	0.5
NaCl	4000	300	0.75
Sucrose	2000	300	1.5
dH2O	-	-	7.25

2.3.4 Agarose gel electrophoresis.

PCR products were generally analysed by running the product in a 1% (w/v) agarose gel in 0.5x TBE buffer (Appendix 4); 5 µl of ethidium bromide (EtBr) 500µg/ml was added to the cooled gel. 1.2 µl of loading dye (1x diluted from 6x); was added to 10 µl of PCR phusion amplified product and mixed. Samples were loaded into the wells with; 2.5 µl of Hhyper ladder I (Bio line, London, Uk) was loaded alongside to enable accurate sizing of products. Gels were run at a constant voltage of 100V for 60 minutes, then imaged using a UV transilluminator. The image were captured by a digital imaging system (Synegene, Cambridge, UK).

2.3.5 RNA Extraction.

QIAGEN RNeasy Plant Mini Kit was used for RNA isolation; young leaves (approximately 100-150 mg) of *Lilium*, barley open flowers, and Arabidopsis leaves were collected and transferred into 1.5 micro centrifuge tubes and snap frozen using liquid nitrogen. Total RNA was extraction following the manufacturers' protocol except that samples were resuspended in 30 µl of Sigma water. Samples were analysed by Nano-Drop and then stored at -80°C.

2.3.6 cDNA synthesis.

Total RNA, was prepared as described in Section 2.3.4, and was used for synthesis of cDNA. 1.5 µg of RNA was added to a 0.6 ml microcentrifuge tube along with 1 µl of 10 mM dNTP, 1 µl of oligo dT and Sigma water (Sigma Aldrich, Gillingham, Uk, W4502) to a total volume of 13 µl. After that, the mixture was incubated in a PCR machine for 5 minutes at 65°C and then transferred quickly into ice for 1 minute. 4 µl 5x First-Strand buffer, 1 µl 0.1M DTT, 1 µl RNase OUT

and 1 µl of Super Script III RT (2200u/ µl) were added and mixed gently by pipetting. The mixture was incubated for 1 hour at 50°C, and then inactivated by heating 15 minutes at 75°C. The cDNA was stored at -80°C until required.

A similar procedure was followed to prepare samples for negative RT-PCR, the same amount of RNA was used for the cDNA synthesis however no reverse transcriptase was added. To check the quality of the barley cDNA samples, the α-Tubulin (House-Keeping) gene was used; 28 cycles of PCR amplification were performed using α-TubF/R primers at 61°C. These primers produced different sized PCR products depending on the amplification template; 200 or 250 bp will be obtained for cDNA or gDNA respectively. This was used to give information about any gDNA contamination in the cDNA samples.

2.3.7 PCR Phusion

Phusion PCR reactions were prepared as described in Table 2.2.

Table 2. 2: PCR phusion reaction mixture

Reaction	**Volume µl for 1 x**
5x HF Buffer	1.2 µl
DNTp2.5mM	0.32 µl
DMSO%100	0.36 µl
Forward primer (20 pmol/ µl)	0.2 µl
Reverse primer (20 pmol/ µl)	0.2 µl
Genomic DNA (30-40 ng/ µl)	1 µl
Phusion DNA polymerase enzyme 2u/ml	0.18 µl
Water dH_2O	6.54 µl
Total volume	**10 µl**

2.3.8 PCR genotyping transgenic analysis.

Putative transgenic plants were screened by PCR analysis using a forward primer from the transgene construct combined with a target gene reverse primer. PCR reactions were prepared as described in Table 2.3 and the temperature cycling conditions as in Table 2.4. A detailed list of primers is given in Appendix 3.

Table 2. 3: PCR reaction mixture for transgenic analysis

Reaction	**Volume µl for 1 x**
Sigma RED Taq Mix	5 µl
Forward primer (20 pmol/ µl)	0.3 µl
Reverse primer (20 pmol/ µl)	0.3 µl
Genomic DNA (30-40 ng/ µl)	1 µl
Water dH_2O	3.4 µl
Total volume	**10 µl**

Table 2. 4: PCR cycling for genotyping analysis.

Steps	**Temperature**	**Time**	**cycles**
Initial Denaturation	94°C	6 minutes	1
Denaturation	94°C	30 seconds	
Annealing	59°C*	30 seconds	35
Extension	72°C	30 seconds	
Final Extension	**72°C**	**6 minutes**	**1**

*** Temperature depends on Tm of primers**

2.3.9 Reverse transcriptase RT-PCR.

After confirmed transgenic lines by PCR, RT-PCR was used for checking gene expression in transgenic lines. Tables 2.5 and 2.6 show the conditions used for the analysis.

Table 2. 5: RT-PCR reaction mixture for transgenic analysis.

Reaction	**Volume µl for 1 x**
Sigma RED Taq Mix	5 µl
Forward primer (20 pmol/ µl)	0.3 µl
Reverse primer (20 pmol/ µl)	0.3 µl
Genomic cDNA(30-40 ng/ µl)	1 µl
Water dH2O	3.4 µl
Total volume	**10 µl**

Table 2. 6: RT-PCR cycling for transgenic line.

Step	**Temperature**	**Time**	**cycles**
Initial Denaturation	94°C	3 minutes	1
Denaturation	94°C	30 seconds	28
Annealing	59°C*	30 seconds	
Extension	72°C	30 seconds	
Final Extension	**72°C**	**6 minutes**	**1**

2.3.10 Quantitative Reverse Transcriptase PCR (qRT-PCR).

qRT-PCR was used to quantify gene expression. Total RNAwas extracted and cDNA synthesis performed as specified in Sections 2.3.5 and 2.3.6 respectively. Analysis was conducted using a Light Cycler® 480 real-time PCR system (Roche Applied Sciences, West Sussex, UK), 1.5 µg of RNA with Brilliant SYBR Green qPCR master mix (Agilent Technologies Inc., Santa Clara, CA). Expression was normalized using the alpha-tubulin gene in barley, or other housekeeping genes.

Table 2. 7: Reaction conditions used for qRT-PCR.

Reaction	**Volume µl for 1 x**
Water dH2O	1.8 µl
Genomic cDNA	0.4 µl
Brilliant SYBR Green Master Mix	4.5 µl
Forward primer (10 pmol/ µl)	0.2 µl
Reverse primer (10 pmol/ µl)	0.2 µl
Total volume	**7.1µl**

Table 2. 8: qRT-PCR cycling programs.

Steps	**Temperature**	**Time**	**cycles**
Preheat	95°C	1 minutes	1
Amplification	94°C	30 seconds	
Annealing	63°C*	30 seconds	50
Extension	72°C	30 seconds	
Final Extension	**72°C**	**6 minutes**	**1**

***Represent Tm annealing temperature specific for each primer pair.**

2.3.11 DNA purification from gel**.**

For DNA isolation from agarose gels, PCR amplified products were excised, minimising UV exposure, and transferred to a 1.5ml microfuge tubes. Dialysis membrane column (Sigma-Aldrich, Gillingham, and Uk D9777) was utilized for DNA extraction. Columns were stored in 1mM EDTA (4˚C); the column was first washed inside and outside with 0.5x TBE buffer (Appendix 4) and clipped on one side. Then 2 ml of 0.5x TBE were added to the column. Immediately, the gel slices were put into dialysis membrane column. The gel slices were placed to one side and the excess buffer solution with air bubbles were carefully removed from tube, and column closed by the second clip. The tube containing the sample was placed into the electrophoresis tank for 1hours at 100V, after which voltage was inverted for 30 sec to avoid the DNA sticking to the membrane wall. Buffer containing the DNA was then removed and transferred to a 1.5 ml tube and reduced by butanol reduction. Butanol reduction started by adding an equivalent amount of 100% butanol to the buffer containing the DNA. After mixing and centrifugation for 3 minutes at 12000rpm the supernatant was removed. This step was repeated 3 or 4 times with a constant reduction of buffer until the volume of the samples were reduced to 100-200 µl. Then, 5 µl of 3M sodium acetate and 120 µl of 100% ethanol were added. The mixture was kept at -20°C for 30 minutes. After that, the sample was centrifuged 12,000rpm for 10 minutes, the supernatant was removed and then 200 µl of 70% (v/v) ethanol was added to clean the DNA pellet. The mixture was centrifuged at 12000rpm for 10 minutes, and then the supernatant was removed, and the pellet air-dried. The pellet was re-suspended in 20 µl of Sigma pure water (Sigma Aldrich, Gillingham, UK, and W4502). Finally, 2 µl of

the DNA was used, to confirm the size and purity of the DNA, by gel electrophoresis after combining with 0.5 µl of loading buffer (6 x).

2.3.12 Calculation of transgene copy number in transgenic lines.

For determination of transgene copy number, qRT-PCR was used (Shepherd *et al.,* 2009) after gDNA was extracted from all lines (Section 2.13), concentration of DNA was obtained from Nano-drop (ND-1000 Spectrophotometer, Nano-Drop Technologies, Inc., and Wilmington, USA). To achieved an equal amount of DNA, each sample was diluted (1/10) and calculated depend on lowest concentration amongst the samples.

Controls samples provided by Dr Jose Fernandez contained one and two copies of the Hygromycin (Hyg) transgene that had been previously determined by Southern blot hybridisation (IDna Genetics Ltd., JIC Norwich). Copy number calculations were based on Shepherd *et al.,* (2009), by comparing the relative transgenic amplification (copy number) with an endogenous control gene that has a known gene copy number

2.4 OVER HANGING OF BLUNT-END PCR PRODUCT AND TOPO-CLONING

As some Taq polymerases produce Blunt ends, a sticky end needed to be added so the product could be cloned into a TOPO vector such as pCR8GW (Life Technologies Ltd, Paisley, UK) (Fig 2.2). An overhanging reaction was performed within a 0.6 ml micro-centrifuge tube where 100-200 ng of purified PCR product was mixed with 2µl of Taq polymerase, 2µl of 2.5mM dATP and 2µl of buffer to

reach a reaction volume of 15µl. Samples were incubated at 72˚C for 15 minutes (Table 2.9 overhang reaction).

Topo cloning occurred in a 6 µl reaction containing 1 µl of Topo cloning, 1 ul of salt water, 100-200 ng of purified PCR product and water up to 6µl. After mixing, reactions were kept at room temperature over-night. After incubation, the solution was transformed into *E.coli DH5ά.*

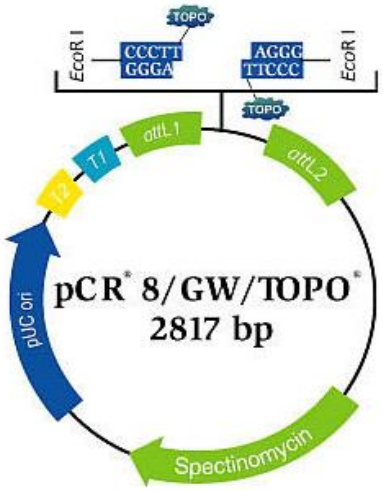


Figure 2. 2: PCR8/GW/ TOPO cloning vector.

https://www.thermofisher.com/order/catalog/product/K250020

Table 2. 9: Overhang reaction.

Reaction	Volume µl for 1 x
PCR product (20ng/µl)	8µl
dATP (10 mM)	2.5 µl
10x Taq reaction buffer	2 µl
Taq DNA polymerase (5U/µl)	2.5µl
Total volume	**15 µl**

Table 2. 10: TOPO cloning reaction.

Reaction	Volume µl for 1 x
PCR8GW TOPO	1µl
PCR product	5 µl
Salt solution (1.2 M NaCl and 0.06 M $MgCl_2$)	1 µl
Total volume	**7 µl**

2. 5 DIGESTION AND LIGATION.

Sac1 and *Xba1* were used to digest the PGKGWY vector (see appendix III). The reaction conditions were as described in Table 2.11.

Table 2. 11: Digestion reaction.

Components	**Insert PCR product (OsERFLP)** **100ng/ml 1x**	**Vector PGKGWY 67.7ng/ml 1x**
Cut smart buffer 10x	1.5 µl	1.5 µl
BSA 100mM	1 µl	1 µl
Template (40-50 ng/ µl)	1.4 µl	1 µl
Water	5.3µl	5.7 µl
Enzyme	0.4 µl	0.4 µl
Enzyme Xbal1	0.4 µl	0.4 µl
Total volume	**10 µl**	**10 µl**

PGKGWY vector was placed in to the PCR for 20 minutes at 80°C, then incubated at 37°C overnight. CIP treatment was conducted (1.67 µl of CIP (0.5 unit) with 3 µl of cut smart 10 x; for 1 hour at 37°C). The PCR product (insert) was placed into the PCR for 20 minute at 80°C, after 40 minutes dephosphorylat ions. Samples were prepared using the. QIA quick® PCR Purification kit. Five volumes of PB buffer were added to one volume of the digested samples, with gentle mixing (250 µl of PB buffer was added to the samples) and then transferred into a binding column and centrifuged at 12000 x g for 1 min. 750 µl of washing buffer PE was added and centrifuged at 12000 x g for 1 min. The, pervious step was then repeated without washing buffer PE. The column was transferred to a new PCR tube

and then 17 µl of pre-heated Sigma water added, and then centrifuged at 12000 x g for 1min.

For ligation, 3 µl of vector (PGKGWY 58ng/ µl), 8 µl of insert, 2 µl of T4 ligase (10X) enzyme and 2 µl of buffer (10X) were mixed together and incubated at 16°C overnight. Samples were transformed into chemically competent *Ecoli* cell as describe in Section 2.6.

2.6 TRANSFORMATION OF CHEMICALLY COMPETENT *E.coli.*

Chemically competent *E. coli* cells (*DH5ά*) (Supplied by Dr. Jose Fernandez) were incubated for 30 minute on ice. 3-4 µl TOPO cloning reaction was added to the chemically competent cells and incubated in a pre-warmed, 42˚C, water bath for 1 minute and a half. The reaction was then transferred onto ice for 1 minute. 250 µl of LB medium were added to the mix and incubated on a rotary 37˚C shaker at 150 rpm for 1.30 hours. After incubation, 50 and 150 µl of the transformed cell culture are spread onto semisolid LB medium (Appendix 4) plates with the appropriate antibiotic, and incubated at 37°C overnight.

2.7 COLONY PCR

Colony PCR was carried out to confirm positive cloning. The reaction conditions are described in Tables 2.12 and 2.13. Primer combination depended on the vector used and gene of interest; one within the vector and the other within the gene of interest. After preparing the master mix, 10 µl were transferred to PCR tubes. Colonies were numbered and a backup plate prepared. Each colony was touched with a thin pipette tip and the colony spread onto to the numbered backup plate

and then to the corresponding labelled PCR tube. This was repeated for every colony.

Table 2. 12: PCR colony reaction mixture.

Reaction	**Volume µl for 1 x**
Sigma RED Taq Mix	5 µl
Forward primer (20 pmol/ µl)	0.3 µl
Reverse primer (20 pmol/ µl)	0.3 µl
Genomic DNA (30-40 ng/ µl)	1 µl
Water dH2O	3.4 µl
Total volume	**10 µl**

Table 2. 13: PCR colony cycling conditions.

Step	**Temperature**	**Time**	**cycles**
Initial Denaturation	94°C	6 mines	1
Denaturation	94°C	30 sec	
Annealing	59°C*	30 sec	36
Extension	72°C	30 sec	
Final Extension	**72°C**	**6 mines**	**1**

*** Represent Tm annealing temperature specific for each primer pair.**

2. 8 PLASMID EXTRACTION FROM *E. COLI* COLONIES

GenElute plasmid mini prep kit was used for plasmid extraction from colony of *E. coli*. 10ml of overnight liquid culture was centrifuged and the pellet was utilized for the isolation of the plasmid by following the manufacturer's protocol supplied by the manufacture that just use except that 30 µl of Sigma water was used rather than buffer for suspending the samples. Quantity and quality of the DNA plasmid samples were checked by Nano-drop, and then stored at -20°C.

2. 9 SEQUENCING PCR PRODUCT.

Purified PCR amplicons (Section 2.3.11) and plasmid DNA (Section 2.8) containing a fragment of interest, were sent for sequencing to Eurofins MWG Operon (London, UK). Samples were prepared as specified by Eurofins guide (https://www.eurofinsgenomics.eu/ sample submission guide, value red tube): A DNA concentration of 50-100 ng/µl was mixed with 1.5 µl of an amplicon/vector specific primer and sigma water (Sigma Aldrich, Gillingham, UK, W4502) to a total a volume of 15 µl.

2. 10 GATEWAY CLONING- L.R REACTION

Gateway cloning technology is a cloning method to transfer DNA sequences into multiple vector systems by using bacteriophage lambda site specific recombination. Entry clones prepared by TOPO cloning as describe in Section 2.13, (Figure 2.3), were recombined into various destination vector by LR reactions as specified by the Manufacturer (https://www.thermofisher.com).The reaction master mixed consisted of: 100 ng/ µl of empty destination vector, 50 ng/ µl of entry clone car-

rying the fragment of interest, 1.5 µl of LR Clonase and Sigma water (Sigma Aldrich, Gillingham, UK, W4502) to 7 µl total reaction volume. The reaction was incubated at 25°C overnight. After incubation, 1.5 µl of proteinase K (2µg/µl) was added incubated in at 37°C for 10 min to terminate the reaction. 5 µl of the reaction was then transferred into the DH5ὰ *E. coli* cells and incubated for 30 minute in ice. The cells were heat shocked at 42°C for 2 minutes and then immediately transferred to ice for 1 minute. 250 µl of LB medium (Appendix 4) then was added it, the sample was incubated on a rotary 37˚Cc shaker at 150 rpm for 1.30 hours, after that, 50 µl were spread onto semisolid LB medium plates supplemented with the appropriate antibiotic depending of the plasmid utilized. Plates were incubated at 37°C overnight prior to plasmid extraction as describe at Section 2.14.

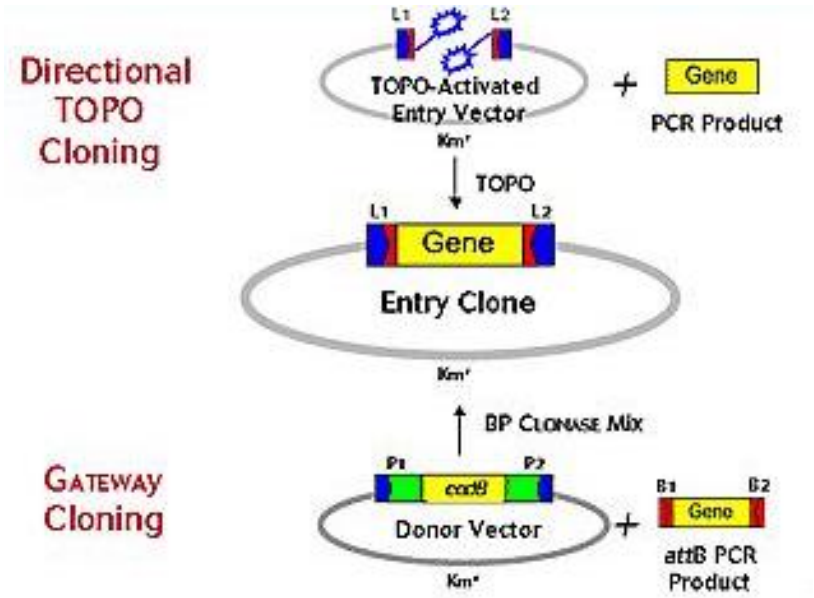


Figure 2. 3: Shows creation of an entry clone by GATEWAY cloning (cloning PCR products into entry clones)

http://homepage.univie.ac.at/nikos.pinotsis/webPP/genetoprotein/cloning_strategy/gateway/gateway_entryclone.html

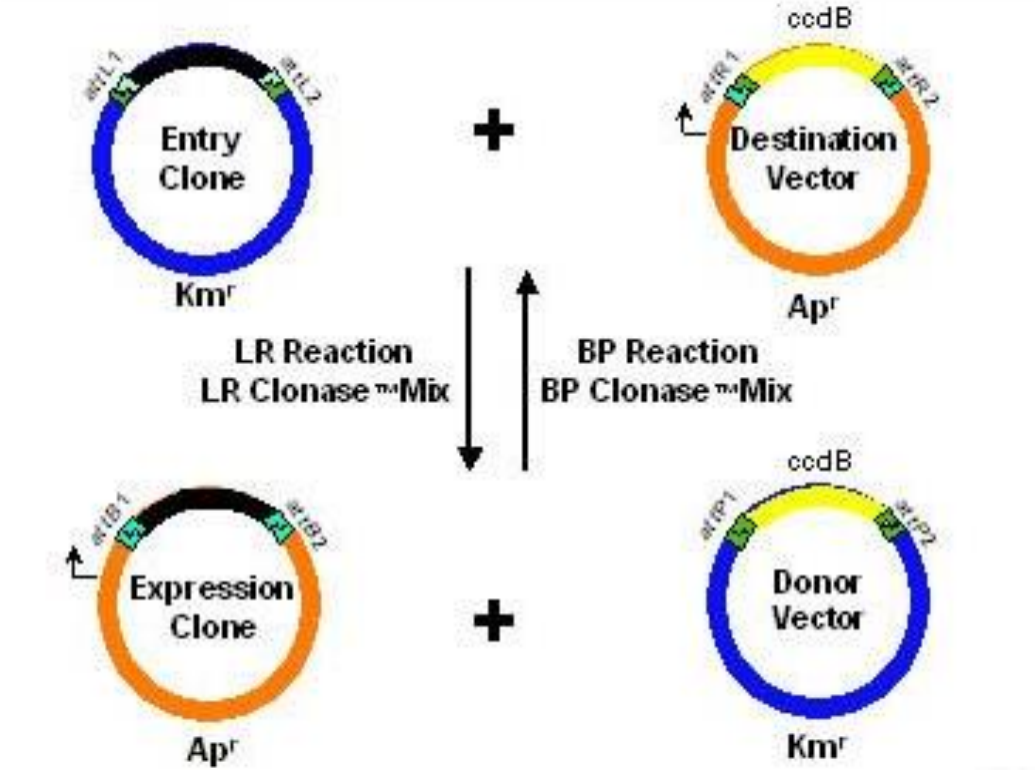


Figure 2. 4: Generated an expression clone by Gateway reaction between an entry clone that is flanked by two recombination site (attL1 and attL2) with a destination vector contains two recombination sites (attR1 and attR2).

https://tools.thermofisher.com/content/online%20seminars/gateway/7.htm

2. 11 GLYCEROL STOCKS PREPARATION.

Liquid culture were prepared by mixing 10 ml of LB (Appendix 4) with the selected positive colony and the appropriated antibiotic for selection, these were incubated overnight at 28˚C for *Agrobacterium*, or 37˚C for *E coli.* on a rotary at 150 rpm overnight. After incubation, and in a clean and sterilized microbiology cabinet, 250µl of 80% (v/v) sterile glycerol was well mixed with 750 µl of the liquid culture in 2 ml screw capped tubes before transferred into liquid nitrogen and then stored at -80° C.

2.12 *AGROBACTERIUM* STRAINS AND PLASMID FOR PLANT TRANSFORMATION.

Two *Agrobacterium tumefaciens* strains were used for transient expression: AGL1 for *Lilium* and Barley, and GV3101 for *Arabidopsis,* these were obtained from the Biosciences genomics facility, The University of Nottingham.

Two destination vectors PGWB3 with GUS, for promoter analysis, and PGWB4 with GFP to create GFP fusions were conducted (Nakagawa, 2002).

PGWB5 destination vectors containing (GFP) gene fused to the CaMV35S promoter and the neomycin phosphotransferase (*npt*II) gene under the control of the nos promoter was used for preparation of over expression construct for lily and *Arabidopsis* (Nakagawa, 2002).

In addition, plasmid pBI121 was used (harbouring the intron- containing β-glucuronidase (GUS) gene fused to the CaMV35S promoter and the neomycin phosphotransferase (*npt*II) gene under the control of the nos promoter also reported by Núñez de Cáceres *et al.,* (2011).

2. 13 *AGROBACTERIUM* ELECTROPORATION

Confirmed positive constructs carrying the gene of interest were transformed into *Agrobacterium* by electroporation. Electro- component cells (AGL1 and GV3101) were obtained from the Biosciences genomics facility, The University of Nottingham. 1 µl (100ng/µl) destination vector and 1 µl (100ng/µl) of helper plasmid (only when using binary vector) were added into Electro- component cells and mixed gently with a pipette tip and transferred into a pre-chilled cuvette. The mixture was electroporated using a Gene Pulser (Bio-rad Laboratories, Ltd, Hemel Hampstead, UK) setting 2500 volts, 25 Mfd, 400 Ohms for 1 second. 250 µl of LB was added into each cuvette, re-suspended and transferred into 1.5 Eppendorf tubes then incubated at 28C in a shaker for 3 hours. 50 µl and 200 µl of the cell culture were spread onto semisolid LB medium plates (Appendix 4) supplemented with the appropriate antibiotic resistant depending of the plasmid and bacteria resistance (for AGL1 and Gv3101 Rifampicin was used (25mg/ml) utilized. Plates were incubated in a 28°C for 3-4days. The positive colonies were developed and the Colony PCR was conducted as specified in (Section 2.7) to confirm the presence of the vector containing the fragment of interest.

2.14 BARLEY *AGROBACTERIUM- MEDIATED* TRANSFORMATION.

A single *Agrobacterium* colony carrying the construct with the target gene (as described in Section 2.11) was utilized to inoculate barley embryos. *Agrobacterium* inoculum was prepared overnight after inoculating 10ml of liquid MG/L medium without antibiotic (Appendix 4). In addition, 75 µl of (10 mg/ µl) Acetocyrogen was added into the medium with mixed by pipetting and then incubated in a shak-

er at 28°C for 20 h with 180 rpm. The *Agrobacterium* culture was then used for embryo inoculation; 200 µl of *Agrobacterium* culture was dropped onto each embryo until just the surface was covered. 25 embryos on co-cultivation medium (Appendix I) plates were inoculated and then tilted for 2 min to allow any excess *Agrobacterium* culture to run off the embryos. After that, embryos inoculated were transferred into a fresh callus induction plate (co-cultivation medium) without any excess culture medium with scutellum side down. The plates then were kept in the dark room 22-23°C for three days.

The embryos were transferred onto fresh callus induction plates (co-cultivation medium) (Appendix 4) containing hygromycin as the selective antibiotic and timentin to remove *Agrobacterium* from the culture. The plates were kept in the dark room 22-23°C for two weeks as selection one. After two weeks, transformed embryos were transferred to the fresh callus induction plates as stated above. After a further two weeks, the embryos were transferred onto callus induction plate as a third selection. It was necessary to reduce the number of embryos per plate at this point since the transformed embryos derived callus started to grow rapidly. After six weeks selection on callus induction medium, the callus derived from embryos was transferred onto transition medium (Appendix 4) with timentin (150mg/ml) and hygromycin (50 µg /ml) for two weeks at 23 °C with low light. In addition, during this period the callus started to produce green areas and small shoots.

After two weeks of transition medium, the green areas were transferred into regeneration medium with timentin (150mg/ml) and hygromycin (50 µg /ml) (Appendix I) to produce roots and shoots and then kept in light at 23-24°C for two

weeks. After roots were formed and shoots were 2-3cm in length, plantlets were carefully transferred into glass tubes with 12ml of callus induction medium without growth regulators see (Appendix 4) with the same concentration of timentin and hygromycin. They were placed in light at 23-24°C for two weeks. When the shoots reached of the top of the tube with strong roots, they were transferred into soil. 5 cm diameter pots with John Innes N°3 compost was used for growing the plants, these were initially covered with plastic sleeves for three to four days to maintain high humidity. After that, plastic sleeves were opened and plants were kept in growth room as describe in Section 2.1.1 (Harwood et *al.*, 2009).

2.15 *AGROBACTERIM -MEDIATED* TRANSFORMATION PROTOCOL FOR LILY (Modified from Nunez de Cacerres *et al.*, 2011)

2.15.1 Pre-Culture (callus induction medium):

Callus induction medium was prepared containing 4.36 g/L of MS basal salts with 30g/l sucrose, pH 5.8, 2.8 g/L phytagel, supplemented with 2.0 mg/L picloram, 1 mg/L zeatin,1mg/L kinetin and 50mM lipoic acid under LAF Chamber sterilization. *Lilium* Star Gazer basal plate discs, of 3-6mm diameter and 0.5-1.5 mm thickness were pre-cultured for 2 h in callus induction medium and under sterile conditions.

2.15.2 Preparation solution

For inoculation and co-culture the MS medium was freshly prepared using 10x Solution A (Macros) and a 10,000x Solution B (Micros) Tables 2.14 and 2.15.

Table 2. 14: Macro elements for preparation solution A.

Macro elements	**Gram per 1L**
Boric Acid (H_3BO_3)	0.062
Magnesium Sulfate ($MgSO_4 \cdot 7H_2O$)	3.700
Ferrous Sulfate ($FeSO_4 \cdot 7H_2O$)	0.278
Manganese Sulfate ($MnSO_4 \cdot 4H_2O$)	0.223
Zinc Sulfate ($ZnSO_4 \cdot 7H_2O$)	0.086
EDTAdisodium ($Na_2EDTA \cdot 2H_2O$)	0.372
Inositol	**1.000**

The chemicals were mixed and double distilled water was used to make this solution up to 1L and then the pH was adjusted to 5.8, autoclaved and kept it in the fridge. This solution was used under sterile conditions.

Table 2. 15: Micro elements for preparation of solution B.

Micro elements	**Gram per 100ml**
Cobalt Chloride ($CoCl_2 \cdot 6H_2O$)	0.025
Cupric Sulfate ($CuSO_4 \cdot 5H_2O$)	0.025
Potasium Iodide (KI)	0.830
Soduim Molybdate ($Na_2MoO4 \cdot 2H_2O$)	0.250
Nicotinic Acid	0.500
Glycine	2.000
Pyridoxine HCl	0.500
Thiamine HCl	**0.100**

The chemicals were mixed and double distilled water was used to make this solution up to 100ml, did not autoclave it and kept it in the fridge with aluminium foil wrapped. This solution was used under sterile condition.

2.15.3 Inoculation solution

100 ml Solution A and 10 µl of solution B (Section 2.6.2) were combined with 3.9g/l of MES and 30g/l of sucrose, the pH was adjusted to 5.8 and then autoclaved, cooled to 55°C and then supplemented with sterile 2.0 mg/L picloram, 1 mg/L zeatin,1mg/L kinetin.

2.15.4 Co-culture medium

For co-culture medium 7.8g/L of MES and sucrose was combined with 200ml of solution A and 20 µl of solution B (Section 2.6.2) the pH adjusted to 5.8, 2.8g/L phytagel semi-solidified added. It was autoclaved, cooled to 55°C and supplemented with sterile 1 mg/L zeatin, 1mg/L kinetin, 2.0 mg/L picloram, and 50 µM of Lipoic acid.

2.15.5 Inoculation of explants

A single colony of *Agrobacterium,* strain AGL1 was cultured overnight on a rotary shaker at 28°C in 50 ml of liquid Luria Broth (Bertani, 1951) at pH 7.0 containing the appropriate antibiotics for both the plasmid and *Agrobacterium* selection (50mg/L kanamycin and 25mg/L Rifampicin).

Four or five hours before to inoculation of explants, Acetosyringone (100 µM) was added into the bacterial suspension culture and then during this time fresh basal plate disc from bulb-lets were prepared and pre-cultured on the pre-culture medium and then left under sterile conditions the LAF chamber for about 2 h without the Petri dish cover. The bacterial culture was then centrifuged at 3500 rpm for 20 min and the supernatant removed, the pellet then re suspended with 25 to 50 ml of inoculation solution as described at Section (2.6.3). 100 µM of Acetosyringone with 100µl of 0.2%(v/v) Sliwet L-77(Lehle Seeds, Round Rock, USA) were added into 50 ml bacterial culture.

The resuspended culture used for inoculation had an OD_{600}= 0.8-1.2; 50µl of bacterial suspension was pipetted on to each explant and left for 3-5 min. After inoculation, the explants were transferred onto co-culture medium as described in Section 2.6.4 and then placed in the dark growth room at 23°C for 2 days.

2.15.6 Transition stage of inoculation tissues (transition medium):

After 2 days co-culture, explants were washed three times in sterile water containing 400mg/L Cefotaxime and 200mg/l of Timentin (Ticarcillin disodium salt) to remove *Agrobacterium* overgrowth. Before transfer onto transition medium, explants were blotted onto sterile filter paper. Transition medium was composed of 4.36g/L of MS basal salts, 60g/L sucrose and 2.5 activated charcoal, this was

mixed, adjusted to pH 5.8 and 2.8g/L of Phytagel added. After that the medium was autoclaved and kept in a water bath at 55°C for 45 min and then the transition medium was supplemented with 1mg/L zeatin, 1mg/L kinetin, 2.0 mg/L picloram, 100 µM Lipoic acid, 100 mg/L kanamycin and 250mg/L timentine under sterile conditions. Explants was cultured in the dark growth room at 23°C for 1-3 weeks and checked daily for any *Agrobacterium* overgrowth.

2.15.7 Selection medium (selection of transformed tissue)

Selection medium was composed of 4.36g/L of MS basal salts, ,60g/L sucrose and 2.5g Activated charcoal, this was mixed and adjusted to pH 5.8 and 2.8g/L of Phytagel semi-solidified was added it. After that the medium was autoclaved and then kept it in water bath at 55°C for 45 min and then was supplemented with 1 mg/L zeatin, 1mg/L kinetin, 2.0 mg/L picloram, 100 µM Lipoic acid, 100 mg/L kanamycin and 250mg/L timentine under sterile conditions. Explants were cultured in the dark growth room at 23°C and checked daily for any *Agrobacterium* over-growth.

2.15.8 Rooting of regenerated transformed shoots

After 28-42 days on selection medium, shoots (1-2 cm in height) were excised from antibiotic-resistant tissues and transferred into screw-capped power round glass jars (Beatson Clark Co., Rotherham UK), each containing 25 ml of rooting medium (composed of 4.36g/L of MS basal salts, 60g/L sucrose and 2.5 Activated charcoal mixed and adjust to pH 5.8; 2.8g/L of Phytagel). The medium was autoclaved and kept in a water bath at 55°C for 45 min and was supplemented with 5mg/L BAP, 2.0 mg/L picloram, 100 µM Lipoic acid, 100 mg/L kanamycin and

250mg/L timentine under sterile conditions. The cultures were incubated at 23°C with 16h photoperiod. After they had 1-2 well-developed leaves they were transferred into a brown paper merchandise bags with some Levington M3 compost (Scotts professional, Ipswich, UK) and then they kept at cold room 4°C for 4 weeks, to break down the dormancy. After this period, the bulblets were transferred into 13cm pots containing Levington M3 compost (Scotts professional, Ipswich, UK); plastic sleeves 27x15 cm were used for covering pot plants which were transferred to the glasshouse under natural light conditions. After 20 days, the sleeves were removed completely.

2.16 ARABIDOPSIS TRANSFORMATION PROTOCOL.

A single colony of *Agrobacterium*, strain GV3101 was cultured overnight on a rotary shaker at 28°C in 100 ml of liquid Luria Broth (Bertani, 1951) at pH 7.0 containing the appropriate antibiotics for both the plasmid and *Agrobacterium* (50µg/ml kanamycin and 25µg/ml Rifampicin). 10 ml of this culture was taken and inoculated into 100 ml of LB without antibiotics and then cultured in rotary shaker at 28°C until an OD = 0.8 -1.2 was reached.

Next, 5 g of sucrose with 50µl of 0.2%(v/v) Silwet L-77(Lehle Seeds, Round Rock, USA) were added into 100 ml of the culture. *Arabidopsis* inflorescences with few siliques were used for floral dipping for 15 second. After dipping, plants were covered with a bag and placed in a clean tray with water under low light for 2 days (Clough and Bent, 1998). Two days after transformation, plants were placed back on the bench and the bags were opened. After plants, were maintained and dried the seeds subsequently collected.

2.17 ALEXANDER STAINING FOR BARLEY ANTHERS

Alexander staining was used to determine pollen viability (Alexander, 1969). This is a rapid and reliable way of checking the viability by using a light microscope (Johnson-Brousseau and McCormick, 2004). Anthers were collected before anthesis stage and placed on a slide and a droplet of the stain added. Pollen grains were released from anthers and covered with a coverslip and observed immediately under a light microscope. Dark purple colour indicated that the grains were viable (Gomez and Wilson, 2014).

2.18 HISTOLOGICAL ANALYSIS OF ANTHER AND POLLEN DEVELOPMENT OF BARLEY.

Barley florets were resin fixed for visualization of anther and pollen development, these were staged according to Fernandez Gomez and Wilson, (2012). Individual florets were selected and detached from spikes and then immediately placed in 4 ml of 4% (v:v) fresh paraformaldehyde solution and kept in ice.(Appendix 4) . Vacuum infiltration then was used in the fume hood for 30 min, to revent air bubbles within the samples, this was then repeated for another 30 min. Then, the fixative was replaced with fresh 3ml of paraformaldehyde solution and then stored overnight in a cold room 4°C with rotating. Tissues were then washed (1hours by 1xPBS solution (30 min each); Appendix 4). The tissues were dehydrated using 4 ml of an ethanol series with increasing concentration (30%, 50% for 1 hour at room temperature) and then samples were kept at 70% (v/v) of ethanol in the

fridge at 4°C overnight. The ethanol series at 90% for 1 hours and then samples where left at 100% of ethanol for 2 hours.

Pre-infiltration the tissue was then carried out by using a mixture of Ethanol/ resin (solution A) as (100 ml of Technovit 7100 resin plus 1 gram of Hardener I) as follows:

Ethanol /resin (2:1) for 1h

Ethanol /resin (1:1) for 1h

Ethanol /resin (1:2) for 1h

Samples were infiltrated using 5 ml of 100% of solution A (resin), then kept overnight in the fridge at 4°C. Tissues were then embedded and placed into the capsules using solution B (375µl of solution A with 70 µl of solution hardener II) was mixed together in an Eppendorf tube. After 1 day, then capsule was removed using hot water or blades and samples were placed into a mould. The cold-curing Technoit resin (Technovit 3040) was used for fix the samples. Samples were ready after 24 hours for sectioning by microtome (Model: 5040; Bright Instrument Company, Huntingdon, UK).

2. 19 GUS ASSAY FOR TRANSFORMATION LILY EXPLANTS.

Histochemical analysis of GUS gene expression was performed based on the method of Jefferson (1987). Tissues were transferred into Petri dishes with 100mM sodium phosphate ($Na_3PO_4.12H_2O$) (pH 7.0) containing 0.1%(v/v) Triton X-100 and 1mM X-gluc (5-bromo-4chloro-3-indolyl-β-glucuronic acid; Melford, Ipswich,UK) (see Table 2.17). After that they were incubated for 2-3 days at 37°C and then explants transferring in to 100% ethanol for facilitating visualization of GUS staining.

Table 2. 16: GUS solution

Material and concentration	Work concentration	solution for 50 ml
0.5M Na3Po4.12H2O	100mM Na3Po4.12H2O (pH 7.0)	10ml
0.1M X-gluc	1mM X-gluc	0.5 ml
Triton X-100	(v/v) Triton X-100	0.05 ml
H2O	Pure	39.45 ml

2. 20 DAPI STAINING FOR LILY.

DAPI (4, 6-diamidino-2-phenylindole) was used for staining live lily pollen by fluorescence microscopy. This stain binds to double strand DNA and can be used to observe nuclear details. Lily anthers of different lengths around 4mm to 35mm were collected and fixed in ethanol: acetic acid (3:1) for 5 minutes in Eppendorf tubes. The top of anthers was cut and placed then into droplet of DAPI stain on a clean slide by gently squeezing to release the pollen into solution and cove ringed with a coverslip. DAPI stain was prepared by combining 300 µl of 10 µg/ml DAPI with 400 µl of 80% glycerol and then up into 1000 µl by water.

2.21 BIOINFORMATICS

2.21.1 Plasmid maps.

All vector maps were created using the Snapgene software package, after finding the sequence for the all destination and entry vectors from the National Centre for Biotechnology Information website (htt://www.ncbi.nlm.nih.gov).

2.21.2 Confirmation of gene sequencing

The biotechnology information (NCBI) database/Global Sequence Alignment Tool was used for checking all sequences. Website (http://blast.st-va.ncbi.nlm.nih.gov)

2.21.3 Primer Design.

Primer3 software website (http://primer3.ut.ee) was utilized for designing primer. 20-21 nucleotides were selected for primers with indicated the condition specify for individual product, and then all primers were tested and confirmed the specify condition using Snapgene software.

CHAPTER 3: ANTHER AND POLLEN DEVELOPMENT OF *LILIUM*; WITH TISSUE CULTURE AND TRANSFORMATION TO MODIFY SENESCENCE BY OVER-EXPRESSION OF *ATIPT*4 AND *ATIPT*8 GENE.

3.1 INTRODUCTION

Demand for ornamental plants has been dramatically increased over recent years, particularly for the cut flower industry. *Lilium* is one of the three major bulbs in the commercial market due to its attractive and fragrant flowers. Bulbs are the main organ of the lily plant, which consists of scales and a basal plate. *Lilium* has been the target for investigated and manipulation of traits for horticultural importance plants, for example flower colour, fragrance and control of male fertility. Male sterility as been selected as a possible route to provide a solution to the problem of the huge amount of pollen production in *Lilium* flower (Stokes and Forrester, 2004; Fitzgerald 2010 and Thompson 2012), which stains and is an undesirable trait for the cut flower industry.

Analysis of pollen development pathways in Lily was therefore conducted using genes previously identified in *Arabidopsis MYB26* for anther dehiscence, with the aim of applying genetic manipulation approaches to target traits that would be difficult to achieve by traditional breeding (Yang *et al.,* 2007b; Rommenes *et al.,* 2007). Tissue culture techniques and regeneration have been used by the floriculture industry for both propagation and breeding, also virus-free stocks can produce by this method (Tanaka *et al.,* 2005 and Cohen, 2011). Propagation of *Lilium* from bulb scales have also been developed; bulblets are created from a tiny

amounts of explants tissue from small part of scales after sub-culture in a sterile nutrient medium with plant growth regulators (Wozniewski *et al.,* 1991; Lian *et al.,* 2009). *Agrobacterium*-mediated genetic transformation has become the main method and technology, for production of genetically manipulated transgenic plants, particularly commercial ornamental plants (Magori 2011 and Gnasekaran *et al.,* 2014).

Nevertheless, transformation in *Lilium* is extremely challenging with only a few examples reported (Brand. 2008). Therefore, there is the need to develop and establish these techniques for routine transformation analysis. *Agrobacterium-mediated* transformation efficiency in *Lilium* using reporter constructs containing GUS and GFP was therefore utilised to test for transformation and to analyse expression in callus and vegetative tissues and thus to show that both transient and stable transformation was occurring.

In addition, in this chapter also includes the development of constructs to manipulate senescence stages in ornamental cut flower Lily plants *(Lilium c.v. Star Gazer)* using expression of the *ATIPT4* and *ATIPT8* genes. Senescence is one of the most important stages for cut flowers in the commercial floriculture sector, since it is the last phase of development and associated with degeneration causing flower death. Longevity of the flower is therefore an important trait for horticultural crops, particularly ornamental cut flowers (Shibuya, 2012).

Currently, production of many ornamental plants has been impacted by economical losses due to senescence, which occurs during many stages of development and growth in the plant (Nooden *et al.,* 1997 and Buchanan-Wollaston 2003,). Therefore, approaches that minimise this impact have been targeted for ornamen-

tal plant improvement and production, approaches that utilise genetic manipulation explored as alternative options to deliver these goals.

Cytokinin levels have been shown to be important in the progression of senescence and the *Isopentenyl transferase (IPT)* gene has been explored as a way to modify cytokinin biosynthesis and thus senescence in higher plants. Over-expression of the *IPT* gene under the CaMV35S promoter, has been used as an approach to manipulate endogenous cytokinin levels in ornamental crops can provided a way to manipulate senescence for potential economical agricultural applications (Gan and Amasino, 1996). This approach was applied to Lily to determine if transgenic Lily plants could be generated that were ectopically expressing the *Arabidopsis IPT4 and IPT8* genes and if so what the impact of this would be on plant development and senescence.

3.2 MATERIALS AND METHODS.

3.2.1 Flower development and DAPI staining for Anther of lily plant.

Process of flower and pollen development was conducted for *Lilium* Stargazer, by analysis of bud, anther lengths during flower development.

For staining lily pollen and development growth, DAPI (4, 6-diamidino-2-phenylindole) was used under in fluorescence microscopy as described in Section 2.20.

3.2.2 Characterization of reporter constructs.

Four promoters were focused on for cloning to express transgenes in a tissue specific manner in *Lilium* and *Arabidopsis*; these included using the promoters *OsERFL, LCHS, Ubi1* and *CaMV35S*. These were cloned into the PCR8GW TOPO entry vector to generate two destination vectors pGWB3 and pGWB4.

Promoters from Rice *OsERFLP* and Lily *LCHS* genes, which are floral specific and Maize Ubi1 and CaMV35S promoters were conducted. Reporter constructs GUS and GFP were also used to analyse the activity of the promoters.

The *OsERFL1* promoter (Kato *et al.*, 2010) was amplified from rice genomic DNA (*Oryza sativa* Japonica ssp Nipponbare cv. Wild type) that was obtained from the Wilson Lab, The University of Nottingham, using PCR Phusion as described in Section 2.3.7; with OsERFLF1 and OsERFLR1 primers; the expected bind size was 1500bp (Appendix 1). The *LCHS* promoter was amplified from lily genomic DNA using LCHSF and LCHSR primers, with the expected band sizes of 900bp, using PCR Phusion as described in Section 2.3.7. The *Ubi1* promoter was amplified from an entry vector generated by Dr Fernandez-Gomez and the

CaMV35S promoter was amplified from the pGKGWC destination vector using PCR Phusion (Section 2.3.7; primers Ubi1 F1 and Ubi1 R1; expected size 2000bp and CaMV35S F1 BstB1 and 35S R1 sal1; expected bind size was 540bp).

The PCR product was then purified and cloned into pCR8/GW/TOPO (Section 2.4; Appendix 1). The insertions were confirmed by PCR (Section 2.3.8) using primer vector M13F with specific gene LCHSprmcolR1; OsERF1colR1; Ubi1 colR1 and 35Ssal1R1 primers respectively. The expected size between M13F and specific gene primer were 850bp; 825bp; 2110bp and 690bp respectively; Tm=59°C; Appendix 2; Fig 3.1). In addition, sequencing was conducted of all constructs (Section 2.9; Appendix 2).

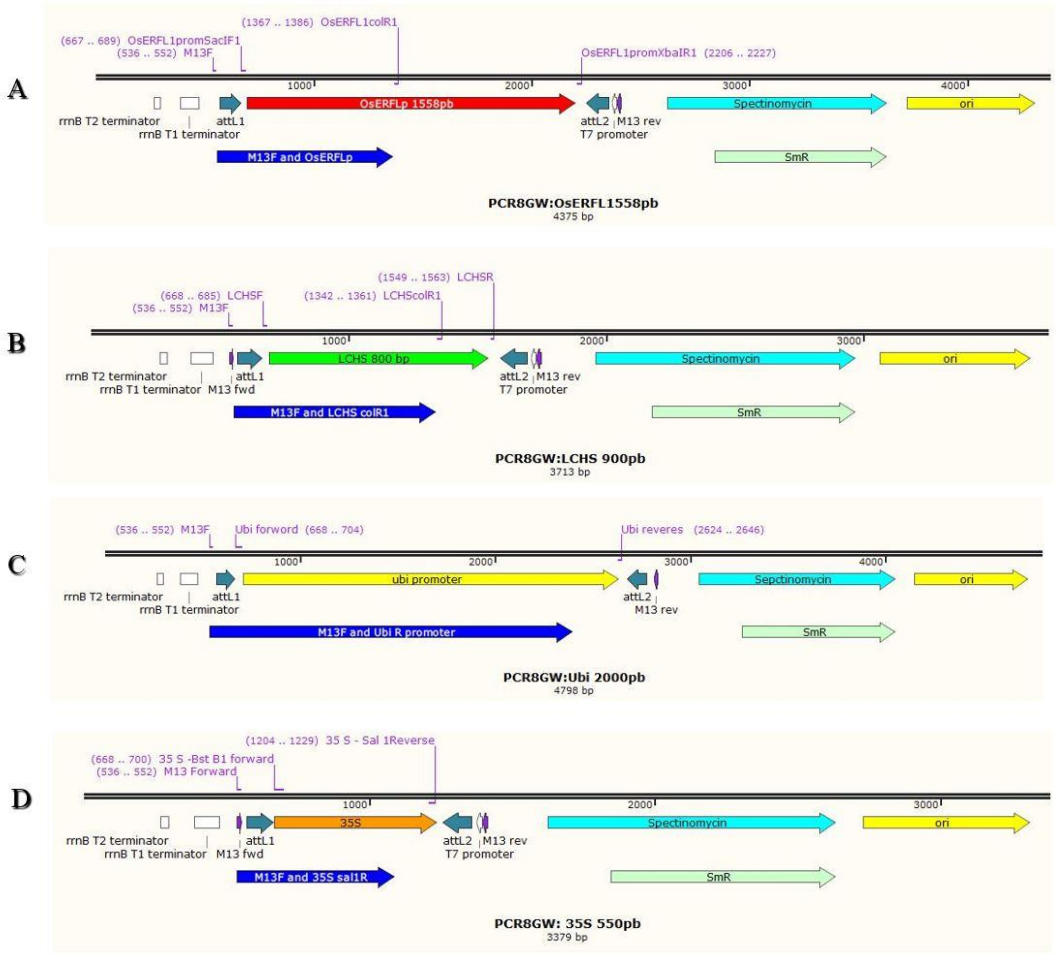


Figure 3. 1: Design of PCR8GW entry vector for OsERFL, LCHS, Ubi1 and CaMV35S promoters.

A: PCR8GW: OsERFLP entry vector, red line is *OsERFLP* promoter (1558bp) and M13F combined with the gene specific OsERFLPcolR1 primers to check the insert (850bp).

B: PCR8GW: LCHS entry vector, green line is *LCHS* promoter (900bp) and M13F combined with the gene specific LCHScolR1 primers to check the insert (825bp).

C: PCR8GW:Ubi1 entry vector, yellow Line *Ubi1* promoter (2000bp) and M13F combined with the gene specific Ubi1R1 primers to check the insert (2110bp).

D: PCR8GW:35S entry vector, orange Line 35S promoter (550bp) and M13F combined with the gene specific 35S sal1R1 primers to check the insert (690bp).

An LR reaction was used to transfer the target promoters into destination vectors (pGWB3 and pGWB4) (Section 2.10). The construct was checked using the primer combinations PGWB3 OsERFLPcolF1; LCHSF; Ubi1F and 35SF with GUSR1. According, to the T-DNA map the size between specific genes with GUSR1 is expected to be 430bp; 1100bp; 2178bp and 750bp respectively Fig 3.2; Appendix 1.

OsERFLP and LCHS entry vectors were also cloned into pGWB4. According to the T-DNA map the size between specific genes OsERFLPcolF1; LCHSF with GFPR1 primers was expected to be 500bp and 1182bp respectively (Fig 3.3; Appendix 1). Confirmed constructs carrying the promoters of interest were transformed into *Agrobacterium* AGL1 and GV3101 strains by electroporation (Section 2.13) after confirmed by Red-Taq PCR and a glycerol stock was made (Section 2.11).

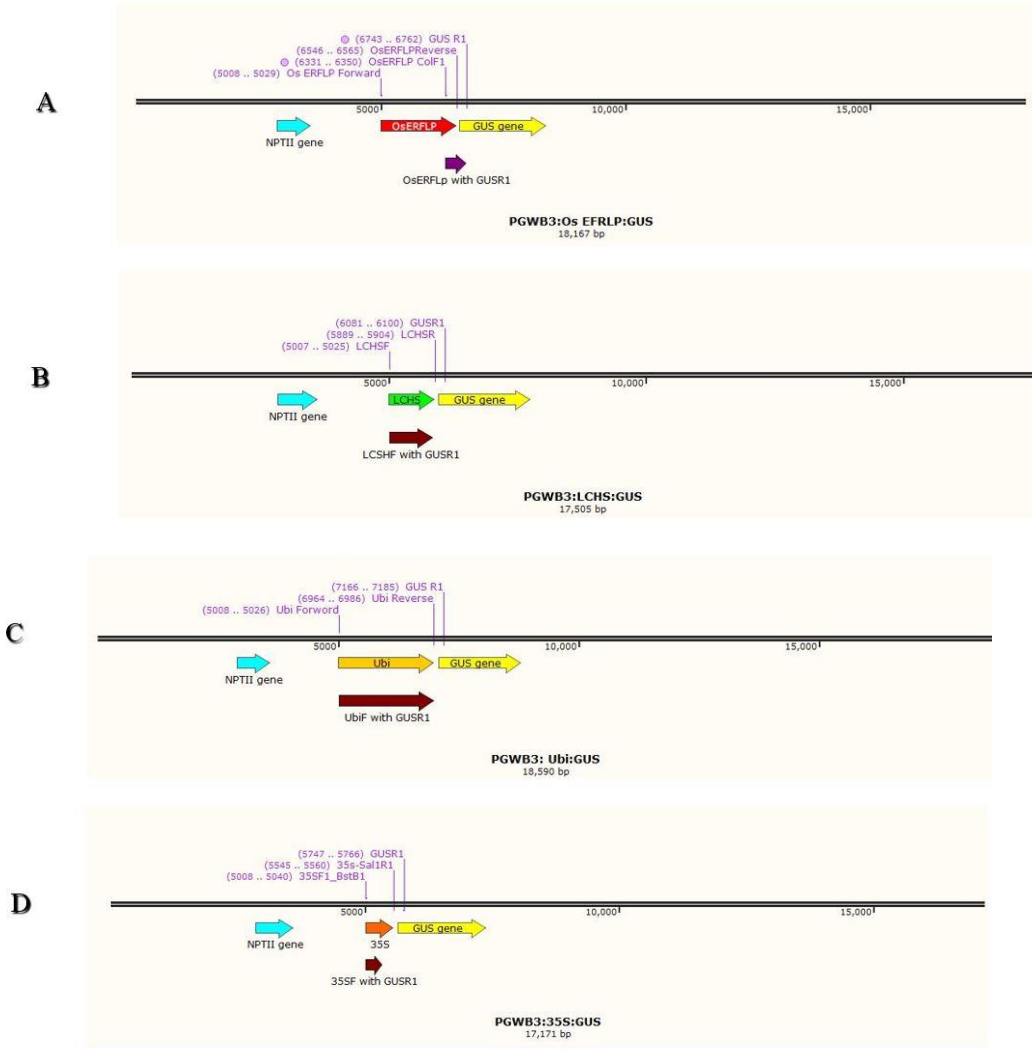


Figure 3. 2: Design of pGWB3 destination vector for OsERFLP, LCHS, Ubi1 and CaMV35S promoters.

A: pGWB3: OsERFLP: *GUS* destination, red line is *OsERFLP* promoter (1558bp) using OsERFLPF1 combined with the GUSR1 gene primers to check the insert (430bp) and its orientation.

B: pGWB3: LCHS: *GUS* destination entry vector, green line is *LCHS* promoter (900bp) using LCHSF1 combined with the GUSR1gene primers to check the insert (1100bp) and its orientation.

C: pGWB3:Ubi1: *GUS* destination vector, yellow Line Ubi*1* promoter (2000bp) using Ubi1F with combined with the GUSR1 gene primers to check the insert (2178bp) and its orientation.

D: PGWB3:35S*: GUS* destination vector, orange Line 35S promoter (550bp) using 35SF with combined the GUSR1 gene primers to check the insert (750bp) and its orientation.

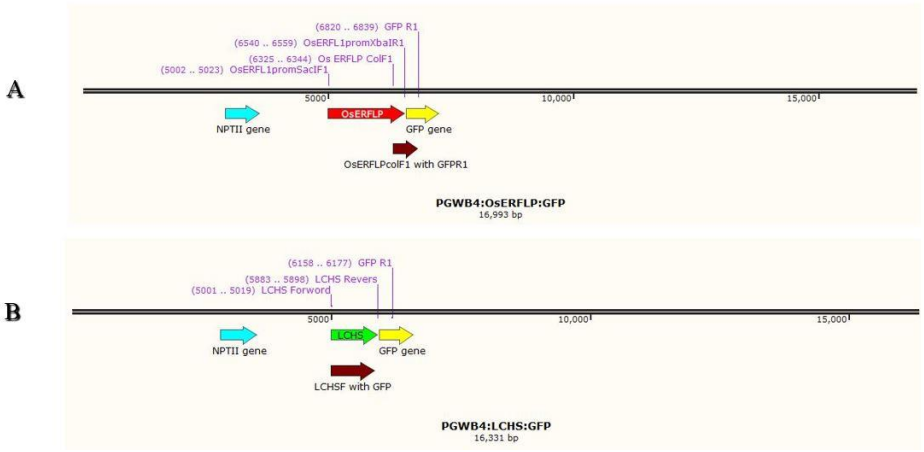


Figure 3. 3: Design of pGWB4 destination vector for OsERFLP, LCHS promoters.

A: pGWB3: OsERFLP: GFP destination, red line is *OsERFLP* promoter (1558bp) using OsERFLPF1 combined with the GFPR1 gene primers to check the insert (500bp) and its orientation.

B: pGWB3: LCHS: GFP destination entry vector, green line is *LCHS* promoter (900bp) using LCHSF1 combined with the GFPR1gene primers to check the insert (1182bp) and its orientation.

3.2.3 Development of constructs containing two floral specific promoters (OsERFLP and LCHS promoter).

First, the anther specific promoter in rice (*OsERFLP*) and Lily flower specific promoter (*LCHS*) were characterized to determine their expression in lily.

OsERFL1 promoter (Kato *et al.*, 2010) was amplified from rice genome as used for pGWB3OsERFLP construct using (OsERFLPSac1F1 and, OsERFLPXbal1R1) primers the expected bind size is 1558bp (See Appendix 3). LCHS promoter was amplified from lily genomic DNA by LCHS spel F and LCHS hindIII R primers, with the expected band sizes of 920bp using PCR

Phusion as explained in Section2.3.7. Both genes then isolated and purified at describe (Section 2.3.11).

Before *OsERFLP* and *LCHs* promoter was then cloned into PGKGWY destination vector, (Sac1 and Xbal1) and (Spel and HindIII) were used to digestion (both promoters and PGKGWY vector) respectively.

The *HvMYB26* entry vector was generated by (Dr Fernandez-Gomez, The University of Nottingham) and then cloned to PGKGWY OsERFLP destination vector by LR. Colony PCR was performed using HvMYB26RNAi F1 and YFP-NY-R1 primers; the expected band size was 1000bp (Fig 3.4).

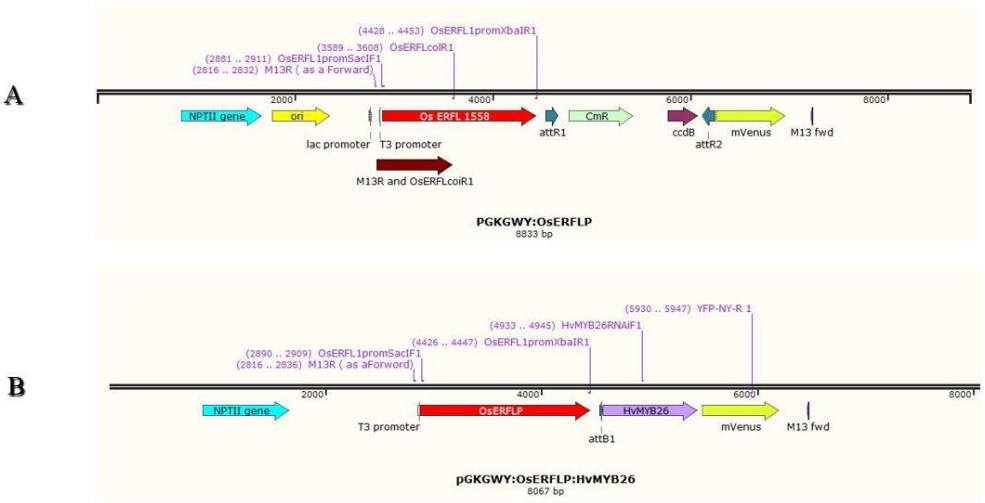


Figure 3. 4: Design of pGKGWY: OsERFLP destination vector.

A: pGKGWY: OsERFLP: destination vector, red line is *OsERFLP* promoter (1558bp) using M13R vector primer (as a forward) combined with the specific gene OsERFLPF1 primer to check the insert (770bp).

B: pGKGWY: OsERFLP: HvMYB26SRDX destination vector, red line is *OsERFLP* promoter (1558bp) and purple line is target gene *(HvMYB26*); using HvMYB26RNAiF1 combined with YFP-NY-R1 primer to check the insert (1000bp).

3.2.4 Over-expression construct.

3.2.4.1 Bioinformatics analysis.

Arabidopsis *IPT* genes *ATIPT4 and ATIPT8* were identified by bioinformatics analysis using the TAIR database (https://www.arabidopsis.org) and the sequences of both sequence genes obtained. Primer3 software website (http://primer3.ut.ee) was utilized for designing PCR primers to amplify the full-length coding regions of both genes (Section 2.21; Appendix 2). Amplification product of 957 bp and 993bp were predicted for the *ATIPT4* and *ATIPT8*, genes with stop codons, respectively.

3.2.4.2 Over-expression construct.

The *ATIPT4* and *ATIPT8* coding sequences were cloned into over-expression constructs to characterize their over-expression analysis. *IPT4* and *IPT8* over-expression constructs (PGWB5:: *ATIPT4*-CDS; PGWB5:: *ATIPT8*-CDS were generated these comprised the destination vector pGWB5 which containing the *GFP* gene fused to the CaMV35S promoter and the neomycin phosphotransferase (*npt*II) gene under the control of the nos promoter.

ATIPT4 CDS and *ATIPT8* CDS genes were amplified using ATIPT4Fw ATIPT4Rw and ATIPT8Fw ATIPT8Rw primers respectively (Appendix 3) from DNA prepared from leaves of *Arabidopsis* ecotype Landsberg *erecta* (L*er*) using Phusion PCR (Section 2.3.7); both genes were isolated and purified as described (Section 2.3.11).

After PCR product purification, an overhanging reaction was conducted and they were cloned into pCR8/GW/TOPO as described in Section 2.4 (Appendix 1).

The insert and its orientation within the entry clone, (PCR8GW:ATIPT4 and PCR8GW:ATIPT8) were confirmed by PCR (Section 2.3.8) using M13F and ATIPT4colR1 and ATIPT8colR1 primers (expected band size 690bp and 660bp respectively; Tm=59°C; Appendix 1); Fig 3.5A and B) In addition, sequencing was conducted to confirm the insertions (Section 2.9; Appendix 2).

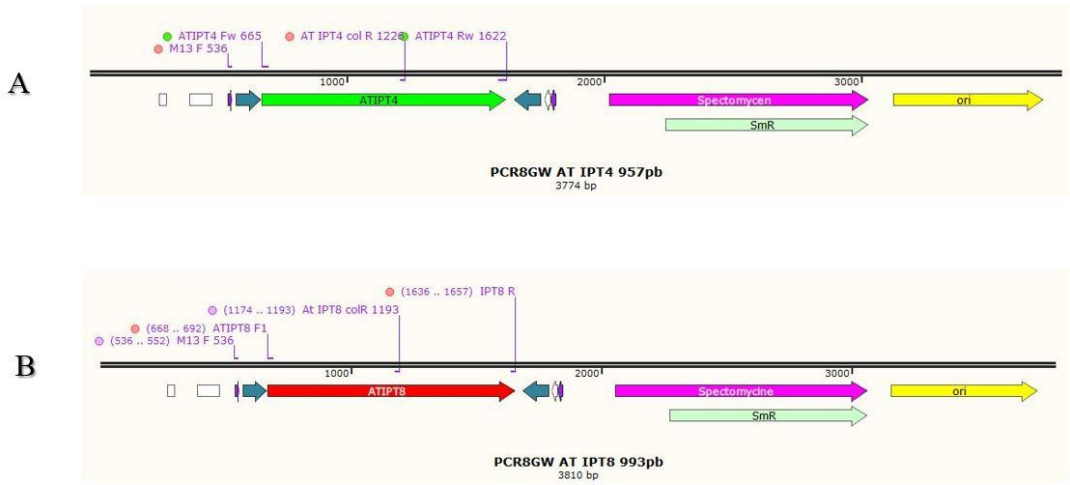


Figure 3. 5: Design of PCR8GW entry clone vector for (ATIPT4 and ATIPT8) genes.

A: PCR8GW:*ATIPT4* entry vector, green line is *ATIPT4* gene (957bp). The M13F primer combined with the gene specific ATIPT4colR primer was used to check the insert (690pb) and its orientation.

B: PCR8GW:*ATIPT8* entry vector, red line is *ATIPT8* gene (993pb). The M13F primer combined with the gene specific ATIPT8colR primers was used to check the insert (660bp) and its orientation.

An LR reaction was then performed to transfer the target genes from the entry vectors (PCR8GW: *ATIPT4* and PCR8GW:*ATIPT8*) into the destination vector (PGWB5) as described in Section 2.10. Constructs were checked by PCR using the primer combination pGWB8F with AtIPT4colR1 and AtIPT8colR1 primers (Tm=59°C; Appendix 2; Fig 3.6A and B) as described in Section 2.3.8.

Constructs carrying the gene of interest were transformed into *Agrobacterium* AGL1 and GV3101 strains by electroporation and used for transformations.

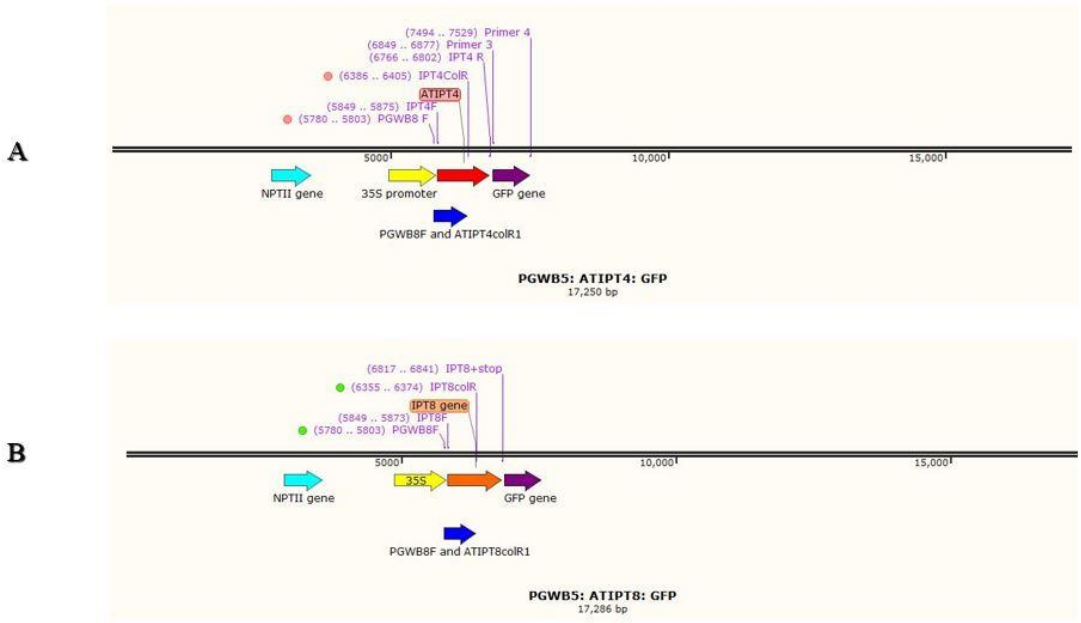


Figure 3. 6: Design of pGWB5 destination vector for (ATIPT4 and ATIPT8) genes.

A: pGWB5: ATIPT4: *GFP* destination, red line is *ATIPT4* gene (957bp) using primer the vector PGWB8F combined with the ATIPT4colR1specific gene primers were used to check the insert (620bp) and its orientation.

B: pGWB5: ATIPT8: GFP destination entry vector, orange line is *ATIPT8* gene (993bp) the vector primer vector PGWB8F combined with the ATIPT8colR1specific gene primers were used to check the insert (600bp) and its orientation.

3.2.5 Tissue culture and lily transformation.

Star Gazer bulbs were obtained from J. Parker Dutch Bulbs Company and they were grown under control conduction as described in Section 2.1.2 and scales used for in *vitro* bulb-let induction (Section 2.2.2).

Agrobacterium containing the constructs AGL::PGWB3::Ubi1::*GUS* and AGL1::PBI121::*GUS* were used for inoculate lily basal plate samples.

Agrobacterium cultures containing the *ATIPT4 and ATIPT8* over-expression vector (AGL1::pGWB5::ATIPT4CDS:*GFP* and AGL1::pGWB5::ATIPT8CDS:*GFP*) were used to inoculate lily basal plate explants as described in Section 2.15.

3.2.6 Analysis of GUS assay and transformed lily explants.

Histochemical analysis of GUS gene expression was performed to confirm the transformation of tissue and activity of Ubi1 promoter based on the method of Jefferson (1987) as described in Section (2.19).

In addition, bulblets that regenerated from transformation with the *AtIPT4* constructs were grown under controlled Glasshouse (24/22°C, 16/18 hours' day/night cycle), and genomic DNA was isolated from young leaves as describe in Section 2.3.2. Putative transgenic plants were confirmed by PCR analysis using the vector specific primer pGWB8Fw and the gene specific primer *ATIPT4colR1*, producing a product 620 bp.

3.2.7 *Arabidopsis* transformation and transgenic analysis

Arabidopsis ecotype Ler was used for transformation as describe at Section 2.16 using *Agrobacterium* containing the GV3101::PGWB3::Ubi1::*GUS* and GV3101::OsERFLP::Ubi1::*GUS* constructs. Seed was then sterilised and screened as described in Section 2.2.3, presence of the transgenes analysed by PCR using (Ubi1proF1 promoter Fw; OsERFLPcolF1 and GUSR1 vector specific Rv), the expected band size was 200bp and 400bp respectively.

Over-expression vector (GV3101::pGWB5::ATIPT4CDS:*GFP* and GV3101::pGWB5::ATIPT8CDS:*GFP*) were also used to transform *Arabidopsis* was by floral dipping as described in Section 2.16.

3.3 RESULTS

3.3.1 Morphological data analysis.

Detailed analysis of the process of flower and pollen development was conducted for *Lilium* Stargazer, by microscopy and analysis of bud, anther lengths during flower development. Bulbs were grown individually in 13cm pots in a controlled growth room. Bud development was checked daily and measured from a bud size of 5mm to 120mm. The relationship between length of buds and anthers development stage was established by analysis of buds of increasing 5mm sizes over a period of 48 days Figure 3.7 and 3.8 shows that the development stages of bud and anther changed rapidly developed from 5mm buds and anthers of 1mm, to 55mm buds with anthers of 32mm, when anther length was reach 30mm started steadily developing and growth. In addition, to size increases, the buds also changed colour (bud 75mm, 33mm anther) from green to pink starting from the bottom to the top (Fig 3.8). However, the full pink colour was only seen during from bud development of 110mm, 33mm anther; flower opening started at bud lengths 115 -120mm.

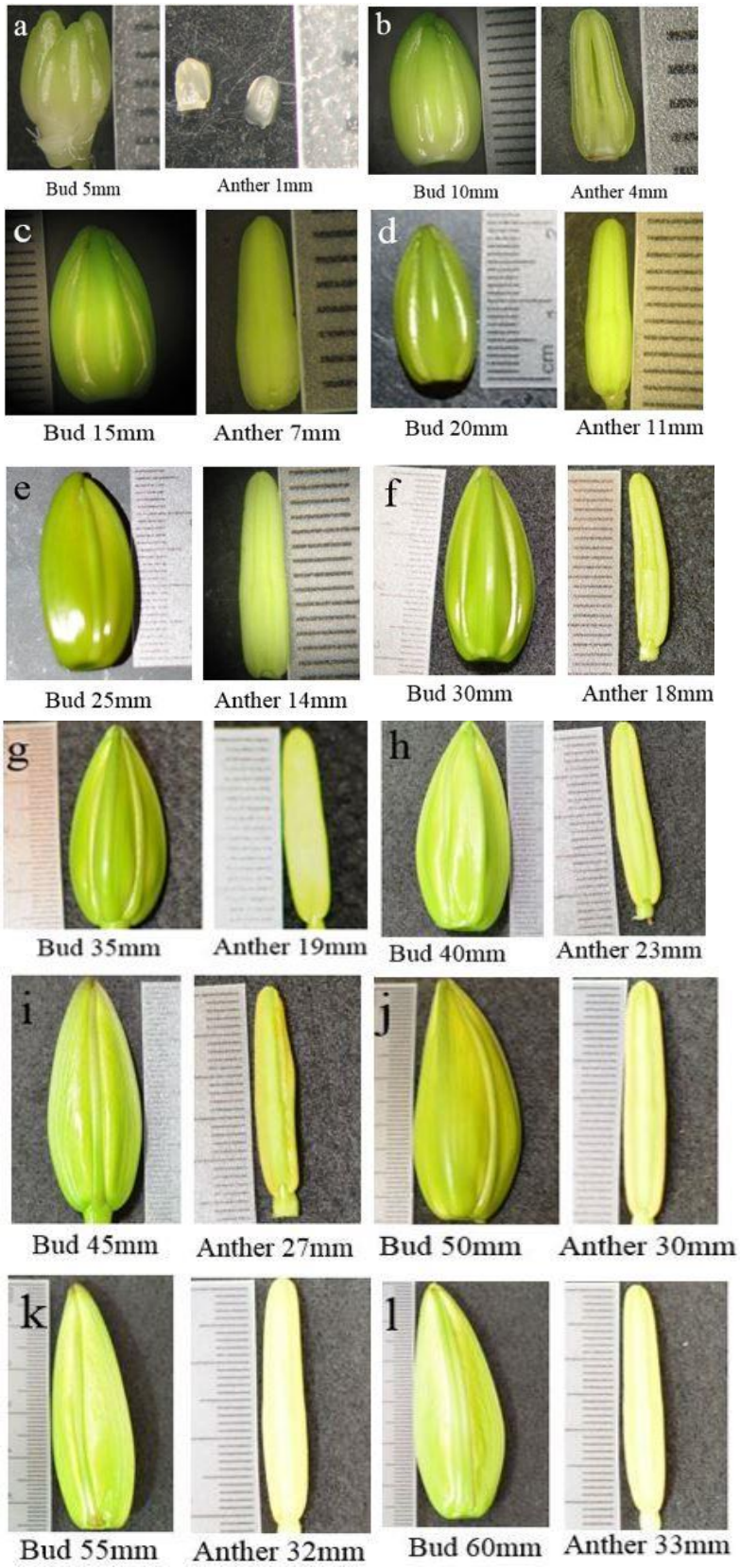


Figure 3. 7: Relationship btween the bud size and anther length/development stage for Lilium Star Gazer (a - l

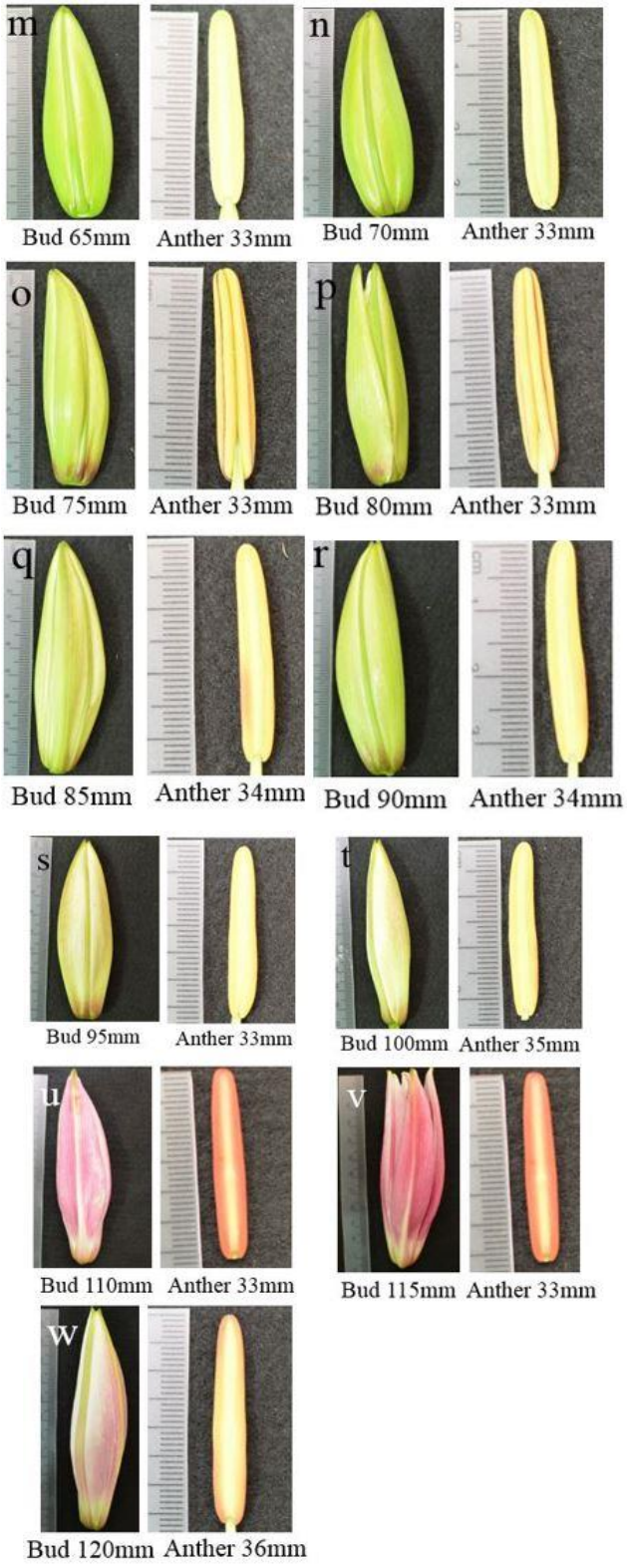


Figure 3. 8: Relationship btween the bud size and anther length/development stage for Lilium Star Gazer during a 48day period of development.

The bud and anther growth data was analysed to determine correlations between growth and development stage. Figure 3.9 shows bud growth over the flowering period; anther size increased until the buds reached 60mm (24 days), after which point they did not increase substantially in length.

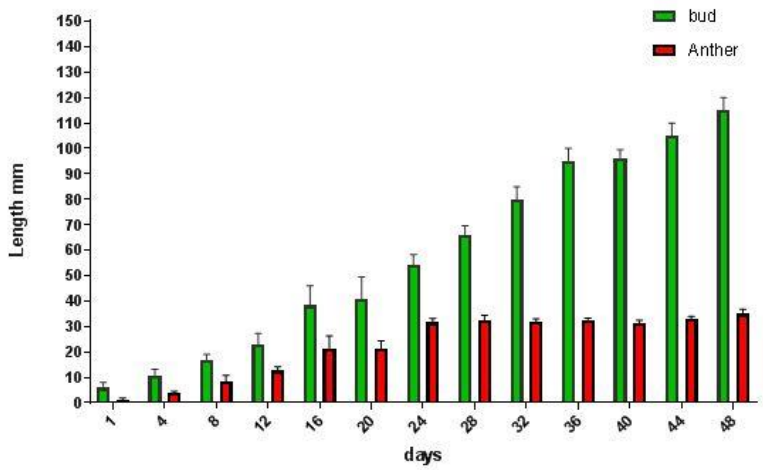


Figure 3. 9: Relationship between bud and anther length and growth over foral development in Lilium Star gazer wild type flowers based on 3 replicates.

3.3.2 Microscopy of Pollen development in *Lilium* cv.Star Gazer wild type.

Microscopy of the different pollen developmental stages was conducted for *Lilium* cv. Star Gazer and use to stage materials relative to bud and anther lengths. The development process starts by division of single diploid microspore mother cells to produce four haploid microspores in a tetrad. Figure 3.10 show these developmental stages; tetrads of haploid microspores were produced at bud lengths of 30mm and anthers of 18mm; mature pollen was released when the bud length was 120mm and anther 36mm.

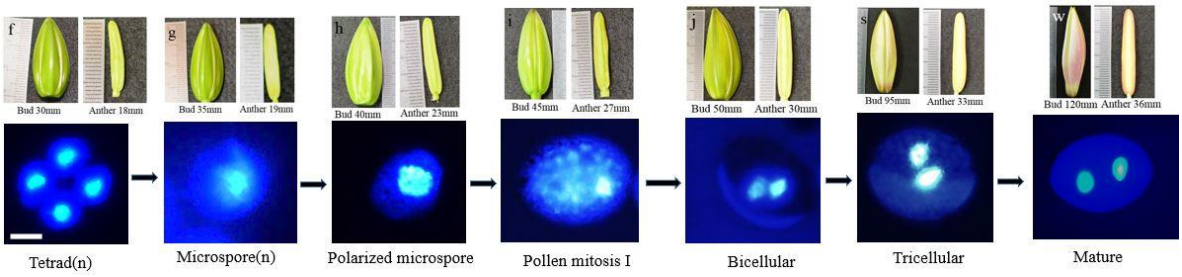


Figure 3. 10: Development of the male gametophyte in in Lilium cv.Star Gazer.

A: *Lilium* Bud and anther lengths

B: DAPI staining and pollen stages. Bar=20µm.

3.3.3 Analysis and activity of different promoters.

Rice OsERFLP and Lily LCHS floral specific promoters and Maize Ubi1 and CaMV35S promoter were prepared. Reporter constructs GUS and GFP were also developed to analysis expression in vegetative tissues in Lily and Arabidopsis plants.

3.3.3.1 Amplification and purification of promoters.

OsERFL1 promoter was amplified using PCR Phusion (Section 2.3.7) from rice genome DNA (*Oryza sativa* Japonica ssp Nipponbare cv. Wild type) using (OsERFLF1, OsERFLR1 primers) with expected band size is 1500bp (Kato *et al.*, 2010). Lily specific primer LCHS F with LCHSR was utilized to amplified from lily genomic DNA, with the expected band sizes of 900bp (Fig 3.10 A and B).

Ubi1 and CaMV35S promoter were amplified using PCR Phusion from destination vectors (pBract214) and PGKGWC (primers Ubi1 F1 and Ubi1 R1; 35S F1 BstB1 and 35S R1 sal1; expected band size 2700bp; 540bp respectively) (Fig 3.11 C and D).

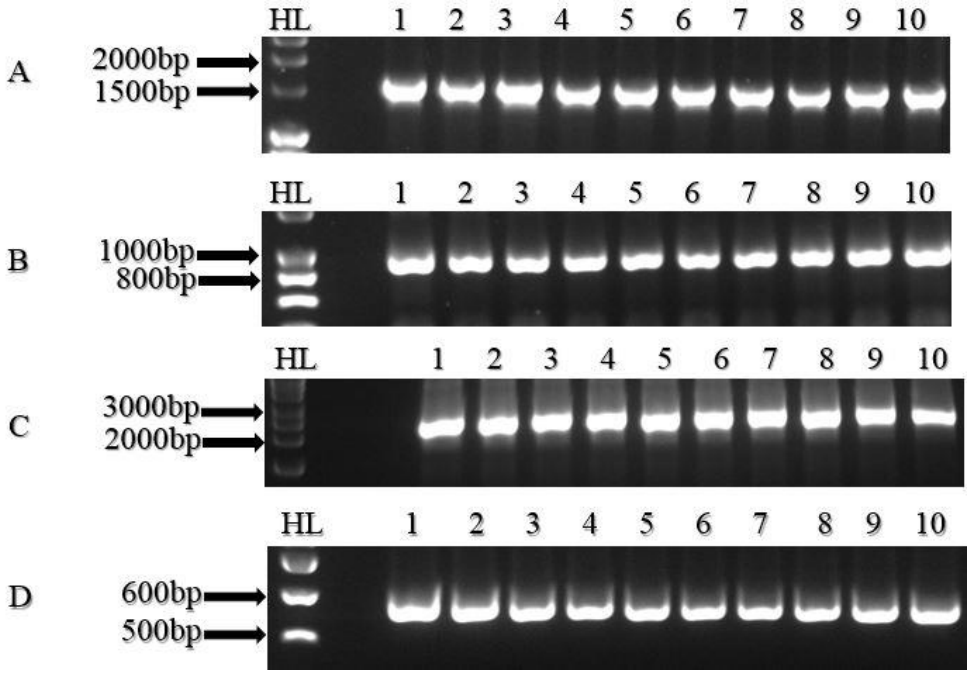


Figure 3. 11: PCR amplification of promoter fragments;

Lanes 1-10; promoter amplification; HL represent Hyper Ladder 1.

A: Amplified OsERFLP promoter using OsERFL1F1, OsERFL1R1 primers; amplification size product was expected to be 1500b.

B: Amplified LCHS promoter using LCHS F, LCHSR primer; amplification size product was expected to be 900bp.

C: Amplified Ubi1 promoter using Ubi1 F1, Ubi1 R1primer; amplification size product is expected to be 2700bp.

D: Amplified CaMV35S promoter CaMV 35S F1 BstB1, 35S R1 sal1 prime; amplification size product is expected to be 540bp.

Four promoters were isolated and purified (Section 2.3.11); Figure 3.12 shows the results achieved with the expected band size for the constructs.

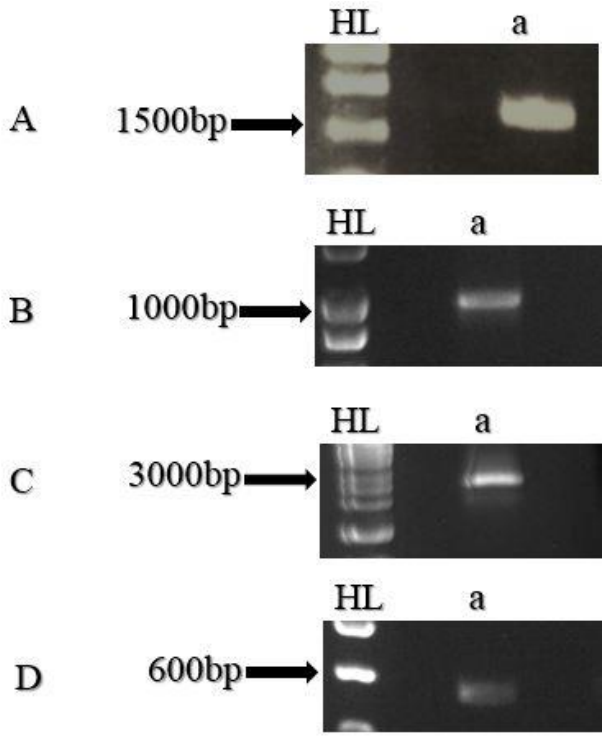


Figure 3. 12: Purification of PCR amplification of promoter fragments.

Line (a) represent PCR product; HL represent Hyper Ladder 1.

A: OsERFLP promoter using OsERFLF1, OsERFLR1 primers; amplification size product was expected to be 1500bp.

B: LCHS promoter using LCHS F, LCHSR primes; amplification size product was expected to be 900bp.

C: Ubi1 promoter using Ubi1 F1, Ubi1 R1primes; amplification size product was expected to be 2700bp.

D: CaMV 35S promoter CaMV 35S F1 BstB1, 35S R1 sal1 primes; amplification size product was expected to be 540bp.

3.3.3.2 Transformation of E. coli cells (DH5 α) with promoter constructs.

The PCR product of promoters (*OsERFLP, LCHS, Ubi1* and *CaMV35S*) were cloned into *E. coli* and 16 randomly colonies were selected for colony PCR using the M13F primer and the vector specific gene primers OsERFL1 colR1; LCH-SpcolR1; Ubi1 R1 and CaMV35S sal1R1 to identify the correctly transformed colonies in the entry vector PCR8GW. According, to the map the size between M13F and specific genes are expected 850bp; 825bp; 2110bp and 690bp respectively (Appendix 1). Figure 3.13 shows that PCR colony screening16 colonies, after sequenced Fig 3.13A lanes 1,10, and 13 were positive for PCR8GW:*Os ERFLP*; Fig 3.13B lanes 4,11 and 12 were positive for PCR8GW: *LCHS*; Fig 3.13C lanes 1,6,9 and 14 were positive for PCR8GW:*Ubi1* and Fig 3.13D lanes 2,10,13 and 16 were positive for PCR8GW: *CaMV35S*; they were then sequenced to confirm identity.

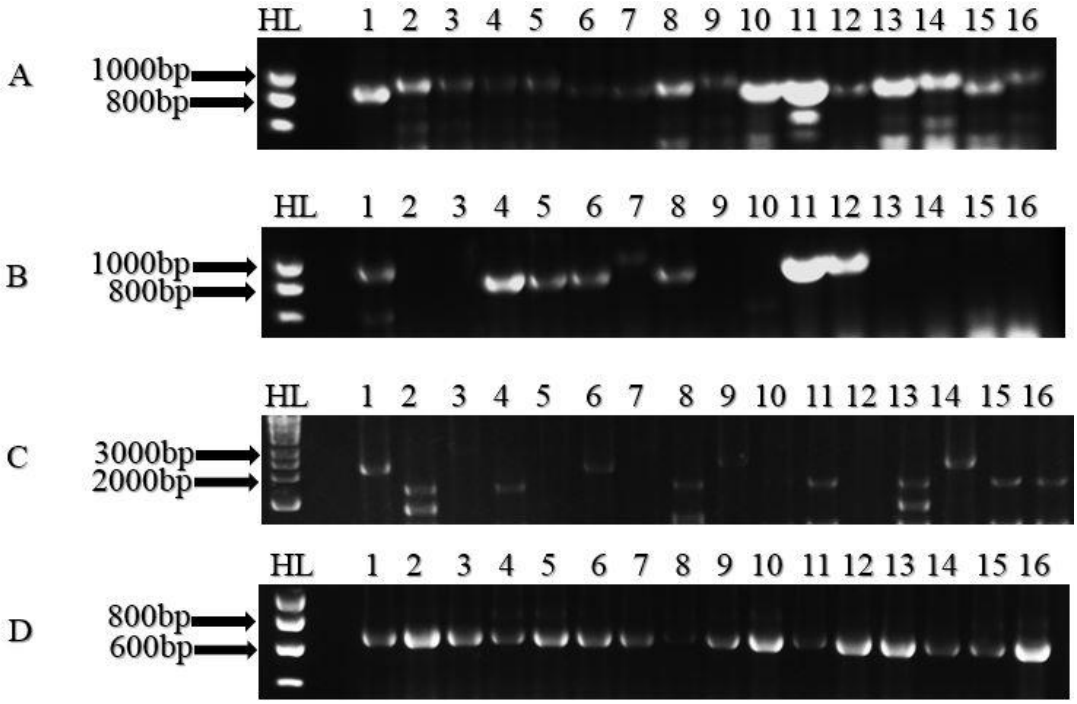


Figure 3. 13: Colony PCR confirming the transformed colonies with OsERFLP, LCHS, and Ubi1 and CaMV35S entry vector PCR8GW.

Lanes 1-16: 16 selected colonies. HL represent Hyper Ladder 1

A: OsERFLP Transformed colony using M13F primer in the vector combined with specific gene OsERFL1 colR1; size product was expected to be 850bp.

B: LCHS Transformed colony using M13F primer in the vector combined with specific gene LCHSpcolR1; size product was expected to be 825bp.

C: Ubi1 Transformed colony using M13F primer in the vector combined with specific gene Ubi1 R1; size product was expected to be 2110bp.

D: CaMV35S Transformed colony using M13F primer in the vector combined with specific gene CaMV35S sal1R; size product was expected to be 690bp.

Positive plasmid of (PCR8GW*Os: ERFLP*); (PCR8GW: *LCHS*); (PCR8GW: *Ubi1*) and (PCR8GW: CaMV*35S*) were LR cloned into destination vector PGWB3. According to the T-DNA map the size between specific gene primers (OsERFLPcolF1; LCHSF1; Ubi1F1 and 35SbstB1F1) with GUSR1 was expected to be 430bp; 1100bp; 2178bp and 750bp respectively (Appendix 1). Transformed colonies were confirmed using PCR Figure 3.14 A (2, 10. 14 and 16) show the positive colonies for pGWB3: OsERFLP; Fig 3.14B all lanes were negative for PGWB3: LCHS; Fig 3.14C 1, 3, 8 and 16 were positive for pGWB3:Ubi1 and Fig 3.14D 7, 12 and 13 were positive for pGWB3:CaMV35S. They were then confirmed by sequencing

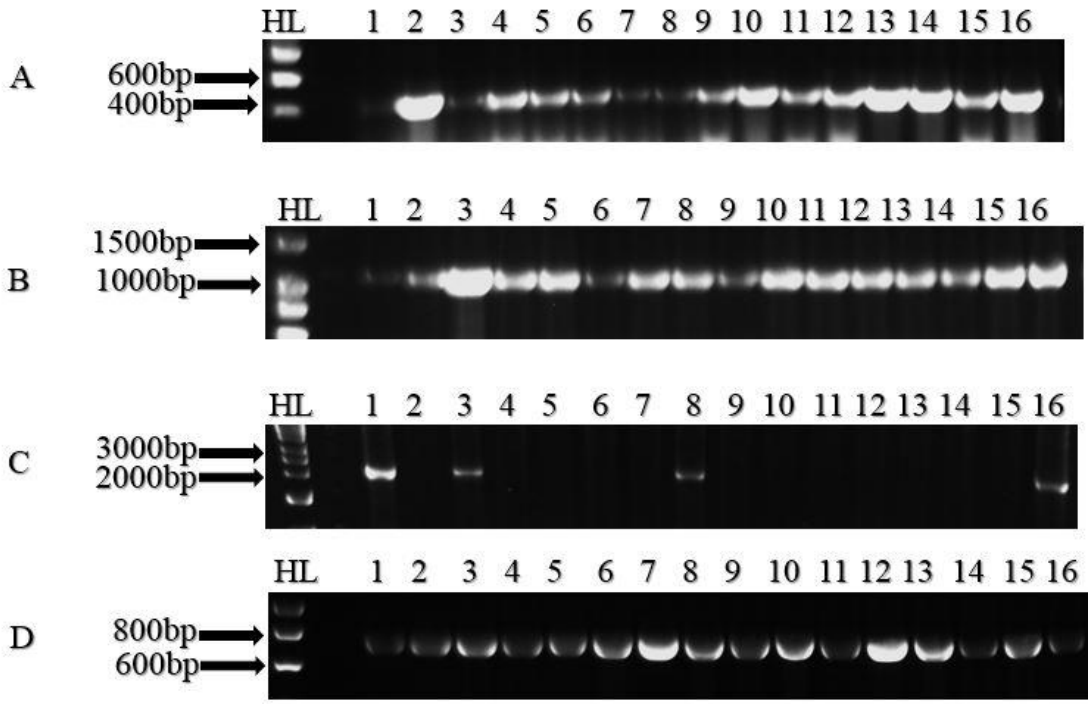


Figure 3. 14: Colony PCR confirming the transformed colonies with (OsERFLP, LCHS, Ubi1 and CaMV35S) destination vector pGWB3.

Lanes (1-16) represent 16 selected colonies; HL, Hyper Ladder 1.

A: OsERFLP Transformed colony using gene specific OsERFLPcolF1 primer combined with GUSR1 in the vector; size product was expected to be 430bp.

B: LCHS Transformed colony using gene specific LCHSF1 primer combined with GUSR1 in the vector; size product was expected to be 1100bp.

C: Ubi1 Transformed colony using gene specific Ubi1F1 primer with combined GUSR1 in the vector; size product was expected to be 2178bp.

D: CaMV35S Transformed colony using gene specific CaMV35SbstB1F1 primer combined with-GUSR1 in the vector; size product was expected to be 750bp.

Positive plasmid of PCR8GW*Os: ERFLP* and PCR8GW: *LCHS* were also LR cloned into destination vector pGWB4. According to the T-DNA map the size between specific genes (OsERFLPcolF1 and LCHSF1) with GFPR1 was expected to be 500bp and 1182bp respectively (Appendix 1). To confirmat the constructs were correctl putatively transformed colonies were PCR analysed using Red Taq; PCR colony screening 16 colonies for each constructed, after sequencing selected colonies. Figure 3.15A show Lanes 1, 2 and 11 were positive for PGWB4: OsERFLP; Fig 3.15B 3 and 6 were positive for PGWB4: LCHS. These were confirmed by sequencing.

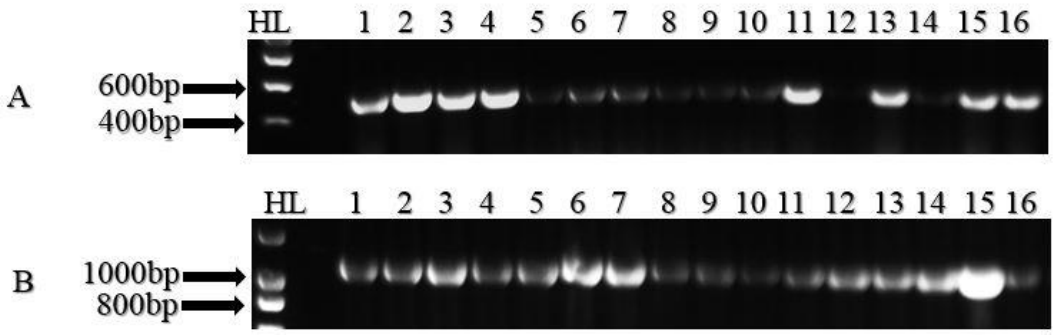


Figure 3. 15: Colony PCR confirming the transformed colonies with (OsERFLP and LCHS) destination vector pGWB4.

Line (1-16) represent 16 selected colonies; HL represent Hyper Ladder 1.

A: OsERFLP Transformed colony using gene specific OsERFLPcolF1 primer combined with GFPR1 in the vector; size product was expected to be 500bp.

B: LCHS Transformed colony using gene specific LCHSF1 primer combined with GFPR1 in the vector; size product was expected to be1182bp.

3.3.3.3 Transformation of Agrobacterium AGLI with Promoter constructs.

Confirmed construct carrying the gene of interest for OsERFLP and *Ubi1* were transformed into *Agrobacterium* AGL1 and GV3101 strains by electroporation and colony PCR was performed for 7 randomly selected colonies. Figure 3.16 illustrated the confirmation of correctly transformed colonies obtained with *OsERFLP* and *Ubi1* expression vector.

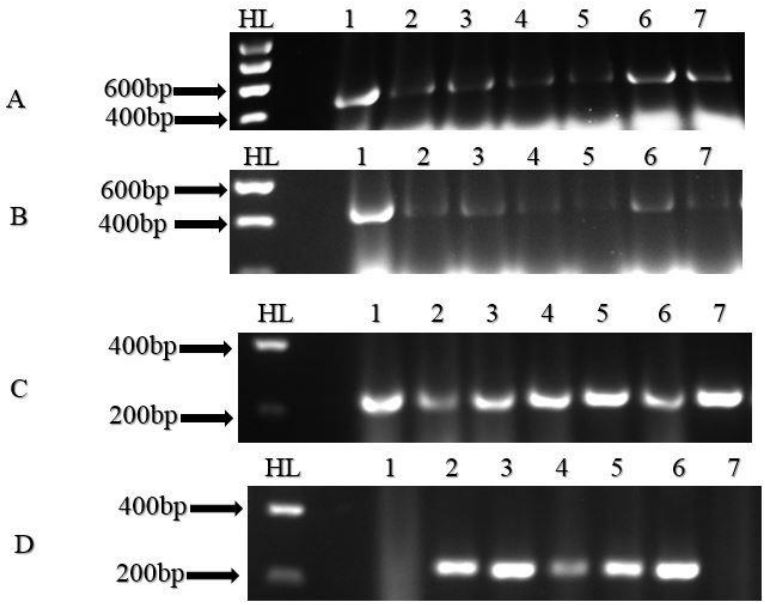


Figure 3. 16: Colony PCR confirming the Agrobactrium transformed colonies with (OsERFLP and Ubi1) expression vector.

Lanes 1-7 represent 7 selected colonies

A and B: pGWB3: OsERFLP transformed into *Agrobacterium* GV3101 and AGL1 strains using gene specific OsERFLPcolF1 primer combined with GUSR1 in the vector; size product was expected to be 400bp.

B and C: pGWB3: Ubi1 transformed into *Agrobacterium* GV3101 and AGL1strains using gene specific Ubi1proF1 primer combined with GUSR1 in the vector; size product was expected to be 200bp.

3.3.4 *HvMYB26* SRDX silencing construct.

HvMYB26SRDx construct was developed by Gateway cloning into the PGKGWY vector, the rice (*OsERFLP*) anther specific promoter was chosen to drive the expression of the Hv*MYB26SRDX*. The *MYB26* gene is critical in *Arabidopsis* for the secondary thickening in the anther endothecium and anther dehiscence.OsERFL1 promoter was amplified using PCR Phusion from rice genome DNA (*Oryza sativa* Japonica ssp Nipponbare cv. Wild type) using (OsERFL1promSacIF1, OsERFL1promXbalIR1 primers) (Kato *et al.*, 2010). Fig 3.17 A shows the isolated and purified products.

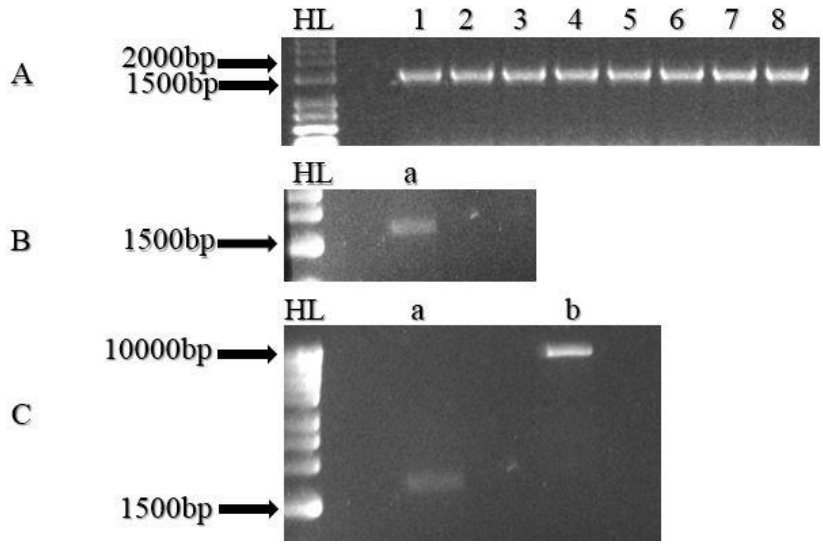


Figure 3. 17: PCR amplified with gel purification digest product for PGKGWY vector with OsERFLP.

Lanes (1-8) represent promoter amplification; HL represent Hyper Ladder 1.

A: Amplified OsERFLP promoter using OsERFL1promSacIF1, OsERFL1promXbalIR1 primers; size product was expected to be1558bp.

B: promoter's purification product; Line (a) represent PCR product; size product was expected to be 1558bp

C: Digestion products; a: promoter digested product b: PGKGWY digested product

OsERFLP promoter was then cloned into the PGKGWY destination vector, Sac1 and Xbal1 was used to digestion (OsERFLP and PGKGWY vector), and then purified by QIAPCR purification (Section 2.5; Fig 3.17C).

100ng/µl of PGKGWY with 100-200ng/ µl OsERFLP promoter was ligated and transferred into DP3.1 *E.coli* cells (Section 2.6). Sixteen colonies were PCR screened; Fig 3.18A shows that lanes 5, 9 and 12 were positive for PGKGWY: OsERFLP.

Hv*MYB26* entry vector was transferred into PGKGWY OsERFLP destination vector (Figure 3.18B; Appendix 1). Colony PCR was performed using Red Taq by (HvMYB26RNAi F1 and YFP-NY-R1) with the expected bind size is to be 1000bp, lane 6 was positive among 16 colonies selected.

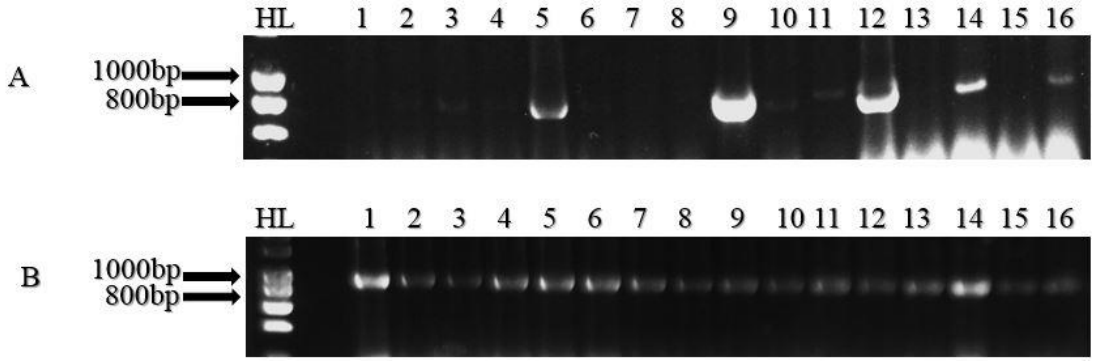


Figure 3. 18: Colony PCR confirming the transformed colonies.

Lanes (1-16) represent 16 selected colonies; HL. Hyper Ladder 1.

A: OsERFLP transformed colony using M13R (as a forward) vector combined with gene specific OsERFLPcolR1 primer; size product was expected to be770bp.

B: HvMYB26SRDx transformed colony using HvMYB26RNAi F1, YFP-NY-R1primers; size product was expected to be1000bp.

3.3.5 Cloning and preparation of over-expression constructs.

Two *Arabidopsis* genes *ATIPT4* and *ATIPT8* were targeted for cloning into pGWB5 vector and transformation into Lily and Arabidopsis plants.

3.3.5.1 Amplification and purification of ATIPT4 and ATIPT8 from Arabidopsis.

ATIPT4Fw ATIPT4Rw and ATIPT8Fw ATIPT8Rw primers (Appendix 3) were used to amplify *ATIPT4* and *ATIPT8* genes respectively from *Arabidopsis* leaves (Fig 3.19); Fig 3.20 shows the gel purification the products from these reactions.

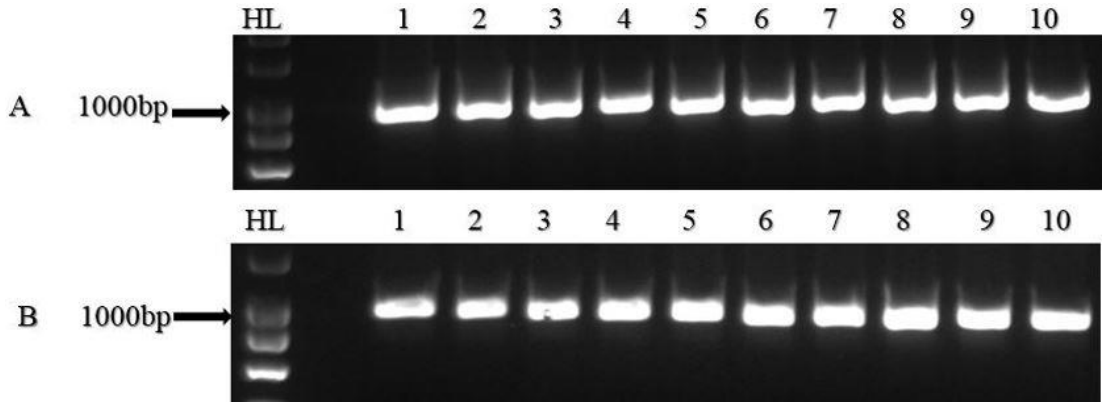


Figure 3. 19: PCR amplification of ATIPT4CDS and ATIPT8CDS from Arabidopsis Ler leaves; Lane (1-10) gene amplification; HL represent Hyper Ladder 1

A: *ATIPT4* gene using primers AtIPT4Fw and AtIPT4Rw, band size of product is 957bp.

B: *ATIPT8* samples using primers AtIPT8Fw AtIPT8Rw, band size is 993bp.

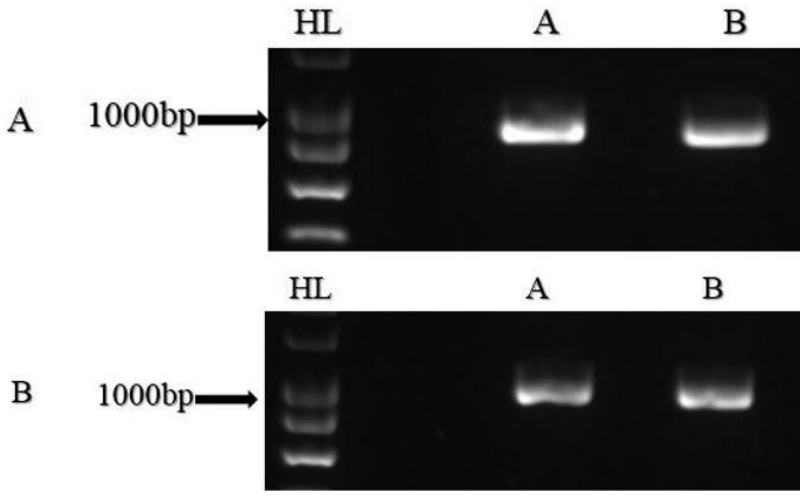


Figure 3. 20: ATIPT4CDS and ATIPT8CDS gel purification products; Lanes (A, B) PCR product; HL Hyper Ladder 1.

A: *ATIPT4* gene (expected size 957bp).

B: *ATIPT8* gene (expected size 993bp).

3.3.5.2 Transformation of E. coli cells (DH5 α) with ATIPT4 and ATIPT8 construct.

The PCR products of the ATIPT4 and *ATIPT8* genes were A-tailed and then TOPO cloned into PCR8GW TOPO and transformed into *E. coli* cells. Sixteen randomly selected colonies were used for colony PCR using the M13F and gene specific primers (*ATIPT4colR1* and ATIPT*8colR1)* to identify the correctly transformed colonies in the entry vector PCR8GW (expected sizes 690bp and 660bp, respectively; Appendix 1). Figure 3.21A shows the PCR from 16 colonies, lanes 3, 4,9,13 and 15 were positive for PCR8GW-*ATIPT4* and lanes 5 and 6 were positive for PCR8GW-*ATIPT8;* Fig 3.21B; sequencing the plasmids using the same primers was used to confirm the sequence identity.

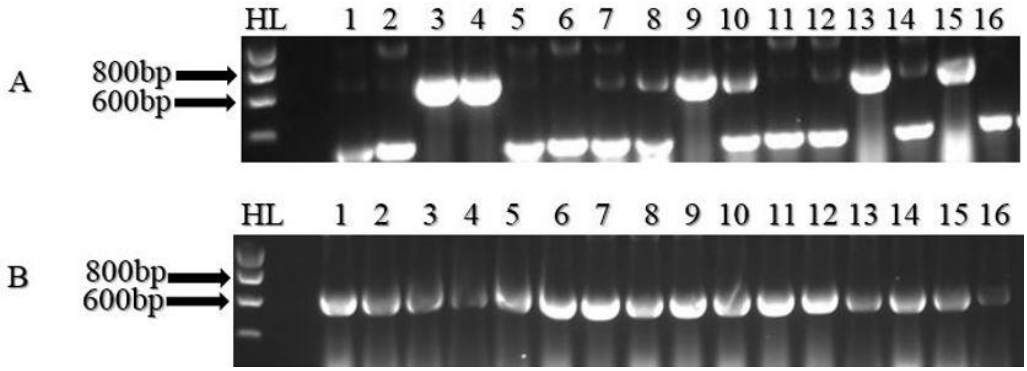


Figure 3. 21: Colony PCR confirming the transformed colonies with ATIPT4CDS and ATIPT8cDS) entry vector PCR8GW.

Lanes 1-16: selected colonies; HL: Hyper Ladder 1

A: ATIPT*4*CDS PCR using M13F and *ATIPT4colR1* primers; expected product was 690bp.

B: ATIPT*8*cDS transformed colony using M13F and ATIPT8colR1 primers; expected size product was 660bp.

Positive entry vectors (PCR8GW: *ATIPT4* and PCR8GW: *ATIPT8*) were L.R cloned into destination vector pGWB5 and confirmed by PCR (gene specific primers *ATIPT4colR1* and ATIPT*8colR1* with pGWB8F vector primer; expected 620bp and 600bp respectively; Appendix1). Figure 3.22 shows PCR screening 16 colonies analysed, that after sequencing lanes 3,6,8,14 and lanes 2,11,12,16 were positive for the transgenes; (pGWB5: *ATIPT4*) and (pGWB5: *ATIPT8)* respectively; these were confirmed by sequencing and used for transformation.

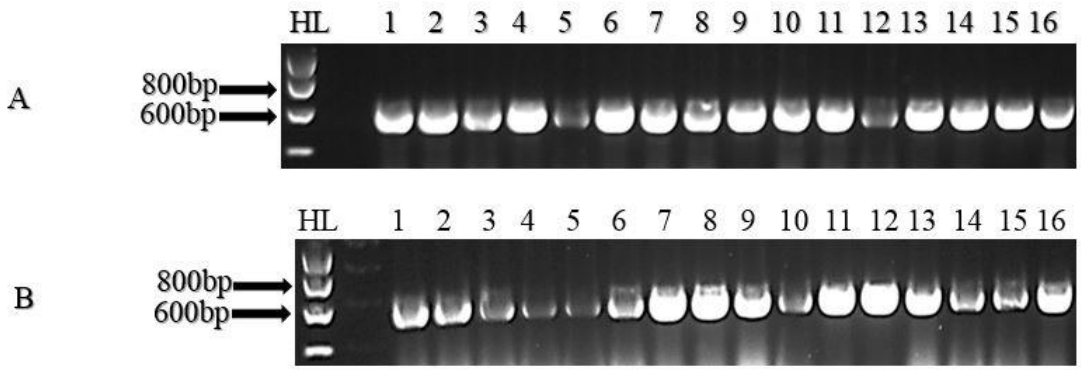


Figure 3. 22: Colony PCR confirming the transformed colonies with (ATIPT4-CDS and ATIPT8-CDS) destination vector pGWB5.

Lanes (1-16) 16 selected colonies; HL: Hyper Ladder 1.

A: PCR of putative ATIPT4CDS transformed colonies using pGWB8F vector primer combined with specific gene primers ATIPT4colR1; size product was expected to be 620bp.

B: PCR of putative of ATIPT8CDS transformed colonies using pGWB8F vector primer combined with specific gene primers ATIPT8colR1; size product was expected to be 600bp.

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3.3.5.3 Transformation of Agrobacterium AGLI and GV3101 with ATIPT4 and ATIPT8 constructs.

Confirmed constructs carrying the gene of interest *(ATIPT4 and ATIPT8)* were electroporated into *Agrobacterium* AGL1 and GV3101 strains (Section 2.13) and transformed colonies were confirmed by PCR (Fig 3.23) and used to transform Lily explants.

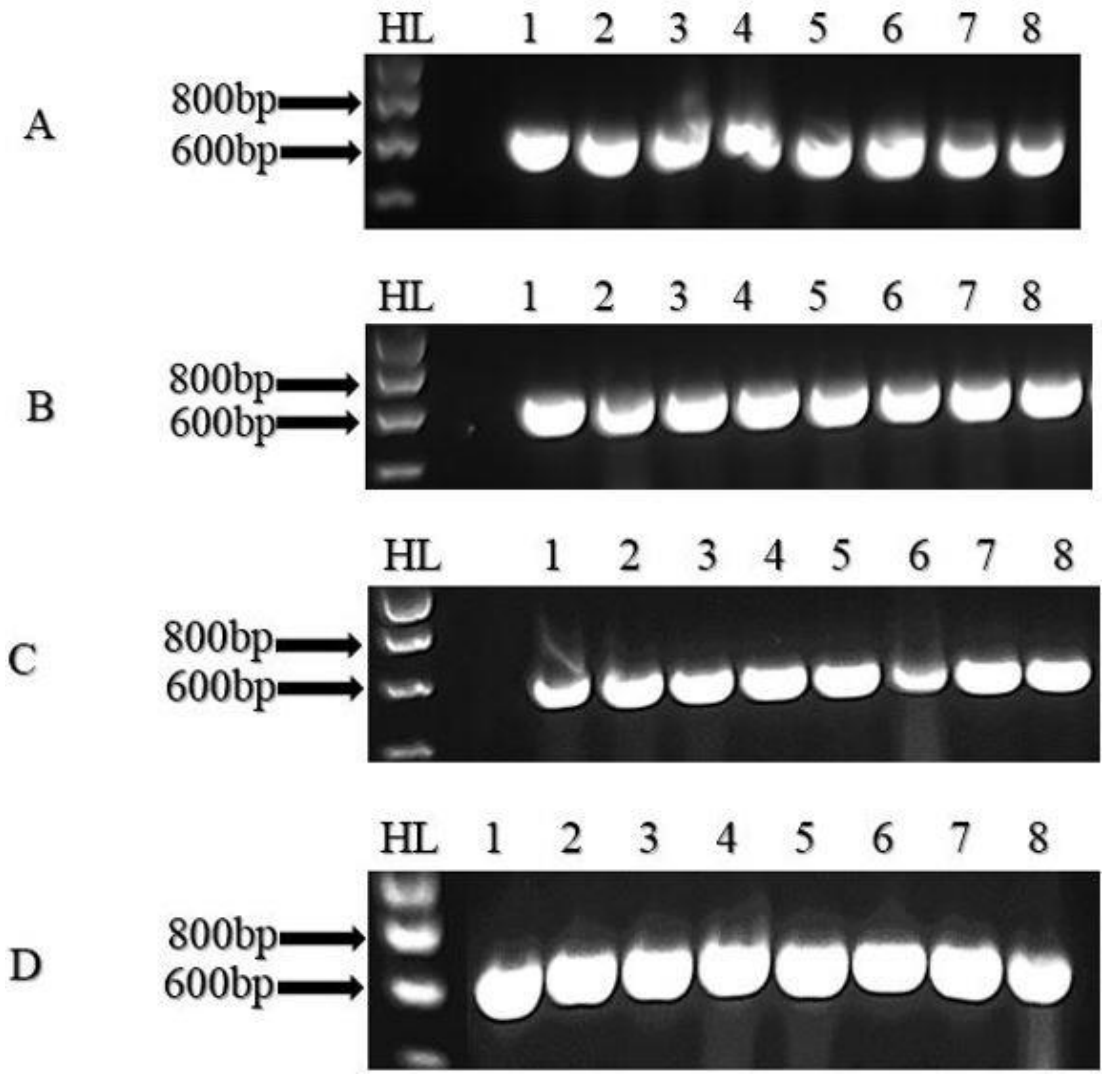


Figure 3. 23: Colony PCR of Agrobacterium putatively transformed colonies containing ATIPT4CDS and ATIPT8CDS.

Lanes 1-8 represent 8 selected colonies; HL: Hyper Ladder1.

(A) and **(B)** pGWB5:*ATIPT4cDS* Transformed into *Agrobacterium* AGL1 and GV3101 respectively and using pGWB8F primer in the vector combined with specific gene primers ATIPT4colR1: size product was expected to be 620bp.

(C) and **(D)** pGWB5:*ATIPT8cDS* Transformed into *Agrobacterium* AGL1 and GV3101; pGWB8F primer in the vector combined with specific gene primers ATIPT8colR1Rv: size product was expected to be 600bp,

3.3.6 Tissue culture and Regeneration of *Lilium.*

Lily bulbs of variety Star Gazer were used to produce bulblets from small explants, particularly parts of sterilized scales (Fig 3.24 b and d), after subculture in the Regeneration medium (Appendix 4) 3-5 small bulblets were created from the edges of scales depending on the size of scales. Fig 3.24c shows the basal plate discs of 3-6mm and 0.5-1.5 mm thick that were excised from standard bulblets (Fig 3.24f); this whole process required at least 3 months for generation of fresh basal plates for transformation.

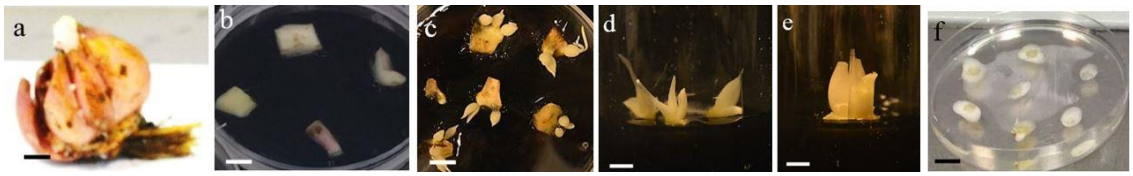


Figure 3. 24: Preparation of basal plate discs from scales from Lily Star Gazer bulbs.

(a) Bulbs as stock starting material; (b) small explant taken from sterilized scales; (c) Small bulblets forming at edges of scales but still attached; (d) detached small bulblets; (e) standard bulblets used to prepare basal plates for transformation; (f) basal plates in co-culture medium. Bar: a = 10mm; b, c, d, and e = 8mm and f= 12mm.

3.3.7 Transformation of *Lilium to analyse the activity of the promoters.*

AGL1::PGWB3::Ubi1::*GUS* was used to transform 50 lily basal plates by *Agrobacterium* transformation (Section 2.15), the AGL1::BPI121::*GUS* construct was used alongside 50 non-transformed basal plates as a control. Histochemical analysis of GUS gene expression was performed to confirm transient expression and the activity of the Ubi1 promoter 4 days after inoculation. Blue regions of GUS

activity with high level of expression were seen (Fig 3.25 b and c) compared to the wild type control (Fig 3.25a). Transformation efficiency was analysed 4days post inoculated and calculated as the number of explants showing transient expressions (transgenic basal plate) divided by the total number of basal plate explants inoculated as summarized in Table 3.1.

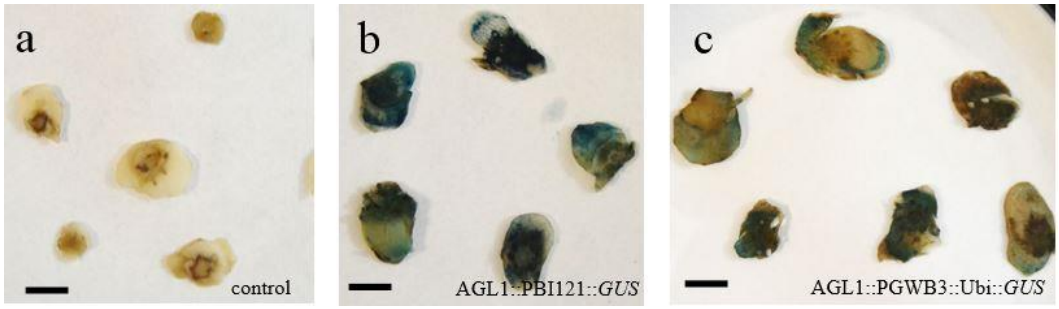


Figure 3. 25: Analysis of GUS expression in basal plate discs 4 days after inoculation for Lilium explants.

(a) wild type as control; (b) Basal plate discs after inoculation using AGL1::BPI121::*GUS* construct (C) Basal plate discs after inoculation using AGL1::PGWB3::Ubi1::*GUS* Bar= 5mm

Table 3. 1: Transformation efficiency for AGL1:: PGWB3::Ubi1::GUS construct.

Name of constructs	**No of basal plate explants inoculated**	**No of transgenic basal plate**	**Transformation efficiency (%)**
Wild type as control	50	0	0
AGL1::BPI121::*GUS*	50	32	64
AGL1::PGWB3::Ubi1::*GUS*	50	25	50

3.3.8 Analysis of *ATIPT4* over-expression lines.

3.3.8.1 Analysis of T_0 Lily ATIPT4 and ATIPT8 over-expression lines.

3.3.8.1.1 Genotypic Analysis for *ATIPT4*

Seven independent putatively transformed plants were regenerated from 150 basal explants from Lily Stargazer (Section 2.15), alongside 50 basal explant that were non-inoculated controls and grown under the same conditions.

After selection, transition and regeneration media, with the presence of kanamycin as selective agent, T_0 plantlets were transferred to pots and placed in an environmental controlled room (Fig 3.26)

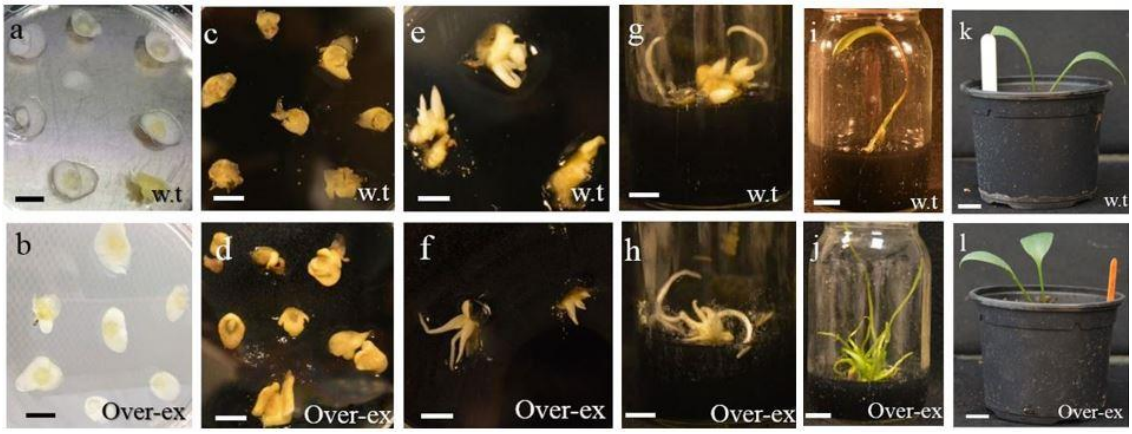


Figure 3. 26: Lily transformation showing the different regeneration stages from basal plate inoculation to plantlets stage for ATIPT4 Overexpression construct, compared with non-inoculated controls.

(a and b) basal plate in co-culture medium; (c and d) Transition medium 2; (e and f) Selection medium; (g and h) Regeneration medium; (i and j) Roots and shoots; (k and l); plantlets growing in soil. Bar: a, b, c, d, e, and f = 8mm; g and h= 6mm; I and j= 10mm; k and l=13 mm

Genomic PCR was used to screen transgenic plants, using PGWB8Fw (vector specific) and the target gene primer *ATIPT4colR1*Rv. Unfortunately, all the re-generated plants were negative for the over-expression transgene (Fig 3.27).

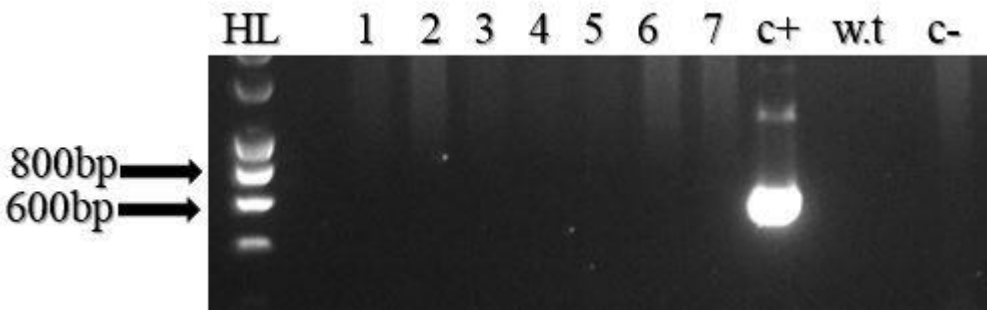


Figure 3. 27: PCR genotyping of T_0 putatively transformed lily plants carrying the ATIPT4 over-expression construct

The vector specific primer PGWB8Fw combined with the gene specific primer *ATIPT4colR1*Rv was used; size product was expected to be 620bp. Lines 1-7 represents transformed plants. (C+) *AtIPT4* over-expression construct plasmid DNA as positive control; (wt.) as negative control; (c-) water negative control; (HL) Hyper Ladder 1.

3.3.8.1.2 Genotypic Analysis for *ATIPT8*

150 basal plates were used for transformation with the *ATIPT8* construct, 50 non-inoculated basal plates were used as controls and grown under the same conditions. Selection and regeneration following the same procedure as used for the *ATIPT4* construct, but unfortunately, none of the inoculated basal plates inoculated regenerated, however control basal plated were successfully regenerated when no antibiotic selection as used.

3.3.9 *Arabidopsis* transformation to analyse promoter activity.

Two constructs, GV3101::pGWB3::Ubi1::*GUS* and GV3101::pGWB3::OsERFLP::*GUS* were used for transformation of *Arabidopsis* L*er*.

3.3.9.1 Genotypic Analysis for pGWB3Ubi1 and pGWB3OsERFLP lines.

pGWB3:Ubi1 and pGWB3: OsERFLP transformed seeds were selected on 50µg/ml kanamycin, Figure 3.28 b and c shows germination of the T_0 transformed seeds on kanamycin selective plates after transformation with the pGWB3:Ubi1 and pGWB3: OsERFLP constructs.



Figure 3. 28: Germination of *Arabidopsis* seeds on control and selective media after transformation with pGWB3:Ubi1 and pGWB3: OsERFLP constructs.

a: Non-transformed *Arabidopsis* seeds without kanamycin.

b: pGWB3:Ubi1 transformed seeds on 50µg/ml kanamycin.

c: pGWB3: OsERFLP transformed seeds on 50µg/ml kanamycin.

Genomic PCR was conducted to confirm T_1 *Arabidopsis* transformed plants, using the Ubi1proF1 promoter Fw; OsERFLPcolF1 primers combined with GUSR1 vector specific Rv primers. Results showed that all selected plants were positive for the pGWB3:Ubi1 and pGWB3: OsERFLP construct (Fig 3.29 A and B respectively).

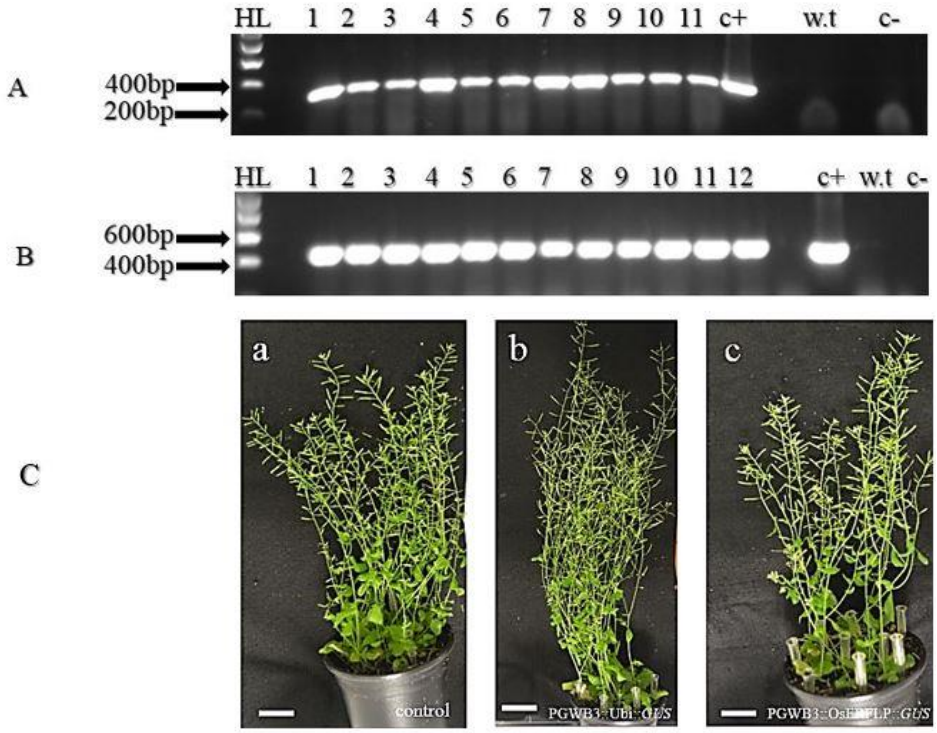


Figure 3. 29: Genotyping of T_1 Transformed plants carrying the pGWB3:Ubi1 and pGWB3: OsERFLP constructs using PCR.

Lanes 1-12 putatively transformed plants; (C+) *pGWB3:Ubi1 and pGWB3:OsERFLP* construct plasmid DNA as positive control; (wt.) wild type as negative control; (c-) water negative control; (HL) Hyper Ladder1.

A: pGWB3:Ubi1 lines using Ubi1 proF1 specific gene primers combined with GUSR1primer in the vector; size product was expected to be 200bp.

B: pGWB3: OsERFLP lines using OsERFLPcolF1 specific gene primers combined with GUSR1primer in the vector; size product was expected to be 400bp

C: Arabidopsis transformed plant: **a:** Non-transformed Arabidopsis plant. **b:** pGWB3:Ubi1 lines

c: pGWB3: OsERFLP lines

The expression of the GUS gene was determined by histochemical analysis to confirm the transformation of leaf tissue *Arabidopsis* plants and activity of Ubi1 promoter. Blue regions of GUS activity with high level of expression of the GUS gene were seen in pGWB3: Ubi1 lines compared to the control samples (Fig 3.30).

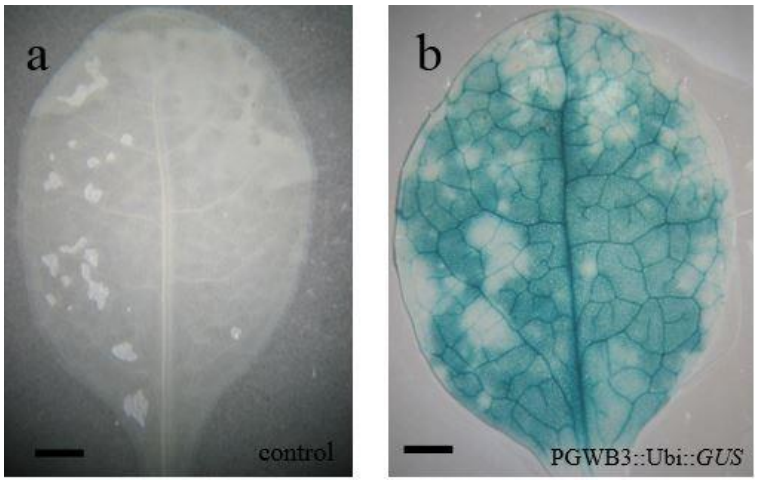


Figure 3. 30: Analysis GUS expression in an Arabidopsis leaf transformed with pGWB3:Ubi1 T_1.

(a) Wild type untransformed control; (b) GV3101::PGWB3::Ubi1::*GUS* Bar= 3mm

3.3.10 Analysis of Arabidopsis T_0 *ATIPT4 and ATIPT8* over-expression lines.

3.3.10.1 Genotypic Analysis for ATIPT4 and ATIPT8

After floral-dip transformation for both the *ATIPT4 and ATIPT8* constructs, the *Arabidopsis* seeds were screened on half MS medium (Appendix 4) containing 50µg/ml kanamycin. Only one seed developed from both *ATIPT4 and ATIPT8* transformations (Fig 3.31 a and b). Transformed plantlets were identified by their robust growth on kanamycin selective plates compared to wild type.

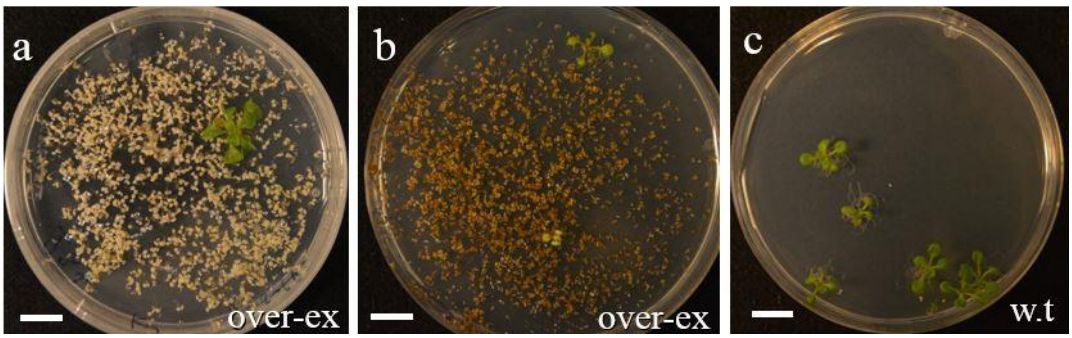


Figure 3. 31: Germination of putatively transformed Arabidopsis seeds on control and selective media:

a: *ATIPT4* transformed seeds on 50µg/ml kanamycin.

b: *ATIPT8* transformed seeds on 50µg/ml kanamycin.

c: Non-transformed *Arabidopsis* seeds without kanamycin.

Genomic PCR was used to screen the *Arabidopsis* transformed plants, using PGWB8 promoter Fw (vector specific) combined with the target gene primer *ATIPT4colR1*Rv and *ATIPT8colR1*Rv. Results showed that regenerated plants were positive for the *ATIPT4* over-expression construct, but negative for *ATIPT8* over-expression construct (Fig 3.32).

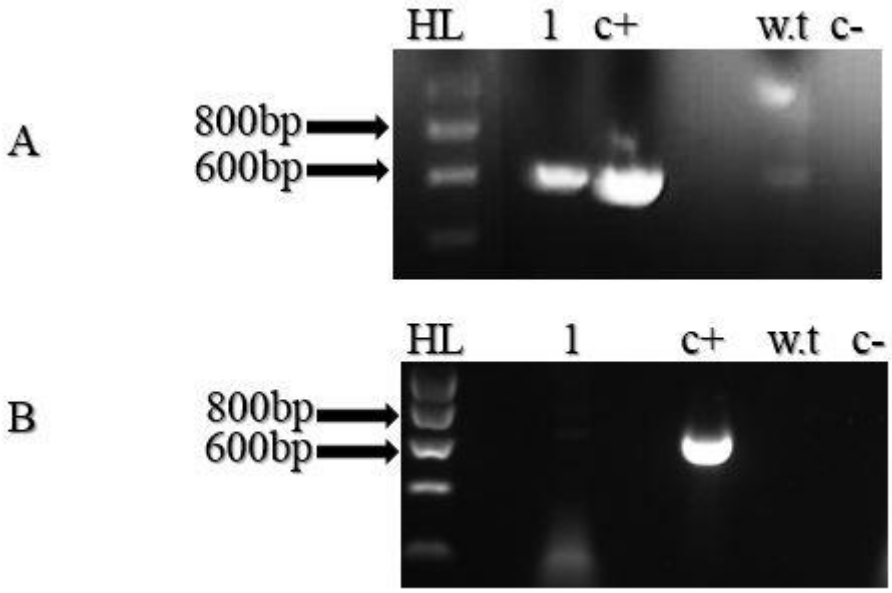


Figure 3. 32: PCR genotyping of T_0 Arabidopsis plants putatively transformed with the ATIPT4 and ATIPT8 over-expression constructs.

(wt.) wild type as negative control; (c-) water negative control; (HL) Hyper Ladder

A: Lane 1 putatively transformed plant; showed positive for pGWB5:*ATIPT4CDS* transgene using PGWB8F primer in the vector combined with specific gene primer ATIPT4colR1; size product was expected to be 620bp. (C+) *AT IPT4* over-expression construct plasmid DNA as positive control.

B: Lines 1 represents transformed plants; showed negative for transgene in pGWB5:*ATIPT8CDS* using pGWB8F primer in the vector combined with specific gene primers ATIPT8colR1: size product was expected to be 600bp results (C+) *AT IPT8* over-expression construct plasmid DNA as positive control

Only one transformed plant was confirmed for the *ATIPT4* construct by kanamycin selection and genomic PCR. Lines derived from this plant were subsequently PCR screened in the T_1 generation; 20 plants were selected (Fig 3.33) but all lines were negative for transgene.

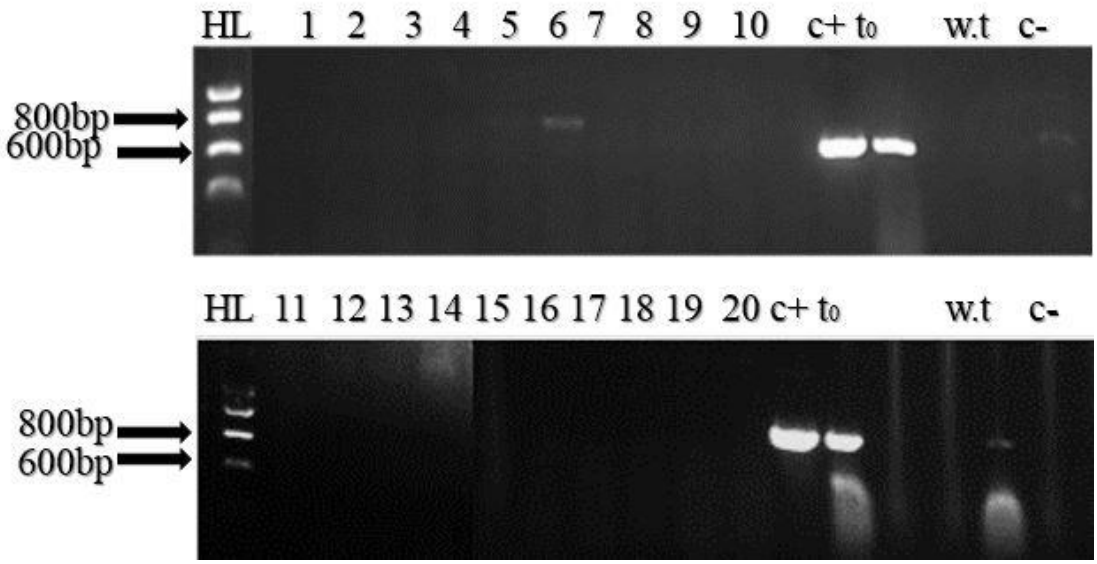


Figure 3. 33: PCR genotyping of T_1 putatively Transformed plants carrying the *ATIPT4* over-expression construct.

Lanes 1-20 putatively transformed plants were negative for the pGWB5:*ATIPT4CDS* transgene using the pGWB8F primer in the vector combined with specific gene primers ATIPT4colR1; size product was expected to be 620bp. (C+) DNA from *AtIPT4* over-expression construct as positive control; (t_0) gDNA from T_0 transformed plant as a positive control; (wt.) wild type as a negative control; (c-) water negative control; (HL) Hyper Ladder1.

3.11 Discussion

The ultimate main aim of this work was to analysis of anther and pollen development pathways in Lily using genes previously identified in *Arabidopsis*, however prior to this a characterisation of pollen development, transformation and expression analysis using tissue specific promoter was required.

The relationship between length of buds and anther development over a period of 48 days was conducted; Figure 3.9 shows that the development stages of bud and anther developed rapidly and that bud length was significantly increased. Microscopy of the different pollen developmental stages was correlated to bud and anther length to enable accurate analysis of pollen development in future transgenic studies (Figure 3.9).

The pGWB3: OsERFLP; Ubi1 and CaMV35S and pGWB4 OsERFLP and LCHS constructs were successfully made and confirmed by colony PCR and sequencing (Fig 3.14 and 3.15) these offer useful resources for future transformation experiments that can be used to target gene expression in specific plant tissues, or throughout the plant. In addition, they can be utilized to drive silencing and over-expression constructs.

The anther specific promoter from rice (Os*ERFLP*) (Kato *et al.,* 2010) was cloned into the PGKGWY vector (Fig 3.18 Appendix 1) along with the barley orthologue of the *Arabidopsis MYB26* gene (*HvMYB26)*; *Arabidopsis MYB26* gene is critical for the secondary thickening in the endothecium and later anther dehiscence (Yang et al., 2007b). The HvMYB26SRDx construct was made by Gateway cloning using pGKGWY vector, the rice (*OsERFLP*) anther specific promoter was chosen to drive the expression of the Hv*MYB26SRDX*, to facilitate targeted ex-

pression of the transgenes in the flowers, and/or anthers with the aim of controlling male fertility. This constructed is prepared and is ready for future transformations. Therefore, transformation of *Lilium* with the *AtMYB26* repressor constrct (SRDX) gene using these constructs may provide a mechanism for tissue specific expression to prevent anther opening in *Lilium*. This could provide a solution to the problem to the large amounts of pollen produced in *Lilium* flowers (Stokes and Forrester, 2004; Fitzgerald 2010; Thompson 2012; Bright *et al.*, 2009), which are undesirable traits for the cut flower industry.

Tissue culture and *Agrobacterium-mediated* genetic transformation have become valuable tools for the improvement of a wide variety of horticulture crops (Magori 2011; Gnasekaran *et al.*, 2014). *Agrobacterium-mediated* transformation efficiency was tested in *Lilium* using reporter constructs containing GUS and GFP, to identify promoter activity. Expression was seen in callus and vegetative tissues in both transient and stable transformations soon after the transformation (~ 4days using the promoters OsERFL, LCHS, Ubi1 and CaMV35S.

GUS reporter constructs PGWB3:Ubi1: GUS driven by the Ubi1 promoter were developed to analysis expression in callus and vegetative tissues in Lily and *Arabidopsis*. High level of GUS expression were seen 4 days after inoculation of *Lilium* basal plates (Fig 3.25 b and c) using the reporter constructs AGL1:PGWB3: Ubi1: *GUS* and AGL::pBI121::*GUS.* Transient transformation efficiencies of 50% and 60% were seen using the AGL1:PGWB3: Ubi1: *GUS* and AGL::pBI121::*GUS* respectively (Table 3.1). Transformation efficiency was calculated based on number of basal plate with GUS activity divides by the total of basal plate inoculated. However, Núñez de Caceres *et al*., (2011) reported the ef-

ficiency calculated based on the number of PCR positive plants compared to the number of explants inoculated which is a more accurate estimate of the stable transformation efficiency. There have also been reports of highly efficient *Agrobacterium* transformation of lily of 20% that is nearly 5 times higher (Ogaki *et al*. 2008). To ensure stable transformation materials must be checked after 11 weeks, however the basal plate transformed here started to show browning and necrosis within 5 days and eventually died after 10 days, suggesting these are only transiently expressing the transgenes and are not stably transformed.

An attempt was made to generate transgenic Lily *(Lilium StarGazer)* and *Arabidopsis* plants that were ectopically expressing the *Arabidopsis IPT4* and *IPT8* genes under the regulation of the CaMV35S promoter.

The pGWB5:*ATIPT4*-CDS and pGWB5:*ATIPT8*-CDS over-expression constructs, containing the transgene overexpression controlled by the CaMV35S promoter with the kanamycin resistance gene as a selectable marker were successfully prepared to express transgenes these genes in Lily and *Arabidopsis* plants. These were used to transform basal plate explants from Lily. Seven putatively independent transformed lily plants were obtained from transformations with the pGWB5:*ATIPT4*-CDS plasmid (Fig 3.26). Unfortunately, these were not confirmed as transgenic by PCR analysis (Fig 3.27). Transformations of lily basal plates with the *ATIPT8* construct did not yield any regenerants.

Arabidopsis transformation of the pGWB3:Ubi1 and pGWB3: OsERFLP constructs showed that all the selected plants were positive for the constructs (Fig 3.29 A and B). GUS gene expression was confirmed for the pGWB3 Ub1 con-

struct in *Arabidopsis* confirming the activity of the promoter and the success of the transformation (Fig 3.30).

In the T_0 generation, only one *Arabidopsis* plantlet was generated for each *ATIPT4* and *ATIPT8* construct after kanamycin selection (Fig 3.31). Genomic PCR confirmed the presence of the *ATIPT4* construct (Fig 3.32 a), but these transformants could not be maintained into the T1 generation. PCR amplification for the *ATIPT8* construct was negative, indicating that non-transgenic plantlets had escaped the kanamycin selection.

CHAPTER 4: CHARACTERISATION OF PUTATIVE BARLEY ORTHOLOGS OF ARABIDOPSIS *DYSFUNCTIONAL TAPETUM1* GENE.

4.1 INTRODUCTION

Genetic manipulation of the genes involved in anther and pollen development can be utilised to create male sterile lines, this can be used to aid understanding of pollen development and dehiscence, which is essential for plant manipulation and hybrid seed production.

Regulation of tapetum development is crucial for normal pollen development and fertility (Section 1.5.2.1). A specific key transcription factor was identified in *Arabidopsis*, *DYSFUNCTIONAL TAPETUM 1 (DYT1)* (Zhang *et al.*, 2006) that plays a central role in tapetum development and viable pollen formation. *DYT1* functions early after initiation of the anther cell layers have occurred (Wilson and Zhang, 2009). It is specifically expressed in the tapetum and encodes for a bHLH transcription factor which is highly expressed in the tapetum during stage 5 to 6, and also, although at lower level, in the meiocytes (Zhang *et al.*, 2006). The *Arabidopsis dyt* mutant exhibits abnormal tapetal cells with enlarged vacuoles and densely stained cytoplasm and microspores that fail to progress after meiotic nuclear division, resulting in the absence of pollen grains and male sterility (Zhang *et al.*, 2006).

DYT1 regulates gene expression within the tapetum around the stage of Pollen Mother Cell meiosis and is essential for tapetum function and male fertility in *Arabidopsis* (Feng *et al.*, 2012). *DYT1* has been proposed as directly regulating at

least 22 genes encoding transcriptional factors, among them *MYB35 (TDF1)*, *AMS* and *MYB80/MS188*, which are essential during early tapetum development, and *MS1* in late tapetum development (Zhu *et al.*, 2011) (see Fig 4.1). DYT1 can indirectly regulate these genes via other transcription factors (Sorensen *et al.*, 2003, Zhu *et al.*, 2008; Wilson *et al.*, 2001 and Zhu *et al.*, 2011). Zhu *et al.*, (2015) suggested that three bHLH TFs (bHLH010/089/091) are important and interact with DYT1 for normal anther development in *Arabidopsis*, and affect the expression of DYT1 and downstream transcription factor (see Fig 4.2).

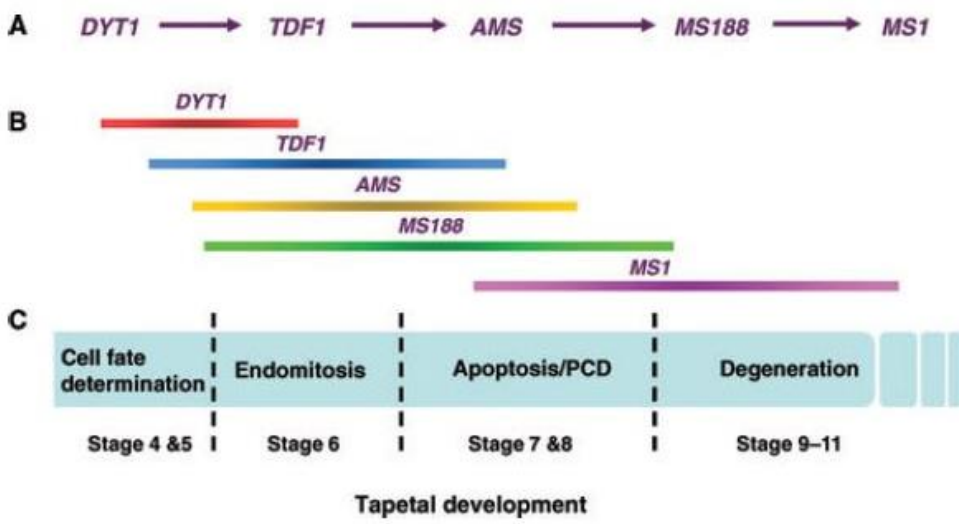


Figure 4. 1: The expression of pattern of transcription factors that are involved in the genetic control of tapetum development (taken from Zhu et al., 2011).

A: The genetic pathway of transcription factors for tapetum development and function.

B: The expression of the transcription factors during tapetum development; the lines with different colours represent the expression pattern of the different transcription factors.

C: Tapetum development with main events at different anther stages including cell fate determination, endomitosis, apopotosis/programmed cell death and degeneration.

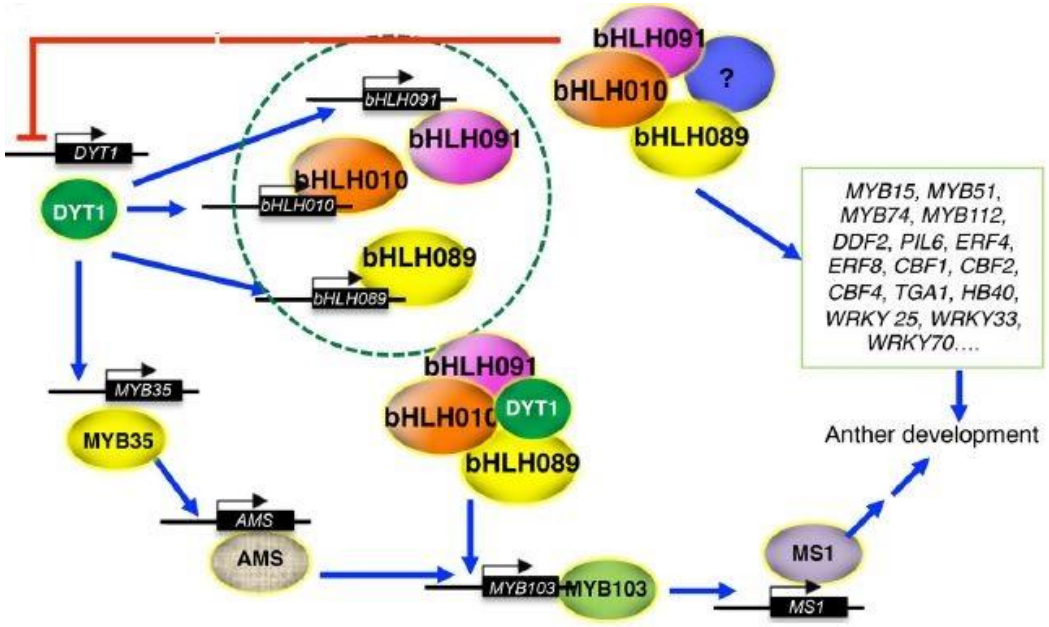


Figure 4. 2: Gene regulatory network model for DYT1 and its interacting proteins bHLH010/bHLH089 and bHLH091 (taken from Zhu et al., 2015).

DYT1 positively regulates anther development by activating the expression of *MYB35*, *AMS*, *MYB103* and *MS1*. In addition, DYT1 activates expression of the three functionally redundant transcription factorsbHLH010, bHLH089 and bHLH091. The bHLH010/bHLH089/bHLH091 proteins form hetero-complexes with DYT1, and subsequently regulate the expression of down-stream genes overlapping with those regulated by DYT1, including *MYB103* and *MS1*. The bHLH010/bHLH089/bHLH091 proteins form homo- or hetero-complexes among themselves, or with other unknown transcription factors, and regulate the expression of DYT1-independent genes. Black lines and arrows represent the promoter and transcribed regions, respectively; blue arrows indicate positive regulation; the red line with blunt end indicates negative regulation; the green dashed circle represents the functional redundancy of bHLH010, bHLH089 and bHLH091

Sequencing of grass genomes such as *Oryza sativa* (International Rice Genome Sequencing Project, 2005), *Brachypodium distachyon* (International Brachypodium Initiative, 2010) *Zea mays* B73 (Schnabel *et al.*, 2009) and the barley genome (Mayer *et al.*, 2012) is enabling the transfer of understanding of these genes to other species, particularly cereal crops. In particular, improvements in cereal transformation, for example in barley (Harwood *et al.*, 2009), wheat (He *et al.*, 2010) and rice (Hiei *et al.*, 2008) are facilitating the translation and characterization of genes involved in pollen development from models to economically important crops such as rice and barley (Wilson and Zhang, 2009 and Gomez *et al.*, 2015).

Rice has shown a high degree of conservation in the regulation of anther and pollen development (Wilson and Zhang, 2009). The *UNDEVELOPED TAPETUM1* gene *(UDT1)* (*Oryza sativa*) was reported by Jung *et al.*, (2005) as the *DYT1* ortholog in rice and has a similar function in maintaining tapetum development during early meiosis. Extension of this knowledge into other cereal crops such as barley is being facilitated by using rice genes as a starting point and the available sequence information from a range of species.

In this chapter, manipulation of fertility was investigated using the tapetum expressed DYT1 transcription factor. Controlling male fertility provides an opportunity as a fundamental tool for researchers and plant breeders in commercially important crops such as barley and wheat, so that increased crop yield and hybrid breeding can be achieved (Gomez and Wilson 2011). Previous research has shown that overexpression of a tapetum-expressed transcription factor (*MALE STERILI-*

TY1) can cause male sterility (Fernandez Gomez and Wilson, 2011). *HvDYT1* over-expression was therefore carried out, alongside analysis of the barley orthologs in Arabidopsis. It was expected that the *HvDYt1* over–expression could be used to recover the phenotype of the Arabidopsis *dyt1* mutant, HvDYT1 RNAi silencing had previously been investigated (Fernandez Gomez and Wilson, un-published), however this did not result in male sterility, but this is likely to be the result of reduced expression rather than complete silencing of the *DYT1* gene, as often seen using RNAi approaches.

4.2 MATERIALS AND METHODS.

4.2.1 Over-expression construct.

HvDYT1 Over-expression construct was provided by Dr. Jose Fernandez Gomez from the University of Nottingham. Construct pBract214: *HvDYT1* CDS over-expression, consists of the gene of interest, *HvDYT1*cds, controlled by the maize Ubi1 promoter, and hygromycin as selection marker controlled by the 35Spromoter (Appendix 1).

An Agrobacterium glycerol stock containing the *HvDYT1* over-expression vector (pBract214:*HvDYT1*cds) was used to inoculate immature barley embryos. Liquid culture was prepared from this glycerol stock and barley immature embryos transformation was conducted as specified in Section (2.11).

The pBract214 vector that uses the maize Ubi1 promoter has been use for cereal transformation particularly in barley (Harwood *et al.,* 2009).

4.2.2 Analysis of transformed plants.

Barley *HvDYT1* over-expression lines were generated as specified in Section 2.2.1. Plants were grown under controlled conditions, (16 hours photoperiod with 15/12°C day/night). Once plants had reached the elongation stage (leaf development nearly 2-3 unfold leaf), genomic DNA was isolated from young leaves as described in Section 2.3.1. Putative transgenic plants were confirmed by PCR analysis using the vector specific primer Ubi1Fw and the gene specific primer *HvDYT1*Rv (Appendix 2), producing a product 400 bp long. PCR was performed using Red Taq as described in Section 2.3.8.

In addition, RNA was extracted from flowers (Section 2.3.5) to analyse *HvDYT1* expression levels in the various transgenic lines at different stages of anther development and the native *HvDYT1* expression pattern in wt. Flower samples were collected at different anther stages from spikes 0.5cm to LFE3 stages (Table 4.1), according to the staging system of Fernandez Gomez and Wilson (2012). Primers *HvDYT1*qpcrFw and Rev (See Appendix 2) were used at a Tm of 59°C using 28 cycles. Control reactions that lacked reverse transcriptase were used to test for contaminated with gDNA; expression was normalised using *HvTubulin* (primers Fw and Rev) (Appendix 2) at a Tm of 61.5˚C with 28 cycles.

Table 4. 1: Barley anther and pollen development stages based on spike size.

Spike size range(cm)	Anther and pollen development stages
0-1	Primary sporogenous cells. Three cell layers surrounding the anther locule
1-2	Secondary sporogenous cells to pollen mother cells. Four layers surrounding the anther locule: epidermis, endothecium, middle layer and tapetum
2-3	Pollen mother cells undergo meiosis. Tapetum layer is prominent
3-4	Microspores released from the tetrad. Tapetum vacuolated
4-5	Free microspores. Middle layer undergoes crushing. The prominent tapetum layer is starting to degenerate.
5-6	Microspores become vacuolated. Tapetum degenerates.
LFE1	Mitosis I. Tapetum degeneration, but still present.
LFE2	
LFE3	Binuclear pollen. Mitosis II occurs
LFE4	Trinuclear pollen. Septum breakage.

4.2.3 Transgene copy number calculation.

gDNA was extracted and concentration equalised to the lowest sample concentration as described in Section 2.3.12. qRT-PCR was conducted using HygFw and Rev primers (Appendix II) at a Tm of 63°C and 50 cycles; controls samples provided by Dr Jose Fernandez contained one and two copies of the Hygromycin (Hyg) transgene that had been previously determined by Southern blot hybridisation (IDna Genetics Ltd., JIC Norwich). Copy number calculations were based on Shepherd *et al.,* (2009) real-time quantitative PCR was used to determine the transgene copy number, by comparing the relative transgenic amplification (copy number) with an endogenous control gene that has a known gene copy number as described in Section 2.3.12.

4.2.4 *HvDYT1* expression pattern in barley.

Flower samples were collected at different anther stages following Fernandez and Wilson (2012) staging system for barley. RNA was extracted and 1.5µg used to synthesise cDNA (Sections 2.3.5 and 2.3.6 respectively); qRT-PCR was performed by using *HvDYT1*qpcrFw and Rev (See Appendix II) as specified at Section 2.3.10.

4.2.5 Analysis of *HvDYT1* putative downstream targets.

To analyse *HvDYT1* downstream targets, qRT-PCR was performed using the Light Cycler 480 real time PCR system as described in Section 2.3.10. Two targets, *HvMYB35* and *HvAMS*, were selected as they have been reported as directly, or indirectly, regulated by DYT1 in other species such as Arabidopsis (Wilson *et al.*, 2001; Zhu *et al.*, 2011and Feng *et al.*, 2012) and Rice (Wilson and Zhang, 2009). Primers *HvMYB35qpcrF2* x *HvMYB35qpcrR2*, and *HvAMSF3* x *HvAMSR3* were used at a Tm of 62°C and 61°C, respectively using 50 cycles, qRT-PCR reaction was normalized by using HvTubulin (Section 2.3.10; see Appendix 2).

4.2.6 Microscopy Analysis.

Anthers were collected from flowers before anthesis from different lines based on Fernandez Gomez and Wilson (2012). Alexander staining was used to check pollen viability (Alexander, 1969), by light microscopy (Johnson-Brousseau and McCormick, 2004) (Section 2.17).

4.3 RESULTS.

4.3.1 BLAST analysis to identify putative ortholog of *Arabidopsis DYT1* gene in barley.

BLAST analysis using the *AtDYT1* CDS and the barley databases did not produce any results. A homologous sequence was identified using the rice *UDT1* (*AtDYT1* orthologue) against the *Brachypodium* and barley databases. These results agreed with the orthologue proposed in the Grammene database, Bradi1g25230. This sequence is 83% similar to the rice orthologue UTD1 and the MLOC_67972 (90% similar to the Brachypodium orthologue). The alignments between the paired sequences, a) Rice x Brachypodium, b) Brachypodium x Barley (a and b) and all three (c) Multiple alignment, are shown (See Appendix 1) (Dr Jose Fernandez Gomez, unpublished data).

4.3.2 *HvDYT1* expression pattern.

RT-PCR and qRT-PCR were performed on cDNA generated from staged bud samples using *HvDYT1* primers (*HvDYT1 qPCRF1* and *HvDYT1qPCRR4*), results showed that *HvDYT1* was expressed at stages B (0-1cm, primary sporogenous), D (2-3cm, PMC meiosis and Stage F (4-5cm, free microspore stage (Fig 4.3). In addition, signal was detected in LFE1 and 2 Samples (Mitosis I stage, tapetum degenerating, but still present) by qRT-PCR analysis, but not by RT-PCR, suggesting a very low level of expression in these stages (Fig 4.3). In addition, very low expression was seen in the vegetative stages that were analysed (coleoptile, leaf, and root) (Fig 4.3 and 4.4).

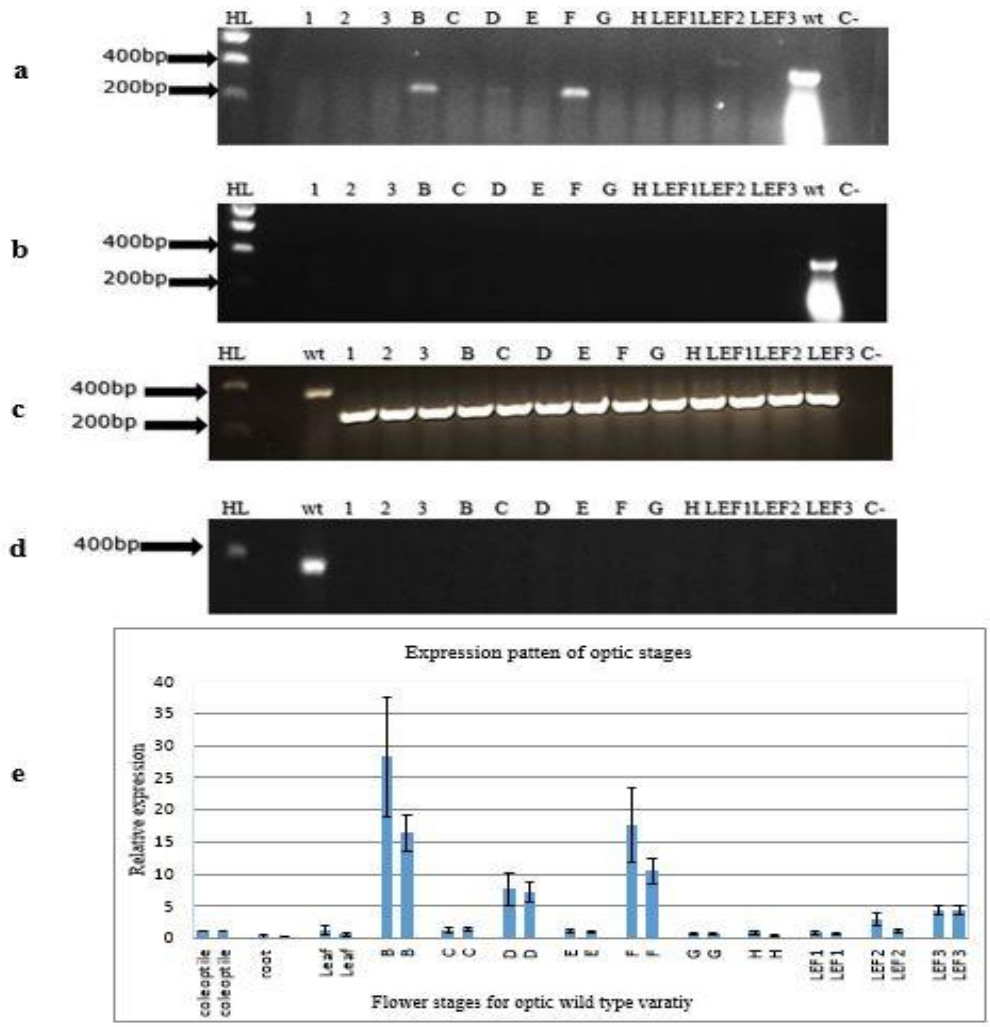


Figure 4. 3: RT and qRT-PCR HvDYT1 expression analysis in barley (var. Optic)

a - d: Reverse Transcriptase (RT)-PCR analysis in Lane (1) coleoptile; (2) root; (3) Leaf; Stage B (0-1cm) primary sporogenous stage, secondary sporogenous; Stage C (1-2-cm) secondary sporogenous stage; Stage D: (2-3cm) PMC meiosis; Stage E (3-4cm) late tetrad stage; Stage F (4-5cm) free microspore stage; Stage G (5-6cm) tapetal degenerate; Stage H (≥6cm) tapetal degenerate/microspore mitosis ; Stage LEF1 mitosis I; Stage LEF2 mitosis II; stage LEF3 mitosis II occurs/ Binuclear pollen; (wt.) wild type as barley genomic DNA; (c-) water negative control and (HL) Hyper Ladder1.

a: RT-PCR analysis of *HvDYT1* (using gene-specific primers qPCRF1 and HvDYT1qPCRR4; expected amplification product is 265bp).

b: Negative RT-PCR without reverse transcriptase for each cDNA template using gene-specific primers qPCRF1 and HvDYT1qPCRR4.

c: RT-PCR analysis of the normalisation control of the *HvTubulin* housekeeping gene for *HvDYT1* overexpression lines (T_1 and F_1), size product is expected 240bp.

d: Negative RT-PCR for the *HvTubulin* housekeeping gene.

e: qRT-PCR amplification (n=3 replicated) (error bars represent standard deviasion).

4.3.3 Analysis of barley *HvDYT1* over-expression lines.

4.3.3.1 Analysis of barley T_0 HvDYT1 over-expression lines.

4.3.3.1.1 Genotypic Analysis

Twenty-one independently transformed plants were regenerated from 100 inoculated immature embryos (Section 2.13; Fig 4.4 b) with 25 immature embryos as a control (Fig 4.4 a). The T_0 lines were selected on transition and regeneration media containing Hygromycin (Section 2.13) (Fig 4.4 c, e, g with d, f, h). The putative transgenic T_0 plantlets were transferred to bigger pots and placed in an environmental controlled room (Fig 4.4 m and n). A transformation efficiency of 21 % was obtained, that was calculated based on Harwood's protocol (Harwood *et al.,* 2009), defined as the number of independent transformed plants as percentage of the original number of immature embryos treated.

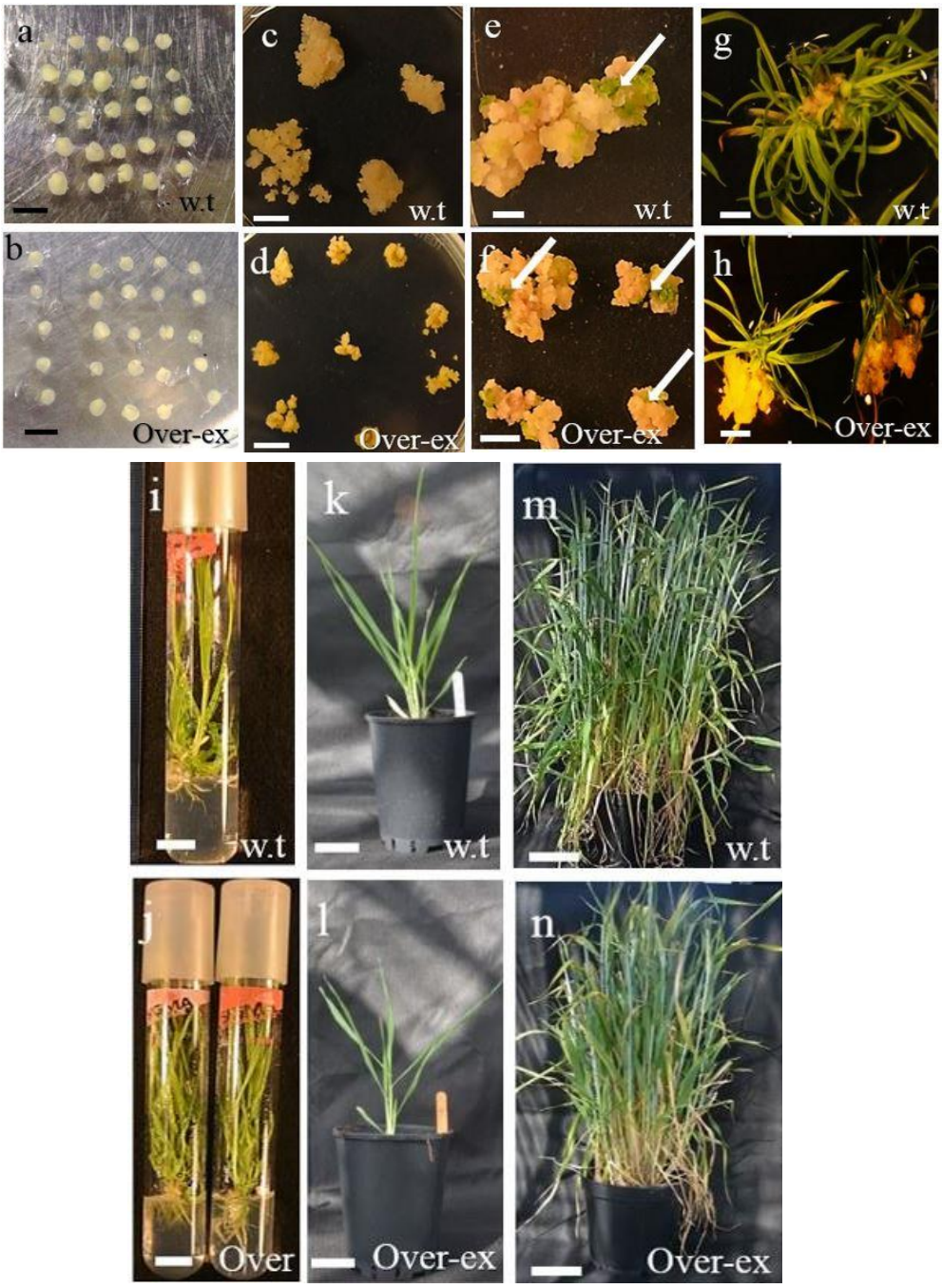


Figure 4. 4: Barley transformation showing the different regeneration stages (see appendix 3) for HvDYT1 Overexpression construct compared with un-inoculated controls from the immature embryo to mature plant stage.

w.t= wild type line that growing without hygromcin antibiotic. Over-ex = over-expression lines that have been grown with hygromcin antibiotic.

(a and b) Immature embryos; (c and d) Selection 2; 4 weeks after inoculation); (e and f) Transition 8 weeks after inoculation; (g and h) Regeneration 10 weeks after inoculation; (i and j) Roots and r 12 weeks after inoculation; (k and l); plantlets are growing in soil 14 weeks; (m and n) mature plant 22-26 weeks; Bar: a, b, c, d, f, I= and j 8mm; e, g and h= 5mm; k, l, m and n =10 mm.

PCR analysis using Ubi1 promoter Fw (vector specific) and the target gene primer *HvDYT1*Rv showed that all regenerated plants were positive for the over-expression transgene except for sample 30 (Fig 4.5) and gave an expected band of 400 bp.

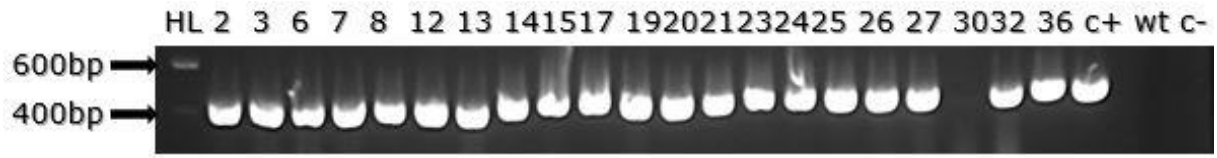


Figure 4. 5: Genotyping of T_0 Transformed plants carrying the HvDYT1 over-expression construct using PCR.

The vector specific primer Ubi1Fw and the gene specific primer *HvDYT1*Rv was used, the expected size product was 400bp. Lanes 2-36 transformed plants; all lines showed positive results except sample 30 which was negative for the transgene. (C+) *HvDYT1* over-expression construct plasmid DNA as positive control; (wt.) wild type as negative control; (c-) water negative control; (HL) Hyper Ladder 1.

Copy number analysis of the hygromycin resistance gene, which is associated with the target gene of interest in the transformation construct, revealed that the number of copies in the transgenic lines varied from 0 to 4. Line 30 was transformed and the *Hyg* gene was detected but the *HvDYT1* target gene was not present, lines 8, 15, 17; 21-25 and 30 had a single copy, whilst line 7 had four copies number; the remaining lines had 2 copies (Fig 4.6).

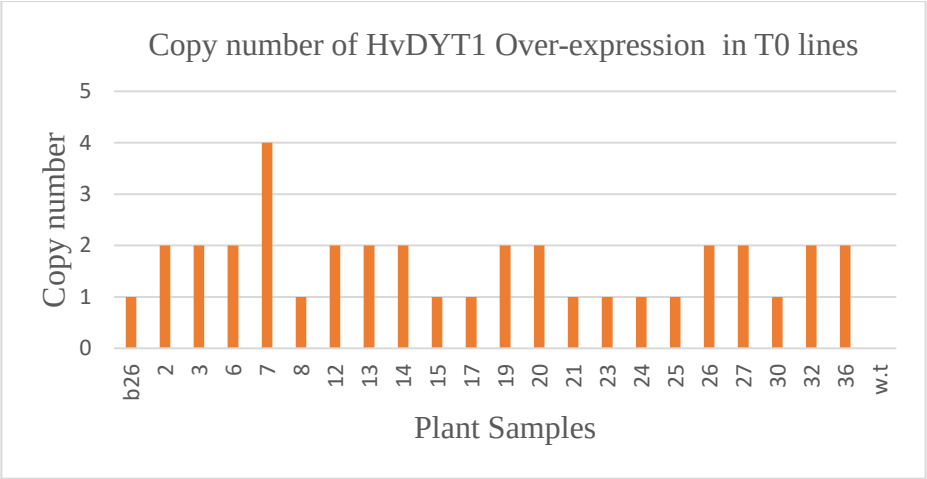


Figure 4. 6: Hygromycin selectable marker copy number in T_0 lines carrying the Ubi:HvDYT1 construct.

B26 is a control standard containing 1 copy number, (2-36) are transformed plant samples, and w.t is wild type control.

4.3.3.1.2 Phenotypic Analysis of barley T_0 lines carrying the *Ubi-HvDYT1* construct.

Phenotypic analysis was conducted after the putative transgenic lines were confirmed by PCR as carrying the transgene (Fig 4.5). Vegetative development showed no difference between w.t and transgenic lines, and spikes and florets developed normally (Fig 4.4 and 4.7), however the anthers were frequently small and undeveloped (Fig 4.7g). Alexander staining revealed an absence of viable pollen in many lines (Fig 4.7h), which were completely male sterile. In addition, some other lines showed partial sterility (Fig 4.7k). Fertility for each spike was recorded as the percentage of seed set divided by floret number (See table 4.2)

Table 4. 2: an example to calculate sterility and fertility of samples.

Sample 21 has 8 tillers	**Number of florets**	**Number of seeds**	**%fertility**	**%sterility**	**average fertility**	**average sterility**
Spike 1	29	12	41.37	58.63		
2	28	9	32.14	67.86		
3	26	8	30.76	69.24		
4	26	11	42.30	57.70		
5	26	11	42.30	57.70		
6	24	1	4.16	95.84		
7	22	7	31.81	68.19		
8	**28**	**14**	**50**	**50**	**34.36**	**65.64**

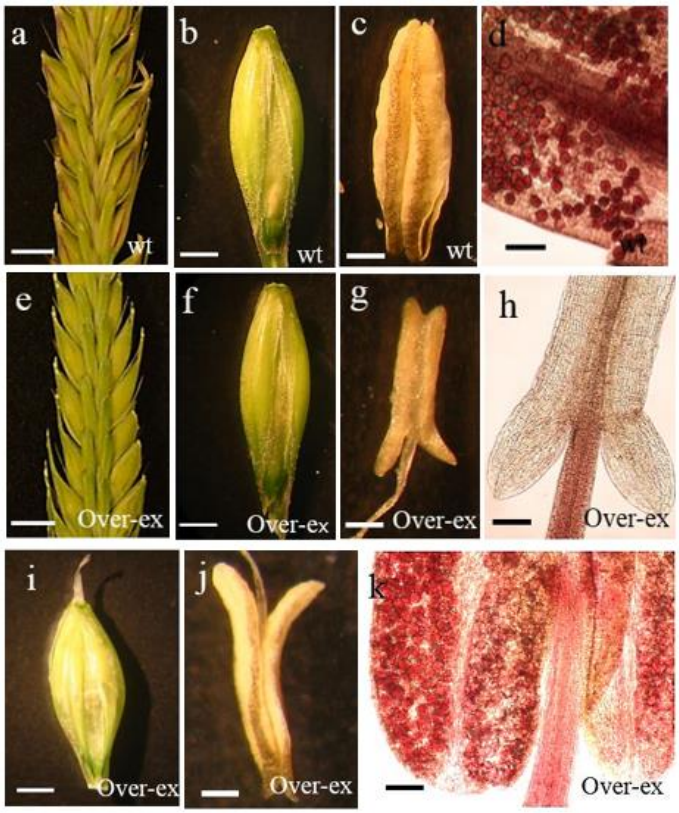


Figure 4. 7: Phenotype analysis of barley carrying the Hv DYT1 over expression construct containing the gene of interest, HvDYT1cds, controlled by the maize Ubi1 promoter compared to wild type.

(a-d) Wild type (e-k overexpression transgenic lines). (a) Wild type spike during grain fill phase; (b) Wild type floret; (c) Dehiscence of wild type anther with abundant visible pollen; (d) Alexander staining of wild type pollen, which is fully viable as indicated by red staining; (e) Spike of transgenic line at grain fill phase; (f and i) Floret of Over-expression line; (g and j) Anthers from the over-expression lines tended to be small and (h) lacked viable pollen, as indicated by Alexander, staining, however some anthers from overexpression lines (k) contained some viable pollen and were partial sterility. Bar; b, f, d, h and k =1mm; c, g, i and j =0.5mm; a and e= 5mm

Comparison between the hygromycin (Hyg) copy number and the male sterility phenotype revealed that plants with two or more copies of the Hyg transgene were completely sterile, whilst those lines with one copy were partially sterile (Fig 4.8).

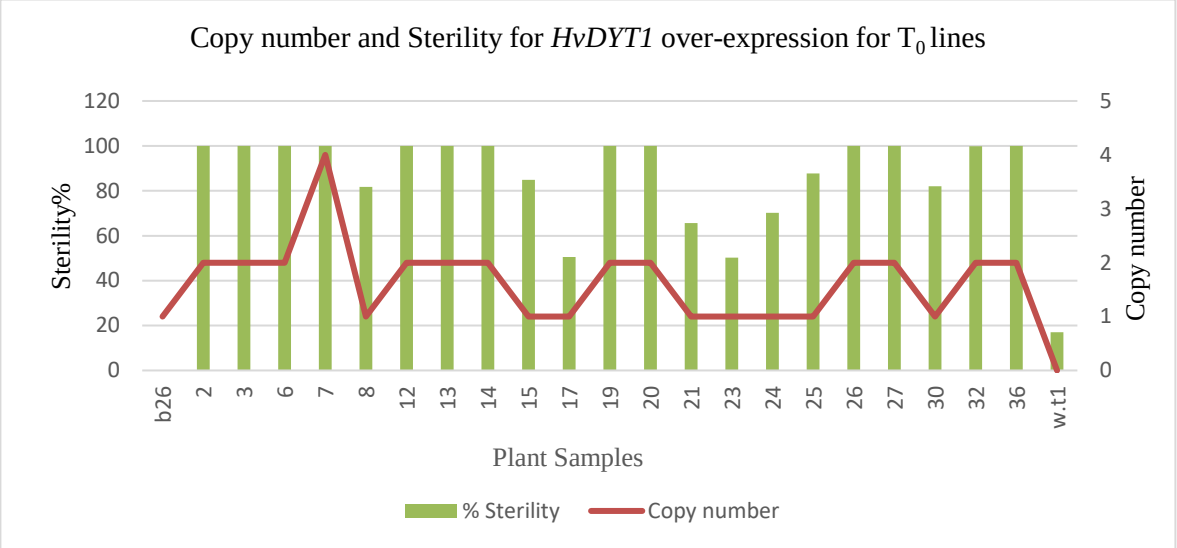


Figure 4. 8: Relationship between the hygromycin selectable marker copy number and sterility in T_0 lines carrying the Ubi:HvDYT1 construct.

B26 is a control standard with 1 copy number, (2-36) are plant samples, and w.t is wild type control.

4.3.3.2 Analysis of T_1 and F_1 HvDYT1 over-expression lines.

F1 lines were generated by crossing T_0 lines 19 and 32 with wt, because they were totally sterile and they need to be maintained; seeds were generated since female fertility was normal.

The result F_1 seeds and those T_1 obtained from partially sterile lines (23 and 24) as well as the negative and non-transgenic control line 30 were selected and grown as described in Section 2.1.1. Sample 30 was selected as a transformation

control, because it was not transformed with the HvDYT1 construct, as confirmed in Fig 4.5, but contained the Hyg resistance gene (Fig 4.5), this could therefore be used to show the phenotype was an effect of the DYT1 over-expression and not because of the transformation process.

4.3.3.2.1 Genotypic Analysis.

The segregating population was analysed for the presence of the over-expression transgene. From the F_1 population, only one plant from Line 19 was negative for the transgene (Fig 4.9), whereas for the other F_1, Line 32, 6 out of 9 plants were positive for the transgene. For the T_1 lines, all plants from Line 24 were positive for the transgene and for Line 23, 5 out of 6 lines were positive; all plants from Line 30 were negative for the transgene (Fig 4.9).

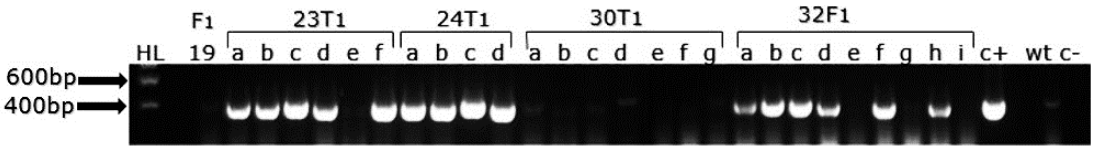


Figure 4. 9: Genotyping of T_1 and F_1 Transformed plants carrying HvDYT1 over-expression construct using PCR.

The vector specific primer Ubi1Fw and the gene specific primer *HvDYT1*Rv was used and the expected product size was 400bp. Lines 19-32i represent transformed plants; all lines showed positive results except sample 19, 32e, 32g and 32i which were not transgenic from the F_1 population and for the T_1 lines sample 23e with all samples in line 30 which were not transgenic. (C+) *HvDYT1* over-expression construct plasmid DNA as positive control; (wt.) wild type as negative control; (c-) water negative control and (HL) represent Hyper Ladder 1.

4.3.3.2.2 Phenotypic analysis of the T_1 and F_1 segregation populations.

Similar sterility phenotype was observed in T_1 and F_1 lines as observed in T_0, however, the relationship between copy number and sterility was different. In T_1 and F_1, one copy of the over-expression transgene was sufficient to produce complete sterility (Fig 4.10). Transgenic lines positive for the *HvDYT1* over-expression transgene (Fig 4.10) such as 23a, 23c, all the line 24 plants and 32a, b, c, d and f were completely sterile with only a copy of the transgene. The T_1 plants from Line 23 b, d, and f, with two copies, were also completely sterile. Only one sample (line 32h) with one copy showed partial sterility (Fig 4.10). However, copy number determined by using the Hyg gene (Section 3.2.3) was misleading as observed in sample 30, since this line is positive for the Hyg selectable marker but is negative for the *HvDYT1* over-expression transgene and thus has normal fertility in the T_1.

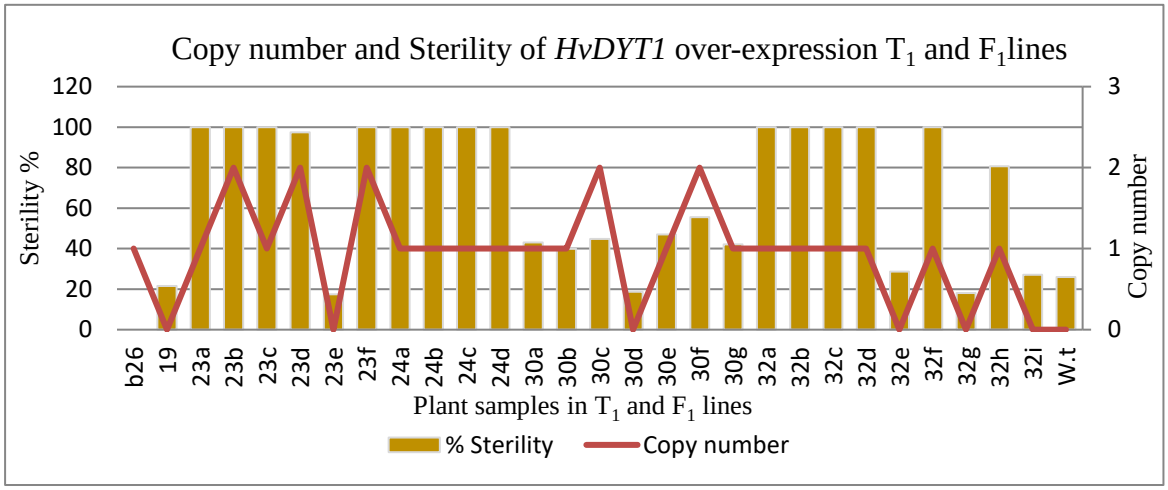


Figure 4. 10: Figure 4.10 Relationship between Hygromycin copy number and sterility in T_1 and F_1 lines carrying the *Ubi:HvDYT1* construct. Line b26 is a control standard with 1 copy of the hygromycin resistance gene, Line 19 and (32a -32i) are F_1 population and the rest are T_1 plants, and (w.t) is wild type control.

To establish the relation between the *HvDYT1* over-expression and the sterility observed in the T_1 and F_1 populations, qRT-PCR was performed using cDNA synthesised from RNA extracted from flowers as described in Section 2.3.5. Spike sizes between 0 and 1cm (*HvDYT1* native expression at the highest) were collected from the different T_1 and F_1 segregation lines. Lines 32f and 23f showed the highest level of *HvDYT1* expression, which was 35 fold and 30 fold higher than the wild type lines respectively (Fig 4.11), whereas lines 23 a-c, 24 a, b, d, and 32 a-d showed between 10 and 15 fold thee expression in w.t (Fig 4.11 C) which is also accompanied by a complete sterile phenotype. Line 32h, showed slightly lower over-expression than line 32h, and this does not appear to be at a sufficient level for the plant to be fully sterile since it is able to produce some seeds, although with a very high sterility. Samples that did not show over-expression, with similar level of *HvDYT1* expression to w.t showed normal fertilityy (Lines 23e, 30b-g and 32e; Fig 4.11).

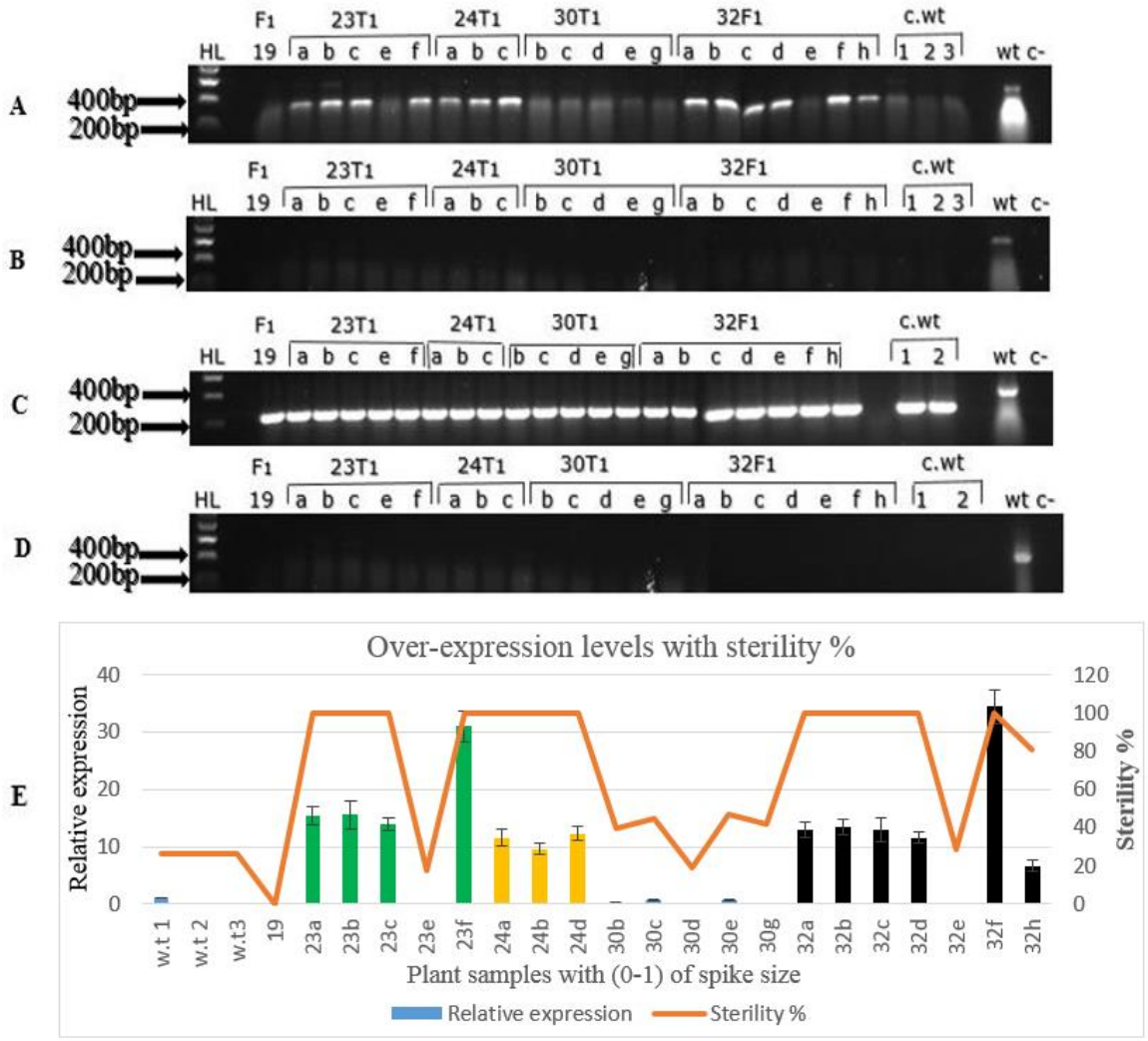


Figure 4. 11: Hv DYT1 Expression analysis in barley T_1 and F_1 segregating lines.

A -D: Reverse Transcriptase (RT)-PCR analysis in w.t., T1 and F1 transgenic lines. Spike size for all lines is between (0-1cm); Line 19a: (0.5 cm); 23a, b and c (0.5cm); 23e: (0.8cm); 23f: (0.6cm); 24a: (0.8cm); 24 b, d: (0.9cm); 30c: (1cm); 30d: (0.9cm); 30e: (0.8cm); 30g: (0.6cm); 32a, c and d: (0.8cm); 32b and h:(0.9cm); 32e: (0.6cm); 32f: (0.7cm); W.t cDNA1 (0.5cm); W.t cDNA 2 (0.8cm); W.t cDNA 3 (1cm); (wt.) wild type as barley genomic DNA; (c-) water negative control and (HL) Hyper Ladder1.

A: RT-PCR analysis of *HvDYT1* expression using gene-specific primers (HvDYT1 qPCRF1 and HvDYT1qPCRR4), size product is expected to be 265bp.

B: Negative RT-PCR for gene-specific primers (HvDYT1 qPCRF1 and HvDYT1qPCRR4).

C: RT-PCR analysis of the normalisation control of the *HvTubulin* housekeeping gene for *HvDYT1* the size product is expected to be 240bp.

D: Negative RT-PCR for the *HvTubulin* housekeeping gene.

E: qRT-PCR amplification with associated levels of sterility in $T_1$and F_1 lines.

4.3.4 Analysis of *HvDYT1* over-expression levels at different stages of anther development.

qRT-PCR using primers *HvDYT1qPCRF1* and *HvDYT1qPCRR4* showed small differences in the level of transgene over-expression at different anther stages (Fig 4.12). Compared with w.t, where higher *HvDYT1* expression was observed in spike size 1.5 cm, which corresponds to secondary sporogenous developmental stage, sample 23f was the highest expressed. *HvDYT1* over-expression in Line 23f remained high between 120-180 fold that of wild type levels throughout the whole of anther development. A high level of expression was also maintained throughout all anther developmental stages for lines 24a and 32h, which showed over-expression levels of between 40-80 and 40-60 fold of wild type levels, respectively. Samples 30d and 32e were negative for the transgene, but had been through the regeneration process and did not show any increase of expression compared to w.t. (Fig 4.12).

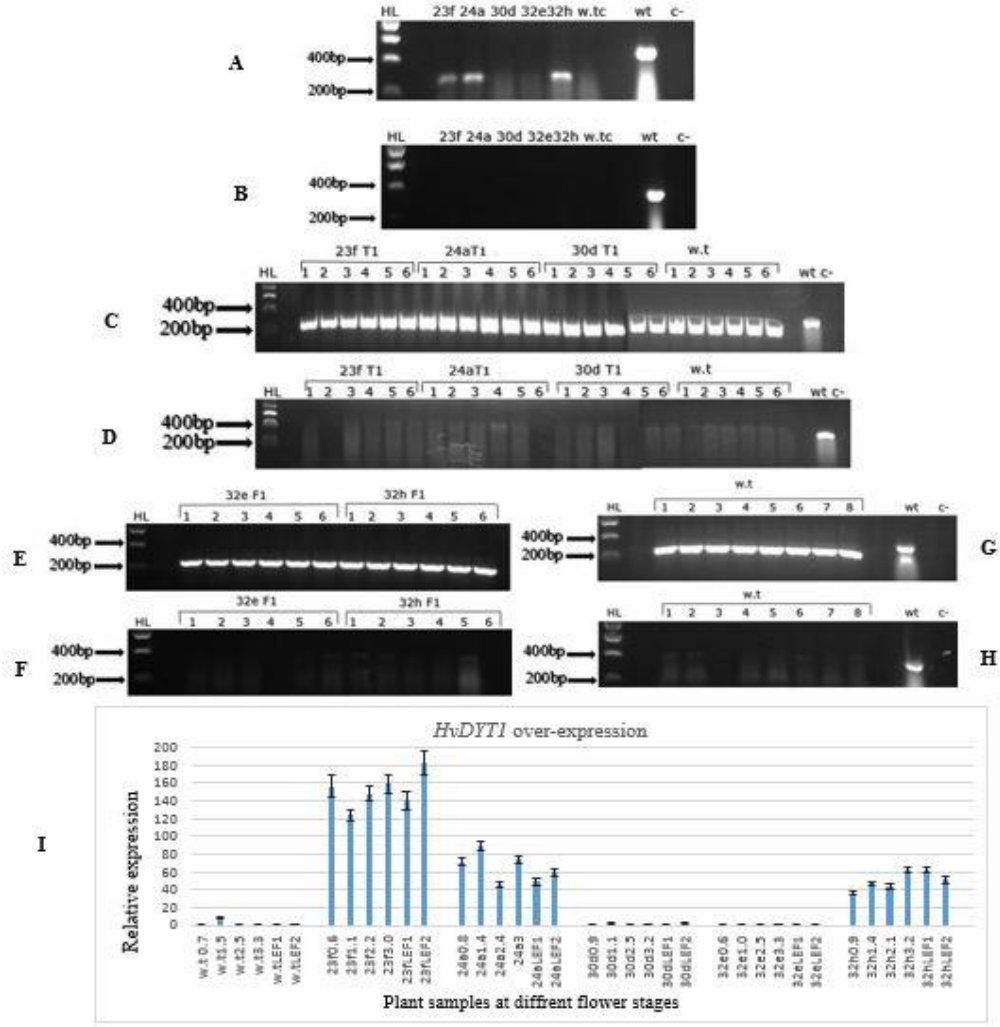


Figure 4. 12: HvDYT1 Over-expression analysis in 5 independent biological replicates of transgenic plants at different stages of anther development.

A -H: Reverse Transcriptase (RT)-PCR analysis in w.t., T1 and F1 transgenic lines. Different spike size of (T_1) Lines: 23F (1-6cm) (0.6cm, 1.1cm, 2.2cm, 3cm, LEF1, and LEF2); 24A (1-6cm) (0.8cm, 1.4cm, 2.4cm, 3cm, LEF1 and LEF2); 30D (1-6) (0.9cm, 1.1cm, 2.5cm, 3.2cm, LEF1, and LEF2); w.t plant (1-6cm) (0.7cm, 1.5cm, 2.5cm, 3.3cm, LEF1and LEF2) and (F_1) lines: Lanes (1-6cm) 32E (0.6cm, 1cm, 2.5cm, 3.3cm, LEF1 and LEF2), lanes (1-6cm) 32H (0.9cm, 1.4cm, 2.1cm, 3.2cm, LEF1, and LEF2), lanes (1-8cm) w.t plant (0.7cm, 0.7cm, 1.5cm, 1.5cm, 2.5cm, 3.3cm, LEF1, and LEF2). (w.tc) wild type as barley cDNA; (wt.) wild type as barley genomic DNA; (c-) water negative control and (HL) represent Hyper Ladder1.

A: RT-PCR analysis of HvDYT1 expression using gene-specific primers (HvDYT1 qPCRF1 and HvDYT1qPCRR4), T_1 lines23f: (0.6cm); 24a (0.8cm); 30d: (0.9cm); F_1 line 32e (0.6cm) and 32h (0.9cm), size product expected to be 265bp.

B: Negative RT-PCR for gene-specific primers (HvDYT1 qPCRF1 and HvDYT1qPCRR4).

C, E and G: RT-PCR analysis of the normalisation control of the *HvTubulin* housekeeping gene for *HvDYT1* overexpression lines (T_1 and F_1), size product expected to be 240bp.

D, F and H Negative RT-PCR for of the *HvTubulin* housekeeping gene.

I: qRT-PCR at different stage of anther development in 5 independent biological replicates of the transgenic lines.

4.3.5 Analysis of *HvDYT1* putative downstream targets.

To analysis *HvDYT1* downstream targets, qRT-PCR was performed as described in Section 2.3.8. Putative *HvMYB35* and *HvAMS* genes were identified by Dr Jose Fernandez Gomez (Unpublished data) and used to design gene specific primers (*HvMYB35qpcrF2* and *HvMYB35qpcrR2*) (*HvAMSF3* and *HvAMSR3*) (see Appendix 2)

The genetic pathway of transcription factors for tapetum development and function including *DYT1, TDF1, AMA, MS188* and *MS1* (see Fig 4.1) was used to help facilitate understanding of the regulation of *DYT1*. *DYT1* has been shown to regulate at least 22 genes encoding transcriptional factors, among them *MYB35 (TDF1)* and *AMS* which are essential for pollen formation and development (Zhu *et al.*, 2008; Zhu *et al.*, 2011)

Expression analysis was conducted for *HvMYB35,* a putative direct target gene of *DYT1*, in 5 independent biological replicate transgenic plants at different stages of anther development (Fig 4.13). An increased level of *HvMYB35* expression was observed for the positive transgenic Lines 23f, 24a and 32h, this higher level of expression started in the early pollen development stages between Secondary sporogenous cells to pollen mother cells stage (1-2cm), expression then started to decrease, but was prolonged compared to wild type, with expression maintained throughout all anther developmental stages. On the other hand, in wild type in Lines 30 and 32e which are negative transgene lines, *MYB35* expression was observed in the equivalent wild type stages (Fig 4.13) with expression seen from the secondary sporogenous cells to pollen mother cells and then decreasing through anther developmental stages.

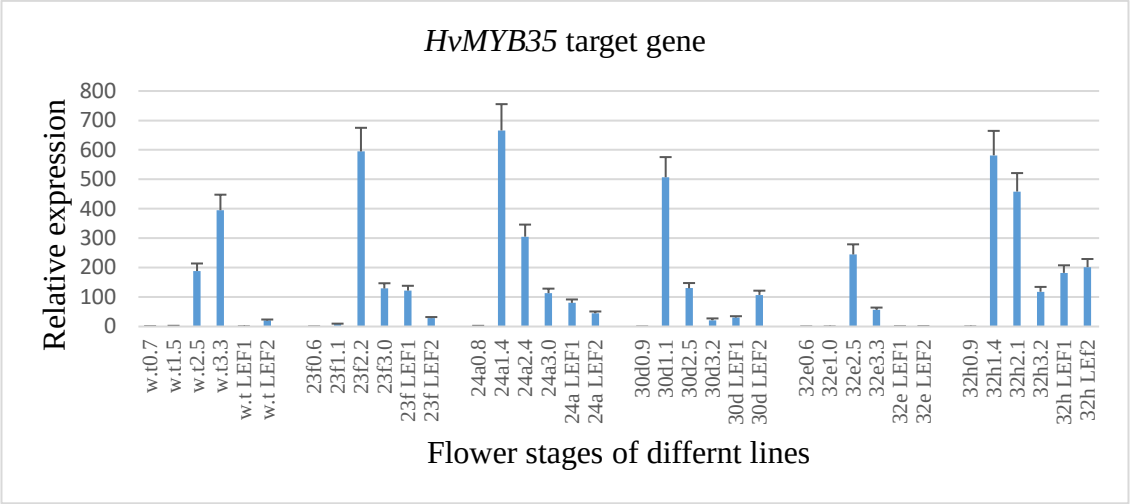


Figure 4. 13: qRT-PCR expression analysis of *HvMYB35* a putative direct target gene of DYT1, based upon the Arabidopsis regulatory network for tapetum development.

5 independent biological replicates of T1 and F1 transgenic plants, at different stages of anther development was used. Different spike size of (T_1) Lines: 23F (1-6cm) (0.6cm, 1.1cm, 2.2cm, 3cm, LEF1, and LEF2); 24A (1-6cm) (0.8cm, 1.4cm, 2.4cm, 3cm, LEF1 and LEF2); 30D (1-6cm) (0.9cm, 1.1cm, 2.5cm, 3.2cm, LEF1, and LEF2); (F_1) lines: Lanes (1-6cm) 32E (0.6cm, 1cm, 2.5cm, 3.3cm, LEF1 and LEF2), lanes (1-6cm) 32H (0.9cm, 1.4cm, 2.1cm, 3.2cm, LEF1, and LEF2), and lanes (1-6cm) w.t plant (0.7cm, 1.5cm ,2.5cm, 3.3cm, LEF1, and LEF2).

Expression analysis was also conducted for *HvAMS,* another putative direct target gene of DYT1, using the same samples as analysed for *HvMYB35* gene expression (Fig 4.14), Levels of expression of *HvAMS* observed for positive transgene lines (23f, 24a and 32h) were similar to that of wild type and the negative transgnic controls (Lines 30 and 32e), however a slightly higher level of expression was seen in Line 32h throughout all staged after the secondary sporogenous cells to pollen mother cell stage

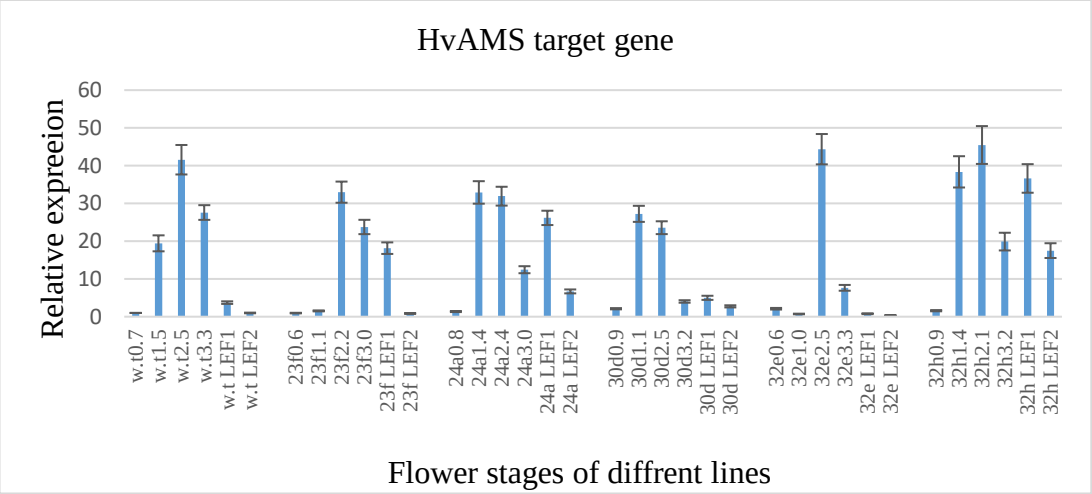


Figure 4. 14: qRT-PCR expression analysis of Hv AMS a putative direct target gene of DYT1, based upon the Arabidopsis regulatory network for tapetum development.

5 independent biological replicates of T1 and F1 transgenic plants, at different stages of anther development was used. Different spike size of (T_1) Lines: 23F (1-6cm) (0.6cm, 1.1cm, 2.2cm, 3cm, LEF1, and LEF2); 24A (1-6cm) (0.8cm, 1.4cm, 2.4cm, 3cm, LEF1 and LEF2); 30D (1-6cm) (0.9cm, 1.1cm, 2.5cm, 3.2cm, LEF1, and LEF2); (F_1) lines: Lanes (1-6cm) 32E (0.6cm, 1cm, 2.5cm, 3.3cm, LEF1 and LEF2), lanes (1-6cm) 32H (0.9cm, 1.4cm, 2.1cm, 3.2cm, LEF1, and LEF2), and lanes (1-6cm) w.t plant (0.7cm, 1.5cm ,2.5cm, 3.3cm, LEF1, and LEF2).

Table 4. 3: Summary of the results of HvDYT1 gene expression, copy number and sterility for the HvDYT1-OEX transgenic lines.

Plant Lines and spike size (0-1 cm)	Copy number of Hyg. gene	Expression of *HvDYT1* gene	% sterility	%fertility
w.t0.7	0	x	26.06	73.94
19a 0.5	0	x	21.60	78.40
23a0.5	1	15 fold	100	0
23b0.5	2	15 fold	100	0
23c0.5	1	12 fold	100	0
23e0.8	0	x	17.46	82.54
23f0.6	2	30 fold	100	0
24a0.8	1	11 fold	100	0
24b0.9	1	10 fold	100	0
24d0.9	1	11 fold	100	0
30b0.9	1	x	39.88	60.12
30c1	2	x	44.75	55.25
30d0.9	0	x	18.68	81.32
30e0.8	1	x	46.94	53.06
30g0.6	1	x	42.15	57.85
32a0.8	1	11 fold	100	0
32b0.9	1	11 fold	100	0
32c0.8	1	11 fold	100	0
32d0.8	1	10fold	100	0
32e0.6	0	x	28.74	71.26
32f0.7	1	35 fold	100	0
32h0.9	1	8 fold	80.60	19.40

The transgene analysis data is summarised in Table 4.2, just only lines 32h that had expression for *HvDYT1* but represented partial sterility.

4.4 Discussion

A putative ortholog of the Arabidopsis *DYT1* gene was identified in barley (Dr Jose Fernandez Gomez, unpublished data); *DYT1* shows a specific expression pattern during anther development in Arabidopsis and is crucial for tapetum development and pollen formation (Zhang *et al.,* 2006; Feng *et al.,* 2012). The possible role of the *HvDYT1* gene was analysed by phenotypic analysis of development by constitutive *HvDYT1* overexpression in transgenic lines under the control of the ubiquitin1 promoter.

ATDYT1 encodes a putative bHLH transcription factor which is highly expressed in the tapetum during stage 5 to 6, and, although at lower level, in the meiocytes (Zhang *et al.,* 2006). The DYT1 homolog in rice, UNDEVELOPED TAPETUM1 (*OsUDT1*) has been identified and shown to be a key regulator of early tapetum development with similar functions in maintaining tapetum development during early meiosis (Jung *et al.,* 2005). This gene is expressed preferentially in anther and starting at the early meiosis stage and then expression level lower at late stages (Jung *et al.,* 2005). The rice Tapetum Degeneration Retardation gene (TDR) is also essential for the molecular network regulating rice tapetum development and degeneration (Li *et al.,* 2006)

In addition, several genes essential for anther cell differentiation and development of tapetal and function have been identified in Arabidopsis (Wilson *et al.,* 2011, Zhang *et al.,* 2006). These include TAPETUM DETERMINANT1 (TPD1) which is essential for specialization of tapetal cells that cause the precursors of tapetal cells to differentiate and develop into microsporocytes instead of tapetum cells (Yang et al., 2003). Regulation of *EMS1/EXS*, also are associated to differentia-

tion of the microsporocytes and tapetal cells (Colcombet et al., 2005; Zhao et al., 2002); thus, TPD1 and EMS1/EXS are involved in same developmental pathway in the Arabidopsis anther (Yang et al., 2003).

Furthermore, Overexpression of TAPETUM DETERMINANT1 alters the cell fates in the Arabidopsis carpel and tapetum via genetic interaction with excess microsporocytes1/extra sporogenous cells *EXS* (Yang et al., 2005). *SERK1* and *SERK2* are required for the formation of tapetum as well that double mutants of both genes are completely male sterile due to a failure in tapetum specification (Albrecht *et al.,* 2005). Millar and Gubler, (2005) reported that double mutant of MYB33 and MYB65 genes are caused of complete male sterility.

Over-expression of *HvDYT1* was used to test for changes in pollen formation and viability and confirm the potential function of this putative orthologue. Overexpression of the barley *MS1* orthologue has been previously shown to cause male sterility (Gomez and Wilson 2011). *The HvDYT1* gene may therefore also provide a tool to analyse the molecular processes of anther and pollen development in agronomic important plants and can be facilitated to the future control of crop fertility, for instance for F1 hybrid lines that is also really important for reduce of genetically modified pollen and an important goal for plant reproductive.

HvDYT1 expression pattern in barley optic variety was identified by analysis in different flower stages (B - LEF3) based upon Gomez and Wilson (2012). Expression is seen at stages: B (0-1cm) primary sporogenous; D: (2-3cm) PMC meiosis and Stage F (4-5cm) free microspore stage (Fig 4.3). *HvDYT1* expression pattern in barley was therefore different from the Arabidopsis *ATDYT1* and *OsUDT1* pat-

tern); *AtDYT1* is highly expressed in the tapetum during stage 5 to 6, and also, although at lower level, in the meiocytes (Zhang *et al.,* 2006). High levels of *UNDEVELOPED TAPETUM1* (*OsUDT1*) expression were preferentially observed in anthers starting at the early meiosis stage and then a lower expression level was seen at the later stages (Jung *et al.,* 2005). Therefore, *OsUDT1* expression pattern different from the HvDYT*1* expression pattern in barley.

Our results demonstrate that *HvDYT1* is preferentially expressed at primary sporogonial and lower at PMC meiosis, and also again higher expressed level in free microspore stage, after that it dramatically dropped throughout the stages. In addition, very low expression was seen in the vegetative stages that were analysed (coleoptile, leaf, and root) (Fig 4.3).

In this study, phenotypic analysis showed that very high levels of male sterility were seen in T_0 lines carrying the Ubi:*HvDYT1* construct in comparison with wild type or negative transgenic lines. Similar male sterility phenotypes were observed in subsequent T_1 and F_1 lines derived from these T_0 lines, however the relationship between hygromycin selectable marker copy number and sterility were different. Copy number calculation is based on Shepherd *et al.,* (2009) that is defined as present in the genome of the transformation, using relative transgenic amplification (copy number) with an endogenous control gene that has a known gene copy number. Copy number knowledge can be very useful for determination what future experiments and the optimal transgenic lines for further analysis (Shepherd *et al.,* 2009). Nevertheless, copy number reflects the selectable marker number rather than the number of transgene targets and also does not take into account insertion position and the impact of flanking sequences on expression levels.

These results confirmed the relationships between failure of pollen development and male sterility with transgenic copy number and *HvDYT1* over expression, however they confirmed that copy number could not be used as a direct indicator of expression level or potential impact, particularly in subsequent generations. Results from T_1 and F_1 positive sterility lines demonstrated that changes in pollen development and thus male fertility directly related to levels of *HvDYT1* over-expression rather than the transgenic copy number (Fig 4.9 and 4.10).

This may be a consequence of changes in the expression level between the generations, or may be a reflection that copy number analysis was performed by amplifying the *Hyg* gene in the genome of transformed plant rather than the *DYT1* transgene.

Analysis of two putative downstream targets, *HvMYB35* and *HvAMS*, were conducted as they have been reported as directly, or indirectly, regulated by DYT1 in other species such as *Arabidopsis* (Wilson *et al.,* 2001; Zhu *et al.,* 2011; Feng *et al.*, 2012) and Rice (Wilson and Zhang, 2009).

From these results, we can conclude that overexpression of the putative *HvDYT1* gene has a significant impact on pollen and anther development, resulting in a completely male sterile phenotype. The vegetative growth and development for both wild type and overexpression lines, did not show any significant changes compared to the wild type or transgenic negative control plants. Analysis of the expression pattern was done in optic variety, because we were using the staging system of Gomez and Wilson (2012) that enables stages of barley optic reproductive developmental based upon external morphology.

In addition, the results showed similarities with other reports for overexpression of transcription factors involved in tapetum development, for example, male sterility was reported by Gomez and Wilson (2011) by overexpression of *HvMS1* gene. A similar result was obtained in *Arabidopsis* by over-expression of *Arabidopsis MS1* gene (Yang et al 2007). In summary, male sterile lines have been created by over-expression of *HvDYT1*without affected the development of the whole plant. Controlling fertility by using a gene involved in anther and pollen development *HvDYT1*, is important for future to control a crop fertility, production hybrid seed and to produce low environmental impact genetically engineered plant

CHAPTER 5: OVER-EXPRESSION OF *ATIPT*4 GENE IN BARLEY FOR SENESCENCE MODIFICATION.

5.1 INTRODUCTION

Senescence is a highly controlled process in plants, which is an important target for increased productivity of crops in the agnomic sector. Regulation of senescence mechanisms and manipulation of this process is a key target for agricultural improvement, particularly for delaying leaf and flower senescence (Zhou and Gan, 2010). Extending the period of photosynthetic productivity is important to improved plant growth, carbohydrate and material deposition in cereal grain yield. Thus, delaying the onset of plant senescence can be advantageous for increasing productivity of cereal crops and increased plant biomass (Verma *et al.,* 2004).

Cytokinins play an important role in development and physiological aspects in plants, particularly to delay the onset of leaf senescence (Clark *et al.,* 2004). Genetic manipulation of the genes involved in plant cytokinin mechanisms can be utilised for the development and improvement of horticultural and agriculturally important crops. The *Isopentenyl transferase* (*IPT*) gene is the key cytokinin biosynthetic enzyme in higher plants (Kieber and Schaller, 2013) (see Section 1.4.2.1), altered expression of *IPT* has therefore been used in a number of species to try to modify the progression and timing of senescence. Previous research reported that the *IPT* gene isolated from *Agrobacterium*, when fused under the control of different promoters and introduced into plants, was able to elevate the levels of endogenous cytokinins (Gan and Amasino, 1996). Initially the senescence-specific promoter from the *Senescence-Associated Gene12* (*SAG12*) was used by Gan and Amasino (1995) fused to the *IPT* gene to observe the role of cytokinin in

leaf senescence; using this construct they reported higher biomass and yield in transgenic tobacco plants. The *SAG12* promoter was then utilized in a wide range of crops to determine the role of cytokinin in prolonging leaf and flower survival (Sykorova et al., 2008; McCabe et al., 2001; Chang et al., 2003; Huynh *et al*., 2005 and Liu *et al.*, 2011).

Regulation of expression of the IPT gene is crucial for achieving increased cytokinin levels and for improvement of agronomic crops. Increasing endogenous cytokinin levels have been significantly affect quality and yield. Enhancing IPT expression and activation depends on the specificity of the promoter, developmental stage and environmental condition (Song *et al.,* 2012). Increased yield/biomass has been demonstrated in some important agronomic crops by transgenic modification of IPT expression including, rice (Gan and Guo, 2014: Hildebrand *et al.*,1998; Rubia *et al.*, 1999; Cao, 2001; Lin *et al.*, 2002; Xu *et al.*, 2010*a*), wheat (Xi *et al.*, 2004; Xia *et al.*, 2006; Bu *et al.*, 2007) maize (Young *et al.*, 2004) and petunia (Jandrew and Clark, 2001; Chang *et al.*, 2003; Clark *et al.*, 2004).

Molecular studies of the genes involved in cytokinin metabolism and signalling, particularly in *Arabidopsis* model plant, have also played a crucial role in identifying and illuminating the role of cytokinins in plant development. In *Arabidopsis*, nine IPT gene were identified and shown to be involved in cytokinin biosynthesis (Lim *et al.,* 2003; Lime *et al.,* 2007; Kieber and Schaller, 2013; Section 1.4.2.1). Among them *ATIPT4* and *ATIPT8* were selected. Therefore, in an attempt to modify the senescence progression, these genes were used under the control of the Ubi promoter to alter cytokinin levels and determine the impact on senescence in barley.

5.2 MATERIALS AND METHODS.

5.2.1 Over-expression construct.

IPT4 and *IPT8* over-expression constructs (pBract214::*ATIPT4*-CDS; pBract214::*ATIPT8*-CDS were prepared as described (chapter 3 Section 3.2.4.2). pBract214 is a destination vector containing the maize Ubi1 promoter that controls the over-expression cassette and hygromycin as the selectable marker controlled by the 35Spromoter.

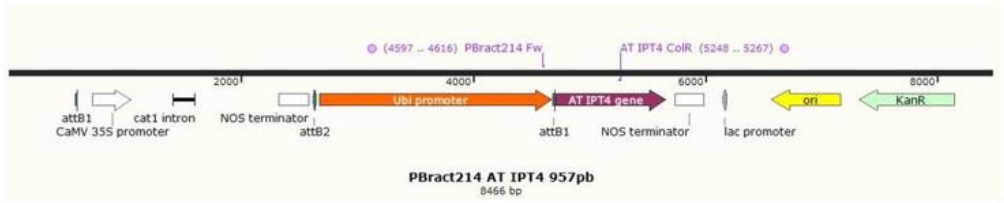
This *ATIPT4* CDS and *ATIPT8* CDS were inserted into the pBract214 destination vector following the same procedure used for pGWB5:*AtIPT4*-CDS and *AtIPT8*-CDS (chapter 3 Section 3.9).

Both constructs were checked by Red-Taq PCR using the primer combination pBract214F with ATIPT4colR1 and ATIPT8colR1 primers (expected band size 670bp and 640bp respectively).

(Tm=59°C; Apeendix 2; Fig 5.1A and B) as described in Section 2.3.8).

Confirmed constructs carrying the gene of interest were then transformed into *Agrobacterium* AGL1 strain by electroporation (Section 2.13).

A



B

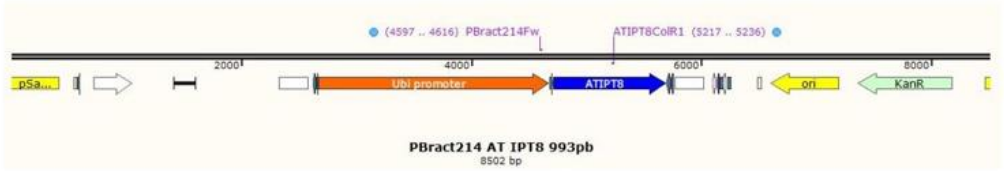


Figure 5. 1: Design of ATIPT4 and ATIPT8 Over-expression cassettes.

A: pBract214:*ATIPT4* distention vector, orange colour indicates the Ubi1 promoter and purple colour the ATIPT4 gene (957bp); the pBract214Fw combined with the gene specific ATIPT4colR primer was used to check the insert (670bp) and its orientation.

B: pBract214:*ATIPT8* distention vector, orange line colour indicates Ubi1 promoter and blue line *ATIPT8* gene (993bp); the pBract214Fw combined with the gene specific ATIPT8colR primer was used to check the insert (640bp) and its orientation.

5.2.2 Analysis of transformed plants.

ATIPT4 and ATIPT8 over-expression lines were generated as specified at Section 2.2.1. Plants were grown under controlled conditions, (16 hours photoperiod with 15°C for day and 12°C for night). Once plants had reached the elongation stage (leaf development nearly 2-3 unfold leaf), genomic DNA was isolated from young leaves as describe in Section 2.3.1. Putative transgenic plants were confirmed by PCR analysis using the vector specific primer Ubi1Fw and the gene specific primer *ATIPT4colR1 and ATIPT8 colR1*, producing a product 670pb and 640pb long respectively. PCR was performed using Red-Taq PCR (Section 2.3.8).

RNA was extracted from leaves (Section 2.3.5) to analyse *ATIPT4* expression. Primers *ATIPT4*qpcrF1Fw and *ATIPT4*qpcrR2 Rev were used at a Tm of 59°C; expected band size was 362bp. Samples were analysed by negative RT-PCR to check for contamination with gDNA; expression was normalised using RT-PCR of HvTubulin Fw and Rev at a Tm of 61.5°C; the expected band size was 240pb.

5.2.3 Morphological data analysis.

5.2.3.1 Measurement of Leaf chlorophyll concertation by Soil and Plant Analyzer Development (SPAD) for T_1ATIPT4 over-expression lines

A SPAD meter (SPAD-502; Minolta, Osaka, Japan) is a hand-held device used for analysis and measurement of leaf chlorophyll concentration (Pask *et al.*, 2012), which was used to determine chlorophyll levels in the transgenic materials. Five reading were taken per plant from the *ATIPT4* and wild type lines, fro main tiller labelled from fully emerged flag leaf twice a week from stage GS41) until GS87.

5.2.3.2 Flag Leaf gas-exchange of photosynthetic rate measurement for ATIPT4 over-expression lines

Biological trait photosynthetic rate, was measured by LiCor6400-XT photosynthesis system (LiCor NE, USA), one measurement was taken from flag leaves of the main tiller per plant at two different growth stages (pre-anthesis and post anthesis).

5.2.3.3 Leaf chlorophyll content (SPAD) during dark and light senescence T_1 ATIPT4 over-expression lines

Leaf chlorophyll concentration was measured using a hand-held device chlorophyll meter (SPAD-502; Minolta, Osaka, Japan) (Pask *et al.,* 2012), in excised leaves from fully emerged flag leaf, from the main tiler at stage GS41-GS45. These were detached and cut into four equal parts (Fig 5.2), and then placed on wet filter paper in Petri dishes. The dishes were then placed in a dark and light room (22°C/0h and 23°C /16h, respectively) for 2 weeks. Flag leaf parts 1and 3 were kept in the dark and 2 and 4 kept in the light; six reading were taken every day for dark, 2 weeks period and 1 week for light.

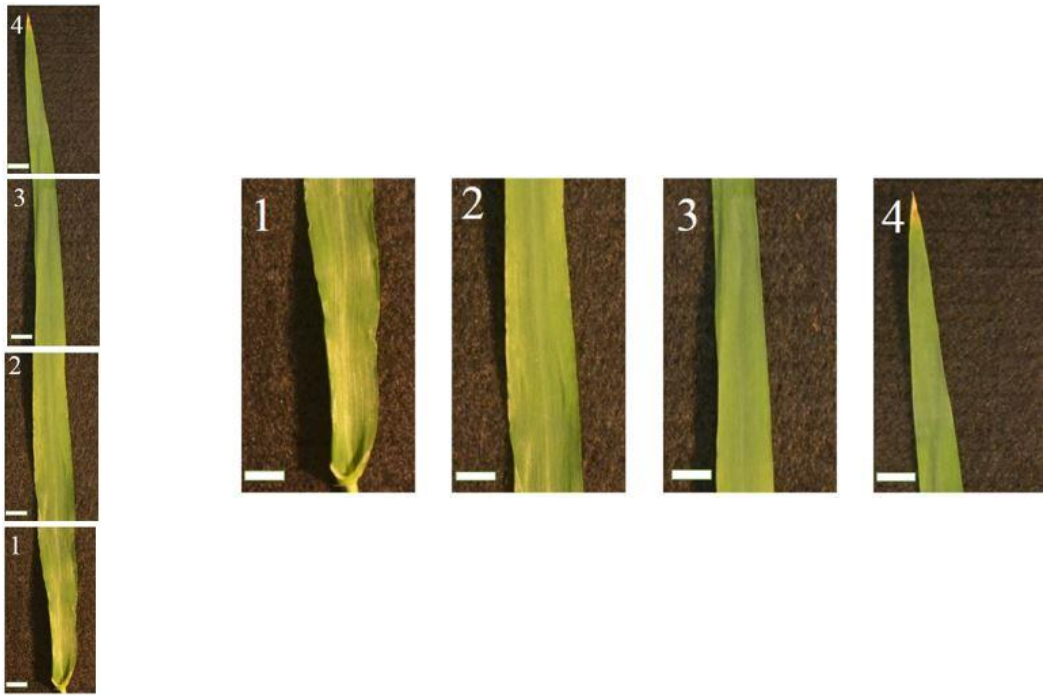


Figure 5. 2: Example showing the cutting and distribution of the flag leaf samples used for dark -induced senescence analysis Bar=3mm.

5.2.3.4 Data statistical analysis.

The Graphpad Prism 7.00 software was used for generating graphs and analysis.

5.3 RESULTS

5.3.1 Cloning and prepared over-expression constructs.

Over expression constructs (pBract214:: *ATIPT4CDS* and pBract214:: *ATIPT8CDS*) were prepared in order to express the *Arabidopsis ATIPT4 and ATIPT8 gene in in barley and Arabidopsis* Plants .

5.3.1.1 Transformation of E. coli cells (DH5 α) with ATIPT4 and ATIPT8 construct.

Positive entry vectore (PCR8GW:*ATIPT4CDS* and PCR8GW:*ATIPT8CDS*) were LR cloned into destination vector (pBract214) and PCR confirmed using pBract 214F primer with gene specific primers *ATIPT4colR1* and ATIPT*8colR1*. According to the T-DNA map the expected PCR band size is 670bp and 640bp respectively (Appendix 1). Fig 5.3 shows the PCR from 16 colonies; lanes (3, 15) were positive for pBract214:: *ATIPT4CDS* and Lane (12) is positive for pBract214:: *ATIPT8CDS*; sequencing the plasmids using the same primers was used to confirm the sequencing identity.

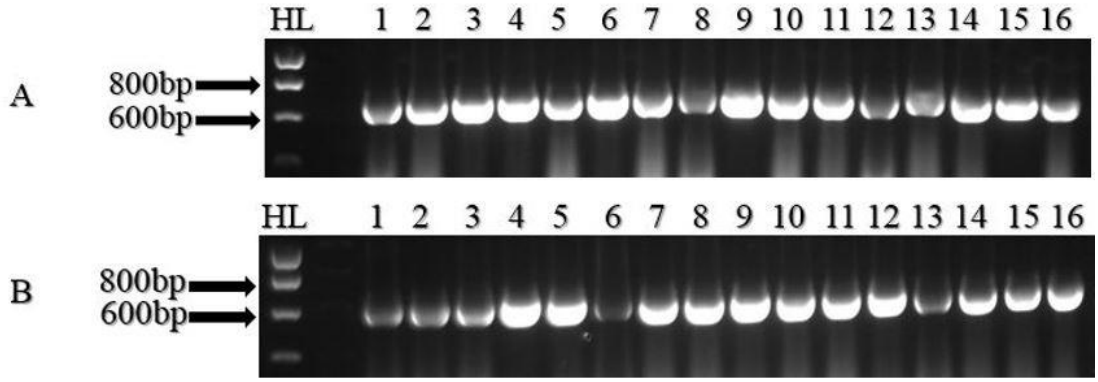


Figure 5. 3: Colony PCR confirming the pBRACT214 transformed colonies with ATIPT4CDS and ATIPT8CDS.

Lanes 1-16: selected colonies; HL: Hyper Ladder 1.

A: *ATIPT4*CDS transformed colony using pBRACT214F vector primer and gene specific gene primer *ATIPT4colR1*; expected product size: 670bp.

B: *ATIPT8*cDS transformed colony using M13F vector primer with specific gene primers *ATIPT8colR1;* expected product size: 640bp.

5.3.1.2 Transformation of Agrobacterium AGLI with ATIPT4 and ATIPT8 construct.

The confirmed constructs *(pBRACT214- ATIPT4* and *ATIPT8)* were transformed into *Agrobacterium* AGL1 strain by electroporation and screened by colony PCR using the same primers for the destination vector; 8 colonies were selected for each construct. Figure 5.4 illustrated the confirmation of correctly transformed colonies obtained with *ATIPT4* and *ATIPT8*.

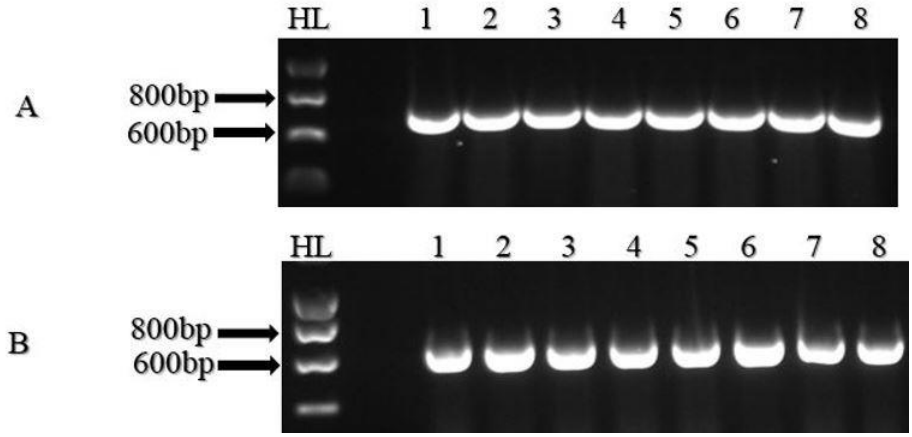


Figure 5. 4: Colony PCR confirming the Agrobacterium AGL1 transformed colonies with (pBRACT214- ATIPT4 and ATIPT8).

Lanes 1-8: selected colonies; HL: Hyper Ladder 1.

A: *pBRACT214:ATIPT4CDS* transformed colony using pBRACT214F vector primer and gene specific gene primer *ATIPT4colR1*; expected product size: 670bp.

B: *pBRACT214:ATIPT8cDS* transformed colony using M13F vector primer with specific gene primers *ATIPT8colR1;* expected product size: 640bp.

pBract214:*ATIPT4* CDS and pBract214:*ATIPT8* CDS over-expression constructs were confirmed by sequencing to create constructs that contained the transgene controlled by the Ubi1 promoter with hygromycin as a selection marker controlled by the 35Spromoter.

5.3.2 Analysis of *ATIPT4* over-expression lines.

5.3.2.1 Analysis of T_0 ATIPT4 and ATIPT8 over-expression lines.

5.3.2.1.1 Genotypic Analysis for ATIPT4

Three independent transform plants were regenerated from 100 immature embryos inoculated with pBract214:*ATIPT4* CDS, alongside 25-30 non-inoculated embryos as a control which underwent the same regeneration protocol. After selection, transition and regeneration media, with the presence of Hygromycin as a selective agent, T_0 plantlets were transferred to bigger pots and placed in an environmental controlled room. The transformation efficiency was 3% and was calculate based on Harwood's protocol (Harwood *et al.,* 2009); (Fig 5.5).

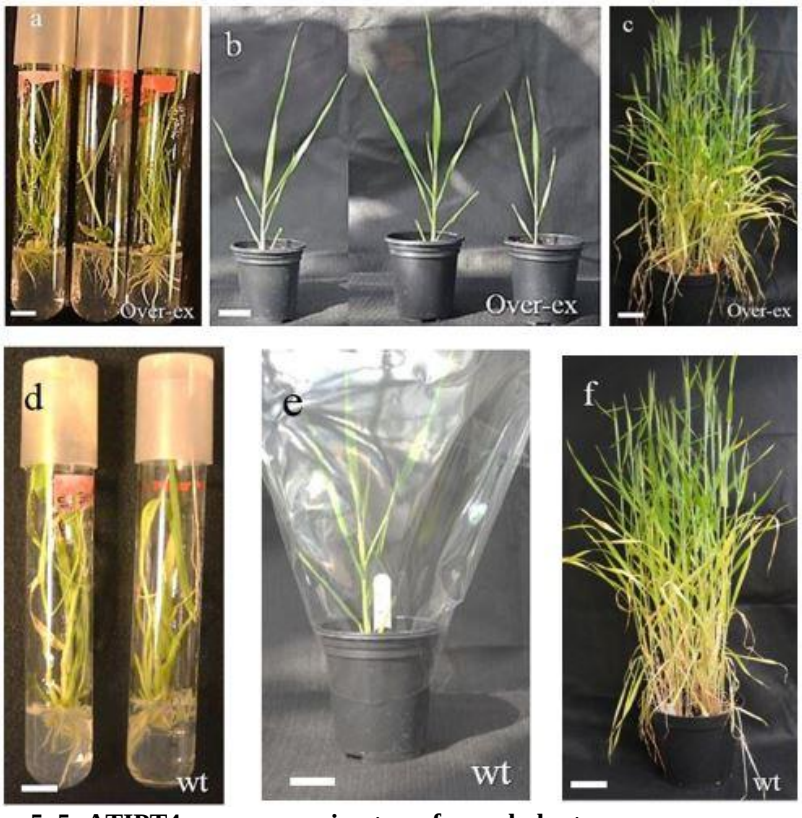


Figure 5. 5: ATIPT4 over-expression transformed plant.

(a and d) Roots and shoots stages; (b and e); plantlets; (c and f) plant growing in the soil. Bar: a, d= 10 mm; b = 6mm; c = 8 mm, e=5mm and f=7mm.

In order to obtain more transgenic plants another transformation expriment was carried out; from this a further 24 independent transform lines were regenerated from 100 immature embryos with 30 non-transformed immature embryos as a control. However, all of these lines failed to form roots in regeneration medium and thus could not be transferred to soil (Fig 5.6).

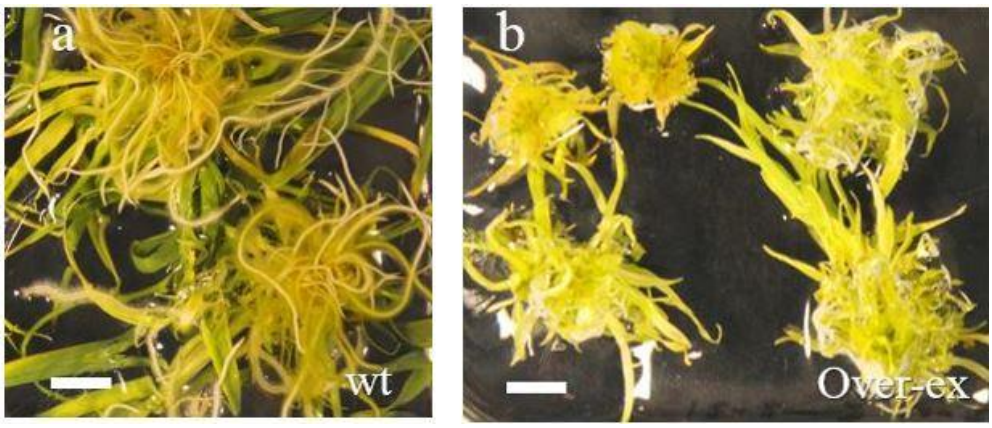


Figure 5. 6: ATIPT4 positive barley material failed to produce roots on regeneration medium.

a: wild type showing normal development of roots.

b: No root development was seen for ATIPT4 overexpression lines. Bar: a, b =5mm.

Genomic PCR was used to screen putative transgenic plants using Ubi1 promoter Fw (vector specific) and the target gene primer *ATIPT4colR1*Rv. Results showed that all regenerated plants were positive for the over-expression transgene (Fig 5.7).

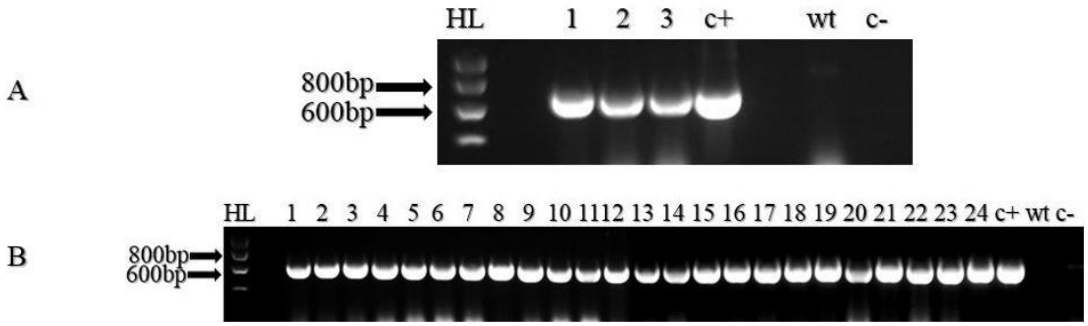


Figure 5. 7: Genotyping of T_0 Transformed plants carrying the AtIPT4 over-expression construct by PCR.

The vector specific primer Ubi1Fw and the gene specific primer *ATIPT4*Rv were used; expected size product 670bp. Lanes 1-24 represents transformed plants; all lines showed positive results. (C+) *AtIPT4* over-expression construct plasmid DNA as positive control; (wt) wild type as negative control; (c-) water negative control; (HL) Hyper Ladder 1.

A: Genotyping of three independent over-expression lines showing normal regeneration.

B: Genotyping of twenty-four independent over-expression lines in which root development was not initiated.

5.3.2.1.2 Phenotypic Analysis of barley T_0 lines carrying the Ubi-ATIPT4 construct.

Phenotypic analysis was conducted on the putative transgenic lines after they were confirmed by PCR as carrying the transgene (Fig 5.10). Seeds were collected from three plants grown normally for phenotypic analysis in the T_1 generation. For those T_0 *ATIPT4* over expression lines without root initiate different concentration of IAA (0.22 mg/L, 0.87 mg/L, 1 mg/L and 5 mg/L) were used in the regeneration medium for about 3 weeks to try to initiate rooting. Fig 5.8 shows that normal root development was not initiated in all concentrations of IAA tested, in comparison to wild type, however Fig 5.8d (0.87 mg/L IAA) and Fig 5.8e (1

mg/L) shows tap root formation with other transgenic lines (Fig 5.8c and 5.8f) and also (Fig5.8b) as a control for transgenic lines.

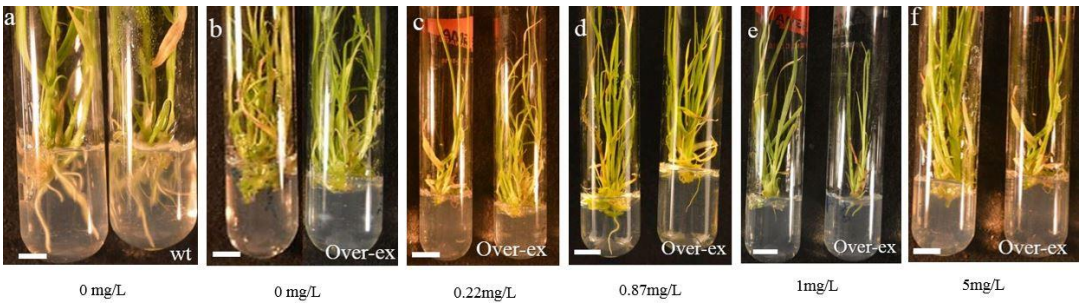


Figure 5. 8: Initiation of root development in T_0 Transformed plants carrying the AtIPT4 over-expression construct by manipulation of different concentration of IAA. (a and b) as control; (c, d, e and f) transgenic lines.

(a) Wild type control showing normal root development; (b) overexpression transgenic lines as control for different concentration lines showed no different with others; (c-f) overexpression transgenic lines showing a small rot tap initiated in d and e; Bar: a =3mm; b, d=10mm; c, e=8mm and f=5mm

5.3.2.1.3 Analysis of AtIPT4 over-expression levels

Gene expression was tested for 3 plants with roots and 5 plantlets without roots at the regeneration stages; RT-PCR and qRT-PCR was performed using ATIPT4qpcrF1 and ATIPT4qpcrR2 (the band size is expected to be 362bp; Fig 5.9A and B). All cDNA samples were tested using the housekeeping gene HvTu-bulin (Fig 5.9 C and D); no expression was seen in -RT which confirmed that there was no contamination with genomic DNA. The RT-PCR results illustrated that all lines were expressed, with different level of ATIPT*4* expression seen between lines (Fig 5.9E). Line 1 and 2 had showed equal expression level, which 1fold and Line 3 0.3fold higher than wild type as (bc). Whereas line 2a was showed higher expression level among lines (1.5fold). It can be seen that expression levels are critical, regarding relationship between different level of expression between lines with root (1, 2 and 3) and without roots (1a -2a). (Fig 5.9E).

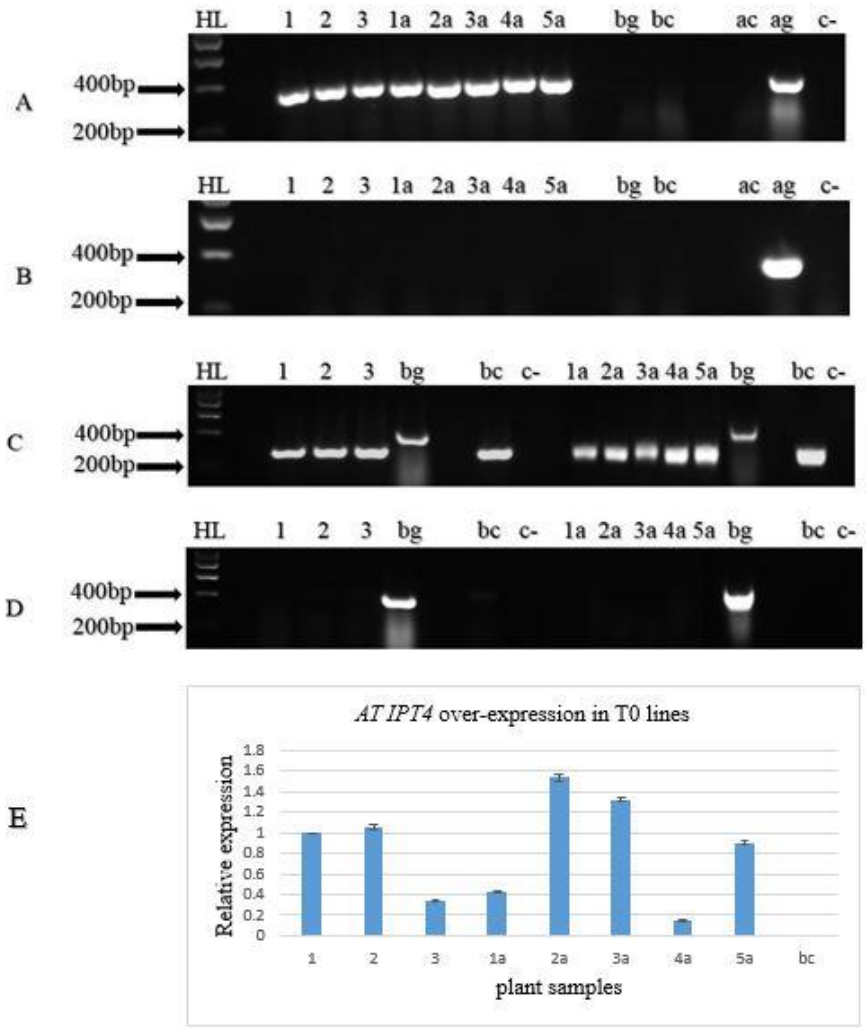


Figure 5. 9: AT IPT4 Expression analysis in barley T_0 lines.

A - D: Reverse Transcriptase (RT)-PCR analysis in w.t., T0 transgenic lines. Lanes 1, 2,3 and 1a-5a: transformed plants; all lines showed positive results. (bg) gDNA of barley plant as negative control; (bc) cDNA of barley plant as negative control; (ac) cDNA of Arabidopsis wild type; (ag) gDNA of Arabidopsis wild type as positive control; (c-) water negative control; (HL) Hyper Ladder 1

A: Positive RT-PCR analysis of the *AtIPT4* overexpression using specific gene (*AtIPT4 qPCRF1* and *AtIPT4qPCRR2*) and size product is expected 362bp.

B: Negative RT-PCR for gene-specific primers (*AtIPT4 qPCRF1* and *AtIPT4qPCRR2*).

C: RT-PCR analysis of the normalisation control of the *HvTubulin* housekeeping gene; expected product size is 240bp.

D: Negative RT-PCR for of the *HvTubulin* housekeeping gene.

E: qRT-PCR amplification in T_0 lines.

5.3.2.1.4 Genotypic Analysis for ATIPT8

Six independently transformed plants were regenerated from 100 immature embryos (Section 2.14), with 25 immature embryos as a control, regeneration followed as the same procedure used for AtIPT4 that all in depended lines showed normal vegetative development. A transformation efficiency of 6% was obtained, based on Harwood's protocol (Harwood *et al.,* 2009).

Genomic PCR was used to screen transgenic plants, using Ubi1 promoter Fw (vector specific) and the target gene primer *ATIPT8colR1*Rv. Results showed that all regenerated plants were negative for the over-expression transgene. Unfortunately, we did not obtain any transformed lines after three repeated analyses (Fig 5.10).



Figure 5. 10: Genotyping of T_0 Transformed plants carrying the AtIPT8 over-expression construct by PCR.

The vector specific primer Ubi1Fw and the gene specific primer *ATIPT8*Rv was used and size product is expected 640bp. Lanes 1-6 represents transformed plants; all lines showed negative results. (C+) *HvDYT1* over-expression construct plasmid DNA as positive control; (wt.) wild type as negative control; (c-) water negative control; (HL) Hyper Ladder 1.

5.3.2.2 Analysis of T_1 ATIPT4 over-expression lines.

Seeds from T_0 lines was obtained and then grown with wild type at the same condition as describe Section 2.1.1.

5.3.2.2.1 Genotypic Analysis.

To analysis segregation population for over-expression transgene from T_1 lines, 14 plants were obtained from each T_0 line. Genomic PCR was used to screen transgenic plants, using Ubi1 promoter Fw (vector specific) with the target gene primer *ATIPT4colR1*Rv. Results illustrated that all regenerated plants were positive for the over-expression transgene, except 1b,1d,1j,1m and1n; 2a,2g, and 2n; 3c,3i,3l and 3m (Fig 5.11; A, B and C) respectively.

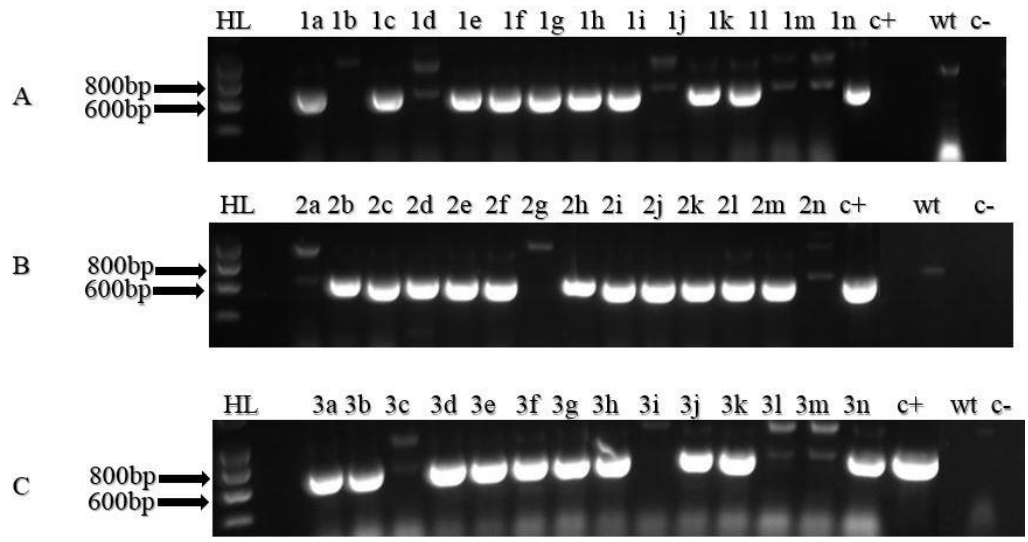


Figure 5. 11: Genotyping of T_1 Transformed plants carrying the AtIPT4 over-expression construct by PCR.

The vector specific primer Ubi1Fw and the gene specific primer *ATIPT4*Rv was used; expected size product 670bp; (C+) *AT IPT4* over-expression construct plasmid DNA as positive control; (wt.) wild type as negative control; (c-) water negative control; (HL) represent Hyper Ladder 1.

A: Lines (1a-1n,) represents transformed plants that all lines showed positive results *AtIPT4* over-expression except (1b,1d,1j,1m and1n) were negative for transgene.

B: Lines (2a-2n,) represents transformed plants that all lines showed positive results AtIPT4 over-expression except (2a,2g and2n) were negative

C: Lines (3a-3n,) represents transformed plants that all lines showed positive results AtIPT4 over-expression except (3c,3i,3l and 3m) were negative

5.3.2.2.2 Analysis of AtIPT4 T_1 expression levels

To analyse the level of transgene expression, RT-PCR and qRT-PCR were performed using cDNA synthesised from RNA extracted from leaves as explained in Section 2.3.5.

Gene expression was tested in 5 plants transformed for each line, among them 1 plant was a control negative transformed line. Specific primers (*ATIPT4qpcr*F1 and *ATIPT4qpc*rR2, expected band size 362bp) were used for checking the gene expression. Fig 5.12 A and B showed that all the T_1 transgenic lines were expressing the transgene except for Lines 1j, 2a and 3i., Samples were shown to have equal template levels using *HvTubulin* housekeeping gene; no amplification was seen in the Negative RT samples (-R) confirming that there was no contamination with genomic DNA in the RNA samples (Fig 5.12 C and D).

The T_1 transgenic lines showed different levels of *AtIPT4* expression, Line 1 samples c, e, h and l, showed an equal expression level (0.5fold) that was segregated from plant 1 in T_0. A slightly higher level of expression was seen in line 2 (plant 2c), whereas the others (plants 2b and 2e) were at a similar level to Line 1. Line 3 showed a similar level of expression except in line 3k and 3n, which were higher (1.5 fold). Samples 1j, 2a and 3j were negative for the transgene, but had been through the regeneration process and did not show any expression, a similar absence of expression was seen in the w.t barley samples as a control (Fig 5.12 E).

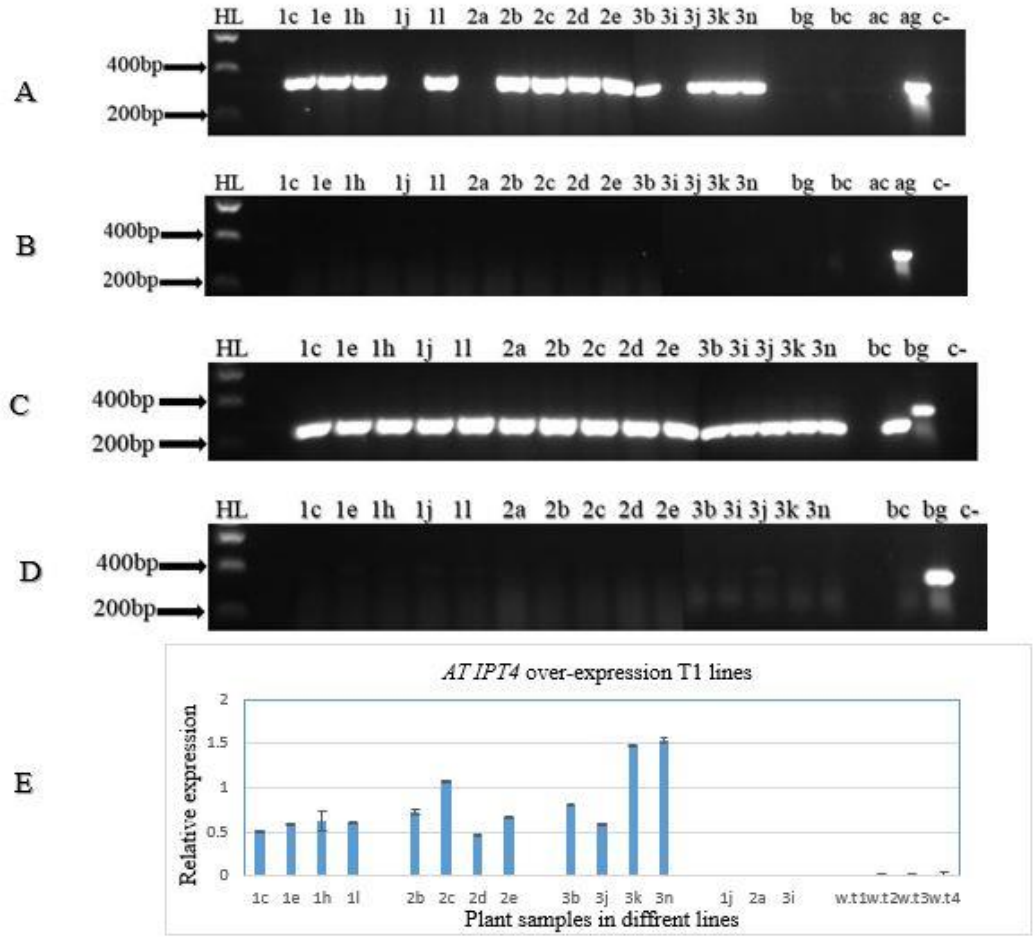


Figure 5. 12: AT IPT4 expression analysis in barley T_1 lines.

A - E: Reverse Transcriptase (RT)-PCR analysis in w.t., T_1 transgenic lines. Lines 1c- 3n are T1 transformed plants; (bg) gDNA of barley plant as negative control; (bc) cDNA of barley plant as negative control; (ac) cDNA of Arabidopsis wild type; (ag) gDNA of Arabidopsis wild type as positive control; (c-) water negative control; (HL) Hyper Ladder 1.

A: Positive RT-PCR analysis of the *AtIPT4* overexpression using specific gene (*AtIPT4 qPCRF1* and *AtIPT4qPCRR2*); and size product is expected 362bp.

B: Negative RT-PCR for gene-specific primers (*AtIPT4 qPCRF1* and *AtIPT4qPCRR2*).

C: RT-PCR analysis of the normalisation control of the *HvTubulin* housekeeping gene; expected product size is 240bp.

D: Negative RT-PCR for of the *HvTubulin* housekeeping gene.

E: qRT-PCR amplification in T_1 lines.

5.3.3 Analysis of physiological traits in barley T_1 *AtIPT4* over-expression lines.

5.3.3.1 Measurement of leaf chlorophyll content in T_1 ATIPT4 over-expression lines under glass house conditions.

Development of chloroplast photosynthetic capacity and leaf nitrogen content can be identified using leaf chlorophyll measurements which is an important paratmeter for general plant health (Ling *et al.*, 2011).

Chlorophyll content was measured using a SPAD meter on the fully emerged flag leaf of the main tiller of each plant, starting at growth stage GS41-45 twice a week (Section 4.2.4.1). (Figure 5.13) shows the decline of chlorophyll content in the flag leaf of all lines over time, this was more severe for the wild type lines compared to those carrying the transgene. From growth stage GS41+25 days the chlorophyll content of the wild type started to decrease whereas this occurred to a lesser extent in all of the transgenic over-expression lines, with chlorophyll levels only starting to drop after GS41+52 days in all of the *AtIPT4-OEx* lines.

According to the qRT-PCR analysis, (Fig 5.12) different levels of expression were observed for the different T_1 AtIPT4 over-expression comparing these results with this from the senescence analysis (Fig 5.13) there appears to be a corelation btween *AtIPT4-OEx* lines and prolonged leaf green (Chlorophyll content) and therfor a potentiall role of cytokinin in delaying leaf senescence by manipulation of *AtIPT4*. futhermore confirmed this results photosynthesis efficiency was analysed using a Li-COR (LI-6400 XT, LI-COR, Lincoln, NE); as explained in next Section, because Chlorophyll content have been directly correlated with Photosynthesis efficiency.

Chlorophyll content was observed to significantly differ over time for all lines ($P<0.001$) See table (5.1). Significant difference were also detected between lines with respect to chlorophyll content ($P= 0.005$). Fisher's protected L.S.D testing showed all lines were significantly hgher that wild type.

Table 5. 1: Leaf chlorophyll content in T_1 ATIPT4 over-expression lines under glass house conditions.

Source	d.f	sums of squares	Mean Squares	F	P
Line	4	2768.82	692	6.10	0.005
Residual	14	1589.20	113.51	7.67	
Time	20	35873.20	1793.66	121.27	< 0.001
Time. Line	80	1413.70	17.67	1.19	0.338
Residual	280	4141.45	14.79		
Total	**398**	**45786.36**			

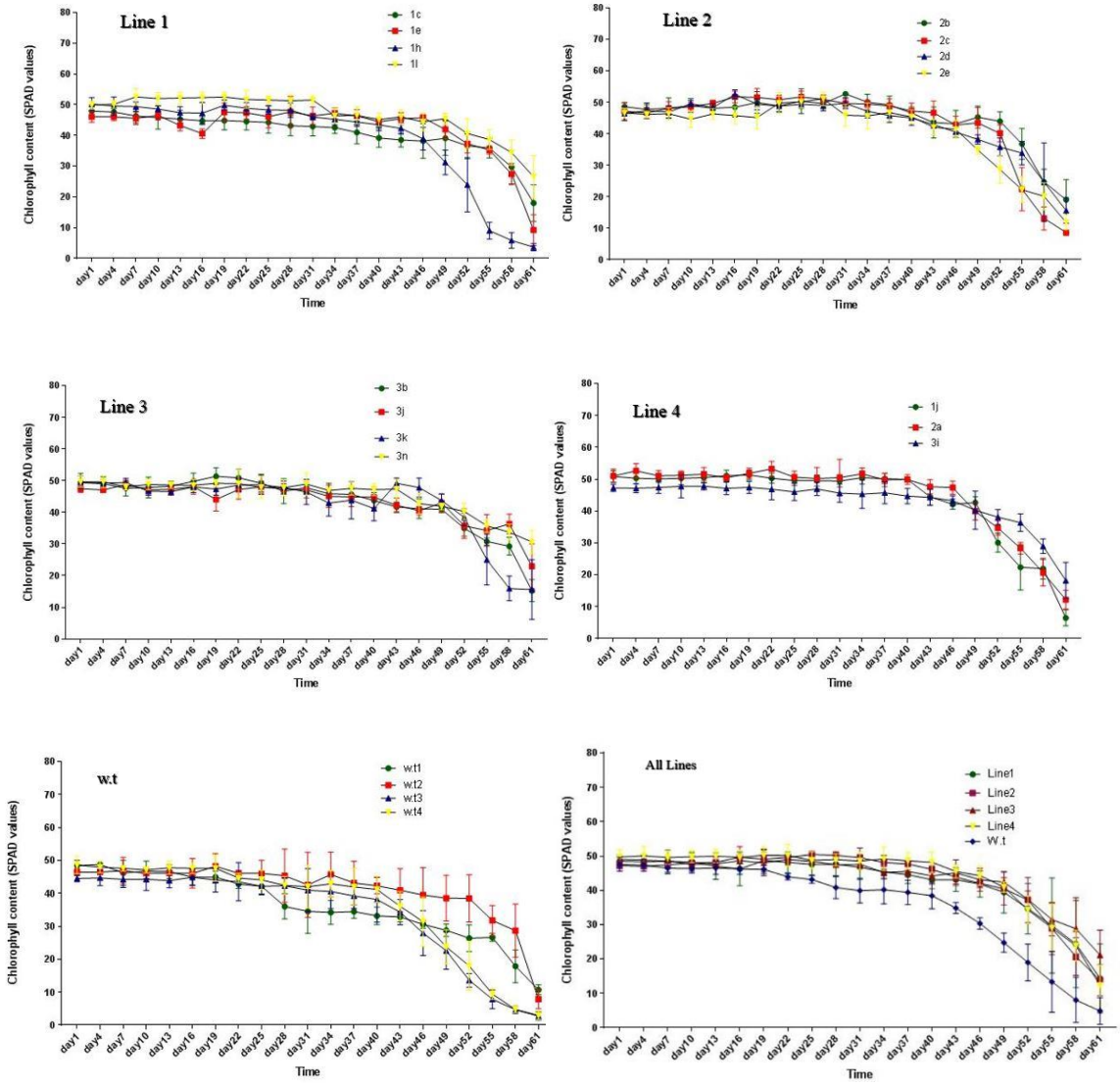


Figure 5. 13: Chlorophyll content (SPAD values) level of barley T_1 AtIPT4 over-expression lines under glasshouse conditions.

Means (±SD) values are shown these are derived from 5 independent SPAD meter readings taken on the main flag leaf of each plant.

Line 1= Four plants (1c,1e,1h and 1l) were positve for transgene; they came from (T_0 Plant 1).

Line 2= Four plants (2b,2c,2d and 2e) were positve for transgene; they came from (T_0 Plant 2).

Line 3= Four plants (3b,3j,3k and 3n) were positve for transgene; they came from (T_0 Plant 3).

Line 4= Three plants (1j, 2a, and 3i) were negative for transgene; they came from (T_1 lines).

w.t = Four wild type plants were selected (w.t1, w.t2, w.t3 and w.t4).

5.3.3.2 Leaf gas-exchange measurements on AtIPT4 over-expression lines under glasshouse conditions.

Photosynthesis efficiency was analysed using a Li-COR (LI-6400 XT, LI-COR, Lincoln, NE); measurements were taken using an open infrared gas-exchange system and a 2 cm^2 leaf chamber with an integral blue red LED light source of 1500 µl moles of photons m^{-2} s^{-1} measurements on flag leaves at two different growth stages (pre-anthesis and post anthesis) for all lines. Figure 5.14 shows assimilation rates for the different lines, these were reduced in post anthesis material, compared to pre-anthesis. The data suggests no significant differences were detected between lines with respect to changed Assimilation rate (P= 0.095), but Fisher's unprotected L.S.D showed the significant difference from wt were observed with line 2 and 3 during pre anthes stage (see Table 5.2).

No significant differences were detected between lines with respect to Change Assimilation rate (P= 0.082), but Fisher's unprotected L.S.D showed significant difference from wt were observed with line 1 and 2 during post anthes stage (see Table 5.3)

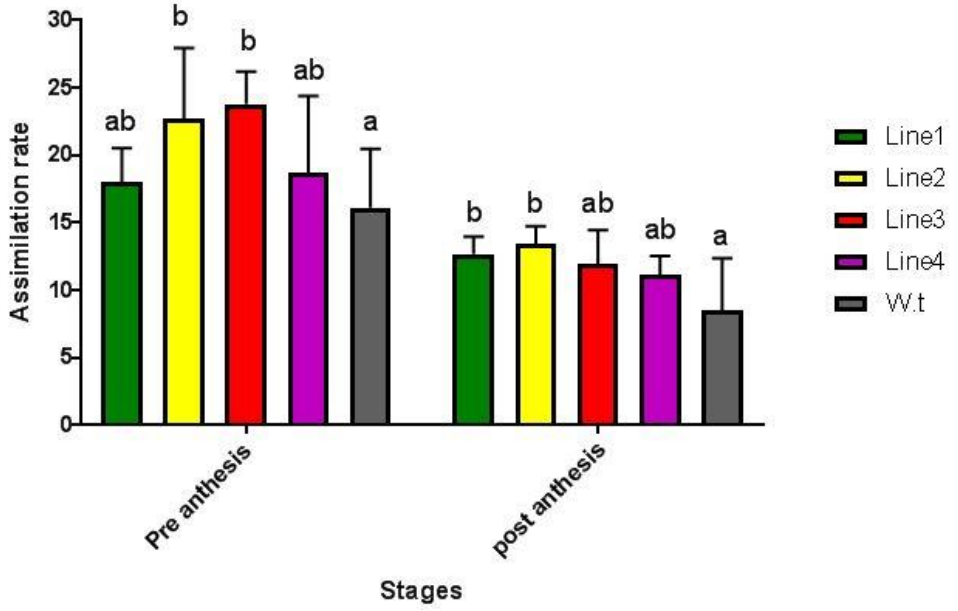


Figure 5. 14: Assimilation rate for T_1 ATIPT4 over-expression lines at two different growth stages (pre-anthesis and post -anthesis).

Assimilation rate data come from same lines as used for Chlorophyll content (SPAD values)

Table 5. 2: Assimilation rate for T_1 ATIPT4 over-expression lines at (pre-anthesis) growth stages

Source	d.f	sums of squares	Mean Squares	F	P
Line	4	168.04	42.01	2.44	0.095
Residual	14	240.59	17.18		
Total	**18**	**408.63**			

Table 5. 3: Assimilation rate for T_1 ATIPT4 over-expression lines at (post-anthesis) growth stages

Source	d.f	sums of squares	Mean Squares	F	P
Line	4	57.967	14.49	2.60	0.082
Residual	14	78.136	5.581		
Total	**18**	**136.103**			

It can be seen that the delayed senescenec phynotype in *AtIPT4* over expression lines was observed. Figure 5.15 shows *AtIPT4* over expression lines (Line1; line 2; and Line3) still green ; whereas as more yellw leaves were observed in wild type as acontrol. However, Line 4 was negative for the transgene; no differences were seen as compered with other lines .

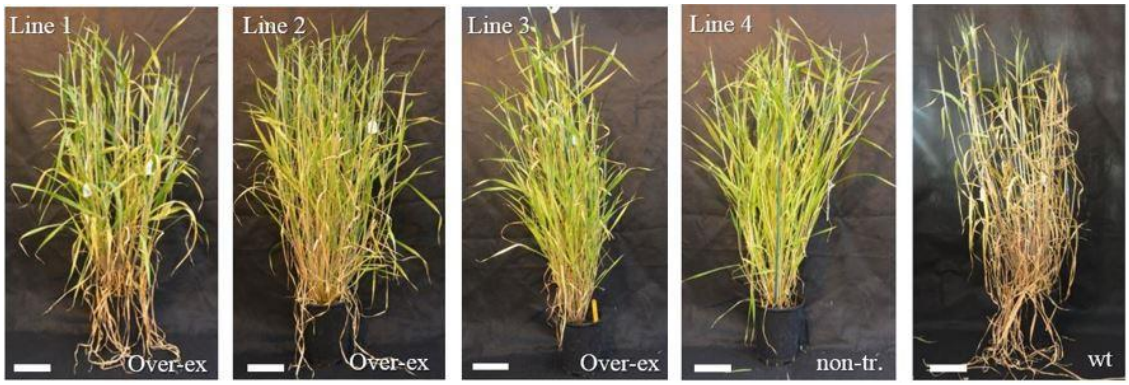


Figure 5. 15: phynotype of whole barley plants at the flowering stage that were over-expressing AtIPT4 .

5.3.3.3 Analysis flag-leaf chlorophyll content (SPAD) during dark and light senescence for T_1 AtIPT4 over-expression lines.

In order to analyse the progression of dark and light- induced senescence in the transgenic T_1 *ATIPT4* over-expression lines, leaf samples were detached from the main flag-leaf and maintained in dark and light conditions (4.2.4.3). The chlorophyll content was then measured (SPAD) meter in these explants over time (Section 4.2.4.1).

In addtion, to anlayse the relationship between the dark and light -induced senescence with the levels of transgene expression, qRT-PCR was performed using cDNA synthesised from RNA extracted from leaves as explained in Section 2.3.5. Different levels of expression were seen between the lines, however all showed increased *TPT4* expression compared to wild type, highest expression was seen in Lines 3d and 3f (Fig 5.16).

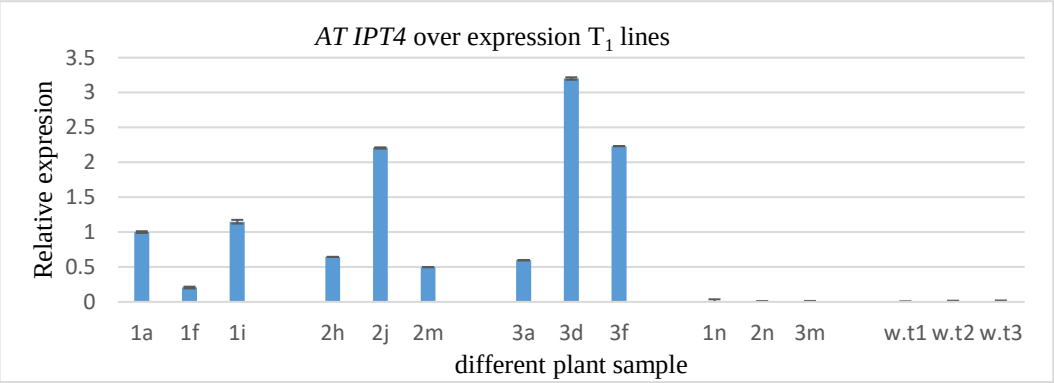


Figure 5. 16: qRT-PCR amplification of *AtIPT4* expression analysis for dark and light -induced senescence barley T_1 lines.

5.3.3.3.1 Changes of chlorophyll content during dark leaf senescence.

Change in chlorophyll content occurring during leaf senescence were investigated on detached flag-leaf sections under dark room conditions (22°C) for T_1 *ATIPT4* over -expressing lines and wild type. (Fig 5.20).

Figures 5.16; different levels of expression were observed for the different T_1 AtIPT4 over-expression lines using qRT-PCR, and comparing with results from (Fig 5.17; 5.18) Statistical analysis of dark-induced senecence was demostrated that no significant differences were detected between lines with respect to chlorophyll content that (P= 0.53). However, chlorophyll content was observed to significantly differ over time for all lines (P<0.001) (See table 5.4).

Table 5. 4: Changes of chlorophyll content during dark leaf senescence.

Source	d.f	sums of squares	Mean Squares	F	P
Line	4	1375.69	343.92	0.84	0.53
Residual	10	4114.88	411.49	15.14	
Time	12	19114.71	1592.89	58.61	< 0.001
Time. Line	48	11226.43	25.55	0.94	0.503
Residual	120	3261.16	27.18		
Total	**194**	**29092.86**			

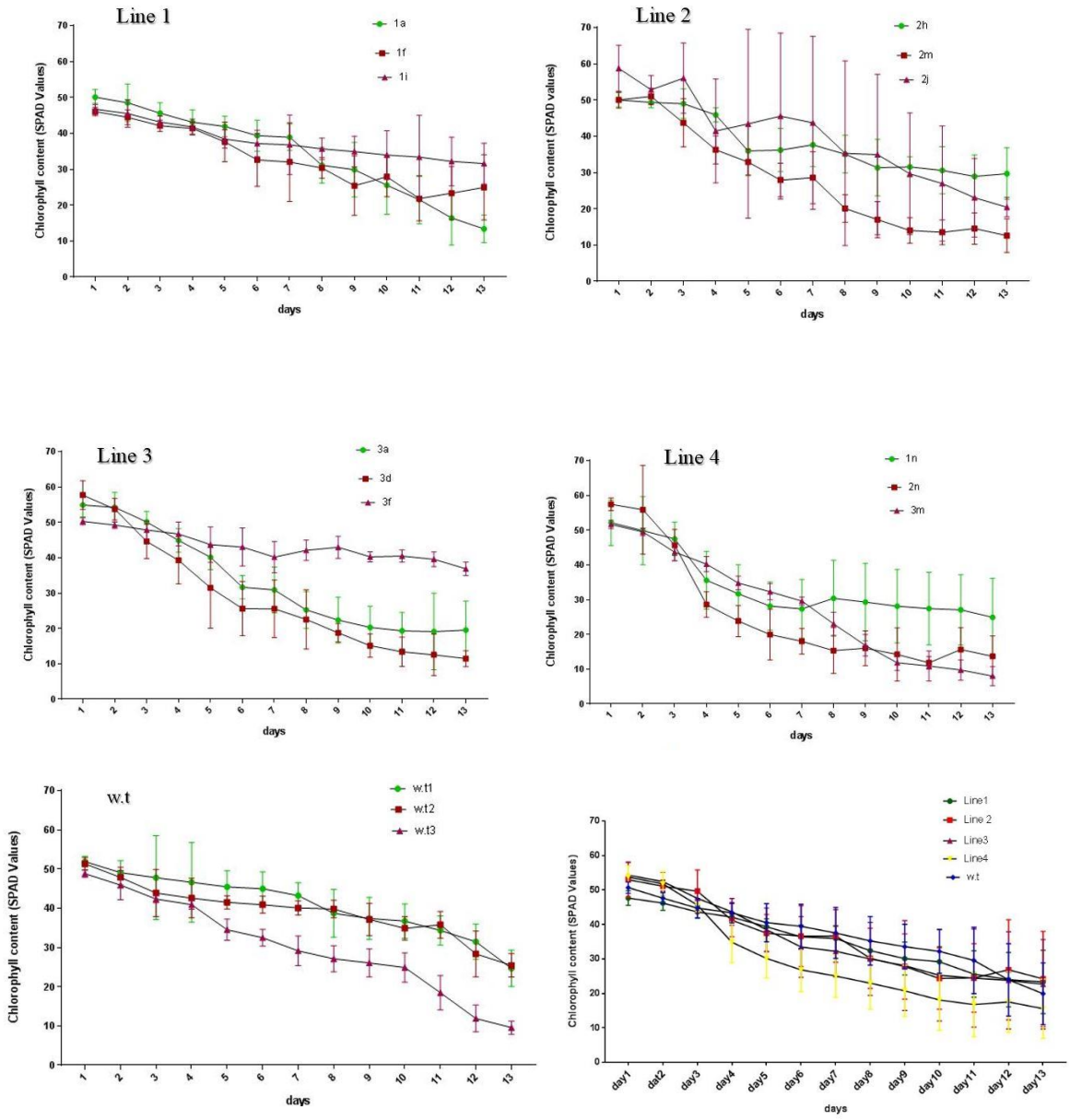


Figure 5. 17: Analysis of dark-induced senecence in detached flag leaves for T_1 AtIPT4 over-expression lines.

Means (±SD) values are shown these are derived from 6 independent SPAD meter readings taken on the main flag leaf of each plant.

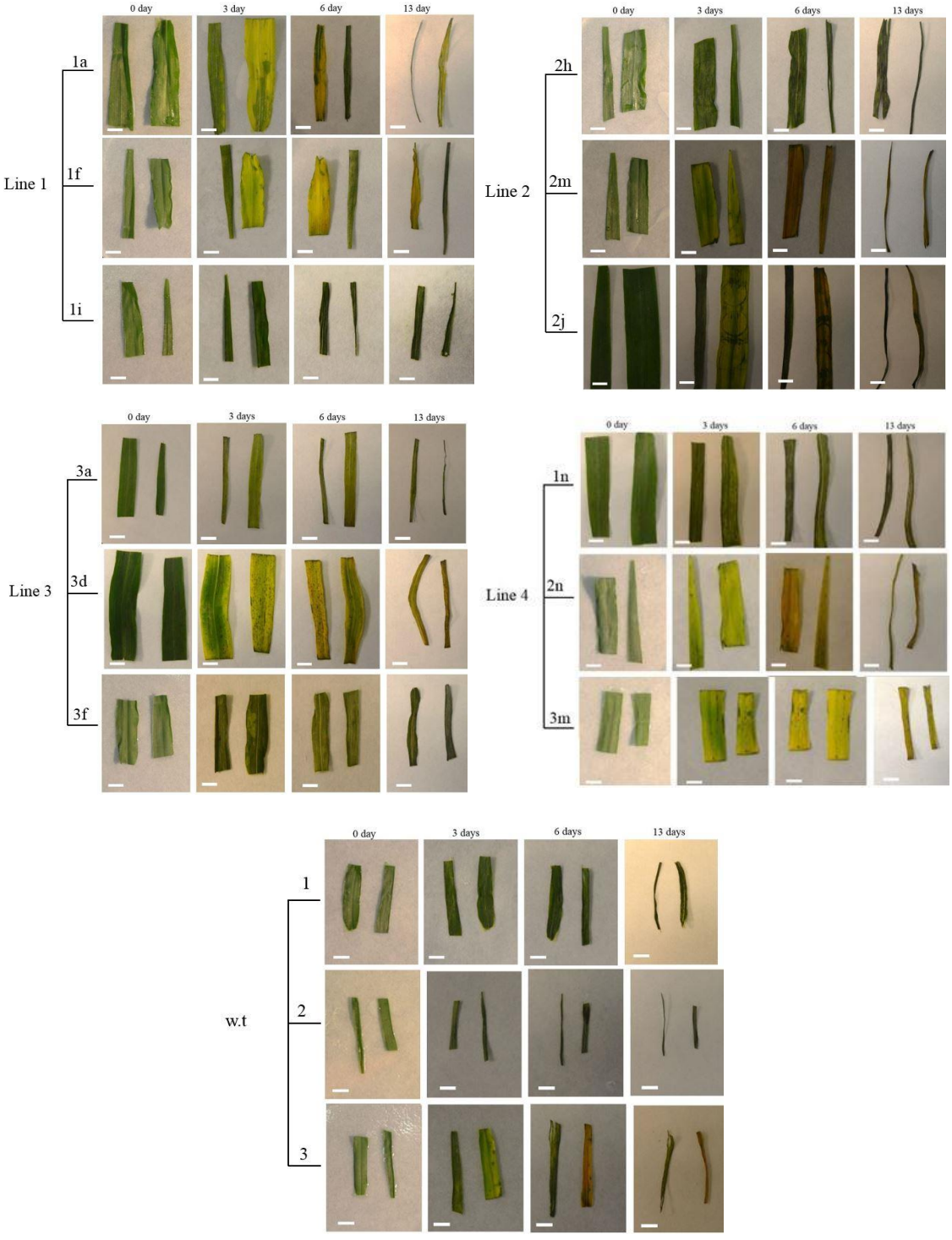


Figure 5. 18: Analysis of the progression of dark-induced senecence in detached flag leaves for T_1 ATIPT4 over-expression lines

5.3.3.3.2 Changes of chlorophyll content during light leaf senescence.

Change in chlorophyll content occurring during leaf senescence were investigated on detached flag -leaf Sections maintained in the growth room under light room condition (23°C /16h) for T_1 *ATIPT4* over -expressing lines with wild type over a one week period (Fig 5.19 and 5.20). Statistical analysis of light-induced senecence was demostrated that Significant differences were detected between lines (P= 0.003). In additon, t significantly different also was observed over time for all lines (P<0.001) (See table 5.5).

The interaction between line and time was also significat (P= 0.024), but Fisher's protected L.S.D showed that line 1 significantly lower than all lines including wt.

Table 5. 5: Changes of chlorophyll content during light leaf senescence.

Source	d.f	sums of squares	Mean Squares	F	P
Line	4	1024.311	256.078	8.21	0.003
Residual	10	311.82	31.183	3.29	
Time	7	34549.606	4935.658	520.01	< 0.001
Time. Line	28	709.365	25.334	2.67	0.024
Residual	70	664.404	9.491		
Total	**119**	**37259.511**			

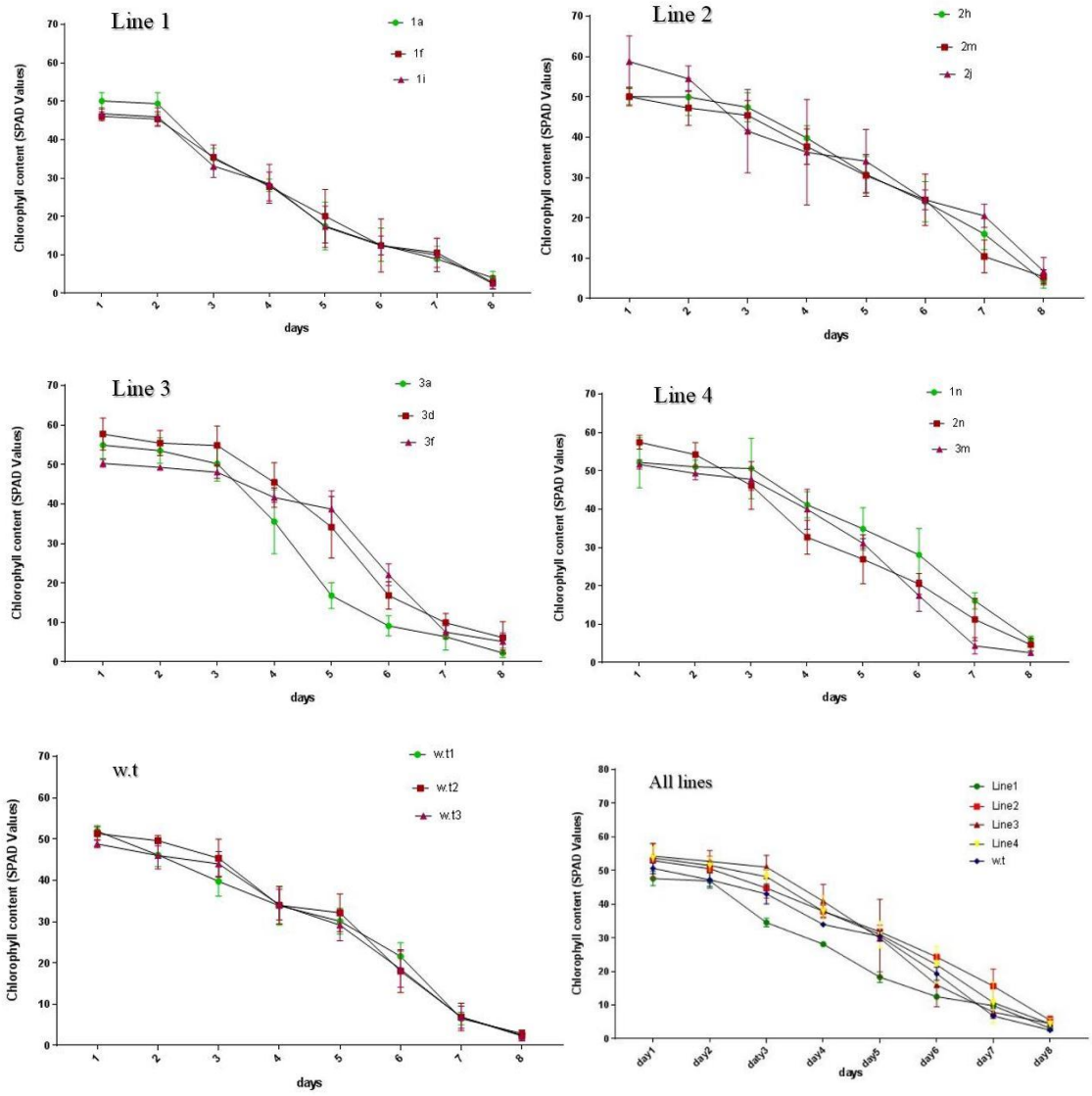


Figure 5. 19: Analysis of chlorophyll breakdown and senecence in detached flag leaves for T_1 ATIPT4 over-expression lines under light conditions.

Means (±SD) values are shown these are derived from 6 independent SPAD meter readings taken on the main flag leaf of each plant

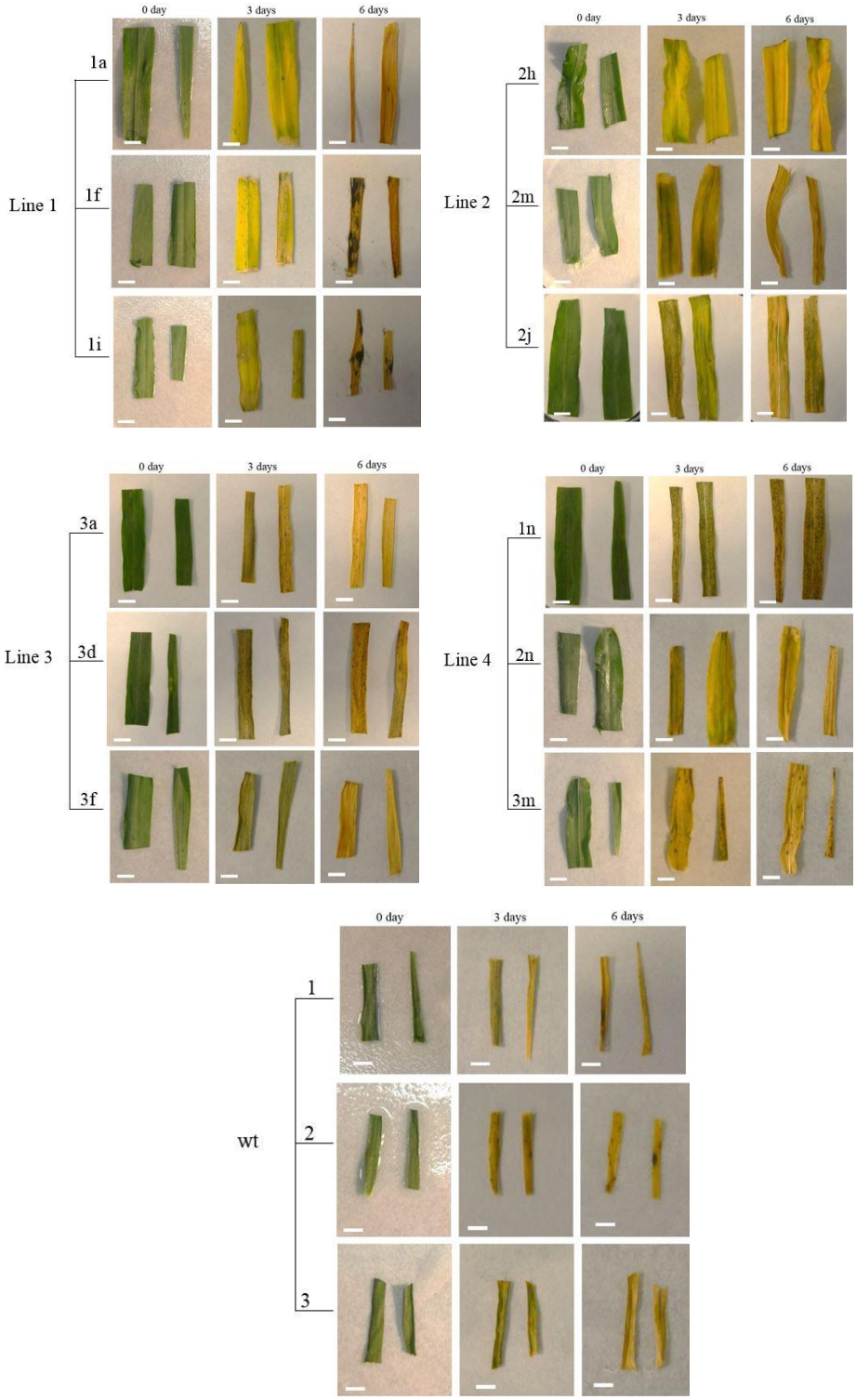


Figure 5. 20: Analysis of the progression light senecence in detached flag leavs for T_1 ATIPT4 over-expression lines.

5.4 Discussion

The main aim of this study was to show whether the over-expression of *ATIPT4* gene in barley was able to alter cytokinin and thus impact upon leaf and plant senescence.

Cytokinin play an important role in development and physiological aspects in higher plants (Clark *et al.,* 2004). Gan and Amasino (1996) demonstrated that the role of cytokinins in controlling plant senescence and that this can be investigated by three approaches, external application studies, secondly measurement of endogenous cytokinin levels before and during senescence and finally by manipulation of endogenous cytokinin levels in transgenic crops.

The key cytokinin biosynthetic enzyme in plants is encoded by the *ISOPENTENYL TRANSFERASE (IPT)* gene family. Therefore, production of endogenous cytokinin can be manipulated by expression of *IPT* gene under different promoters. These studies provide a way to manipulated senescence for potential agricultural applications, particularly to improve of agronomic important traits such as delay leaf senescence, which can impact on quality and quality of yield.

Previous research reported manipulation of cytokinin levels based upon the *IPT* gene isolated from *Agrobacterium* (Gan and Amasino, 1996). *Arabidopsis* has been used to play a crucial role in identifying and characterising the role of cytokinins; nine *IPT* genes have been identified in cytokinin biosynthesis in *Arabidopsis* (Lim *et al.,* 2003; Lime *et al.,* 2007; Kieber and Schaller, 2013)

P$_{SAG}$*-IPT* gene was previously used for manipulation of senescence-related processes; higher biomass and yield were reported in transgenic tobacco plant using the SAG12 promoter (Senescence-specific promoter,) with *IPT* gene to delay leaf senescence (Gan and Amasino, 1995). It has also been used to prolonged leaf development and flowering in several crops (Sykorova et al., 2008; McCabe et al., 2001; Chang et al., 2003; Huynh *et al*., 2005 and Liu *et al.*, 2011).

In this experiment *ATIPT4* gene was used under the control Ubi promoter, to manipulate senescence in barley. Different number of transgenic plants were obtaned from AGLI::pBract214::*ATIPT4* CDS. 3% of transformation efficiency was obtained in the first reformed experiment (Fig 5.5). A transformation efficiency as describe the number of independent transformed plants as a percentage of the original number of immature embryos treated (Harwood et al., 2009). However, a higher transformation efficiency was obtained with further experiment with rates of 24%. Unfortunately, during the regeneration protocol, roots were not initiated and developed in rooting medium for all 24 lines (Figure 5.6 and 5.8). However, they confirmed by genotyping (Fig 5.7) and 5 plants among them were confirmed by RT-PCR and qRT-PCR (Fig 5.9)

Furthermore, different concentration of (IAA) (Section 5.3.2.1.2) were used for those T_0 ATIPT4 over expression lines which failed to initiate roots. However, root initiation did still not occur. This may be a consequence of over-expression of the ATIPT*4* gene, which may be altering the hormonal levels within the plant and in particular it may be repressing IAA and auxin levels and thus preventing root differentiation (Medford et al., 1989; Kuderova et al 2008; Ruzicka et al 2009)

Nevertheless, to reduce the cytokinin level different concentration of IAA were used during regeneration medium, small tap roots were seen Fig 5.8d and Fig 5.8e when explants were maintained on medium containing using 0.87 mg/L and 1 mg/L of IAA concentration. However, eventually these plantlets died after being transferred in to pots. It may be that development root system is the specific time point related to cytokinin level and the concentration of cytokinin and auxin temporally work at specific stages of root development (Ruzicka et al 2009). Therefore, suggests that regeneration may be possible, however a more favourable approach might be the use of a specific promoter to drive *IPT* expression, for example a senescence-related promoter (Gan and Amasino, 1995)

Different expression levels were seen in T_0 *ATIPT4* lines between lines (line 1, 2, 3) that grew normally and lines without root initiation (lines1a - 5a) (Fig 5.9E). Overproduction of cytokinin level by overexpression of IPT gene or exogenous application can cause inhibition root growth in *Arabidopsis* plant (Medford et al., 1989 ; Kuderova et al 2008) with decreases of cytokinin level via over expression have shown the opposite effect (Werner et al., 2003 ; Ruzicka et al 2009). On the other hand, modification of cytokinin level or cytokinin signalling leads to alter auxin efflux carrier genes that have direct impact on root development (Ruzicka et al 2009). Thus, our results do not allow us to draw a clear conclusion about the effect of over production of cytokinin level by over expression of *ATIPT4* gene on root development.

Six independent transgenic plants were regenerated from 100 immature embryos using AGLI:: pBract214::*ATIPT8* CDS construct. However, genotyping (Fig 5.10) showed that all lines were negative for the *ATIPT8* gene

In summary, Three independent transform plants were regenerated from *AtIPT4* construct in T_0. Seeds from 3 lines was independently sown and then 14 plants were obtained from each T_0 line to analysis segregation population of over-expression transgene in T_1 lines. Genotyping showed all regenerated plants were positive for the over-expression transgene, except (1b,1d,1j,1m and1n; from line 1); 2a, 2g, and 2n; from line 2); (3c, 3i, 3l and 3m; From line 3) (Fig 5.11; A, B and C); qRT-PCR then was performed for *ATIPT4* T_1 segregation population (Fig 5.12).

Regulation expression of the IPT gene is an option for altering cytokinin levels and then for improvement of agronomic crops. Increasing endogenous cytokinins have been used to increase quality and yield by enhancing of IPT expression, however this process is dependent on the specificity promoter, developmental stage and environmental conditions (Song *et al.,* 2012). This has been previously demonstrated through increasing the yield /biomass in some important transgenic agronomic crops by IPT expression (Gan and Guo, 2014), including; rice (Hilde-brand *et al.*,1998; Rubia *et al.*, 1999; Cao, 2001; Lin *et al.*, 2002; Xu *et al.*, 2010*a*), wheat (Xi *et al.*, 2004; Xia *et al.*, 2006; Bu *et al.*, 2007) maize (Young *et al.*, 2004) petunia (Jandrew and Clark, 2001; Chang *et al.*, 2003; Clark *et al.*, 2004). The impact of this in barley is as yet not fully determined, however given the apparent delay in whole plant chlorophyll breakdown it will be interesting to conduct further analysis on the cytokinin levels and yield of the *AtIPT4* transgenic lines. According, (Figure 5.13; all lines) Significant differences were detected be-tween lines with respect to chlorophyll content that ($P= 0.005$). Fisher's protected L.S.D testing showed all lines as being significantly higher that wild type.In addi-tion, chlorophyll content was observed to significantly differ over time for all

lines (P<0.001) See table (5.1). On the other hand, different levels of expression were observed for the different T_1 AtIPT4 over-expression lines using qRT-PCR (Fig 5.12E). Generally, it can be seen that have corelation btween *AtIPT4-OEx* lines and (Chlorophyll content); consequently, manipulated of *AtIPT4* gene that have been an important role in cytokinin pathway to produce of cytokinin as results reduce leaf senescence (Takei *et al.,* 2004). Chlorophyll content have been directly correlated with Photosynthesis system in the plant. Therefore, Photosynthesis efficiency was analysed; that no significant differences were detected between lines with respect changed Assimilation rate (P= 0.095), but Fisher's unprotected L.S.D showed the significant differences from wt with line 2 and 3 during pre anthesis stage (See Fig 5.14 and Table 5.2). In addition, during post anthesis stage no significant differences were detected between lines with respect to change Assimilation rate (P= 0.082), but Fisher's unprotected L.S.D showed difference from with line 1 and 2 (See Fig 5.14 and Table 5.3). Thus, leaves will remain photosynthetically active for longer during delay leaf senescence and the flowering period will be extended (Gan and Amasino, 1995; Gan and Amasino, 1996; Bu *et al.*, 2007; Fu *et al.*, 2009*b*; Xu *et al.*, 2010*a*). In addition, extending period of the photosynthetic production is an opportunity to improve plant growth, carbohydrate and material in cereal grain seed yield. Thus, plant delaying the onset senescence can be advantageous for increasing productivity of cereal crops and increased plant biomass, (Verma *et al.,* 2004). Fethuremore, dark and light-induced senescence was investigated for difreent T_1 *ATIPT4* over-expression lines. (Section 5.2.4.3). Different levels of expresion were observed for all *TPT4* over expression lines by qRt-PCR (Fig 5.16); highest expression was seen in Lines 3d and 3f, however, all lines showed increased compared to wild type. It

can be notes that change in chlorophyll content occurring during dark and light - induced senensce for over period (Fig 5.17; 5.18; 5.19 and 5.20) Chlorophyll content steadily decreased during this time for all lines, statistical analysis of dark-induced senecence was demonstrated that no significant differences were seen between transgenic lines and the wild type (P= 0.53). However, chloroghyll content was observed to significantly differ over time for all lines (P<0.001) (See Fig 5.17 and table 5.4).

Howvere, significant differences were detected between lines (P= 0.003) during light-induced senecence and significantly different also was observed over time for all lines (P<0.001) See Fig 5.19 and table 5.5). The intraction between line and time was also signigicat (P= 0.024), but Fisher's protected L.S.D showed that line 1 significantly lower than all lines including wt.

In conclusion, this work suggests that production of endogenous cytokinin can be manipulated by expression of *IPT* gene under different promoters. These studies therefore provide an opportunity to manipulate senescence for potential agricultural applications, to improve of agronomic important traits such as delayed leaf senescence, that it is crucial studied type of senescence due to its impact on quality and quality of yield.

CHAPTER 6: GENERAL CONCLUSIONS AND DISCUSSION

The main aim of this project was to manipulate key traits of agronomic and horticultural importance in crops such Lily (*Lilium* Stargazer*)* and barley (*Hordeum vulgare L.*) using genetic engineering. Manipulation of pollen development (male fertility) and plant senescence (delayed senescence) are two agronomically important traits that were focused on. Agronomically important crops and ornamental plants are critical for economic development in the agricultural and horticultural sector and will impact on life and future generations around the world (Chandrasekaran *et al.,* 2010; Tester and Langridege, 2010), Therefore, targeting crop improvement through biotechnology to control fertility for hybrid seed development, or to reduce damage of horticultural products during senescence are important approach for sustainability of agriculture and human consumption (Subburaj *et al.,* 2016). Genetic manipulation and transcriptomics are providing opportunities for development and improvement food and horticulture production (Subburaj *et al.,* 2016).

6.1 Transformation and analysis of the pollen development pathway in *Lilium.*

Analysis of anther and pollen development pathways in Lily using genes previously identified in *Arabidopsis* were the main goal of this aspect of work. However, transformation and expression analysis using tissue specific promoters were essential for the characterisation of pollen development.

Anther and pollen development pathway in lily can be analysed and manipulated by many approaches. For example, tissue culture and *Agrobacterium*-mediated genetic transformation have become very significant tools for the creation of novel varieties of ornamental cut flowers that are difficult to achieve by traditional breeding (Rommenes and Kishore, 2000; Magori., 2011; Gnasekaran et al 2014). Nevertheless transformation of *Lilium* is challenging, therefore, there is a need to test *Agrobacterium*-mediated transformation efficiency and develop efficient approaches for transformation analysis in *Lilium* using reporter constructs containing GUS and GFP.

In addition, in order to understand the progression of pollen development in lily the length of developing buds and anthers, and the relationship between them was determined over a period of 48 days. Anther development proceeded rapidly until a bud length of 30mm was reached and then the rate decreased, however the anther still continued development and growth, whereas bud length continued the process of rapid development (Figure 3.9). Moreover, microscopy demonstrated that tetrads of haploid microspores were observed in bud lengths of 30 mm and anthers of 18mm; whereas mature pollen was released when the bud length was 120mm and anther 36mm (Fig 3.10). These results enable rapid assessment of development and staging for collection of lily materials for analysis that can be used to investigate the lily anther and pollen pathway with the target of expressing transgene in *Lilium*. pGWB3: OsERFLP and pGWB3:Ubi1 constructs were successfully prepared (Fig 3.15 and 3.16) and used to test for transformation and analyse expression in *Lilium* callus and vegetative tissues. High level of expression, determined by intense blue GUS staining, was observed after 4 days inoculation for AGL1:PGWB3: Ubi1: *GUS* construct. (Fig 3.25 b and c). This construct,

*(*AGL: pBI121: *GUS)* had been previously used by Núñez de Caceres *et al*., (2011) and was used to confirm to confirm successful transient transformation. Transformation efficiencies of 50% and 60%, determined by blue GUS staining, were obtained using the AGL1:PGWB3:Ubi1:*GUS* and AGL:pBI121:*GUS* respectively (Table 3.1). However, previously efficiency of transformation was calculated based on the number of PCR positive plants compared to the number of explants inoculated *(*Núñez de Caceres *et al*., 2011); other reports also suggested a higher efficiency of *Agrobacterium* transformation had been obtained of 20%: nearly 5 times higher that seen here (Ogaki *et al*. 2008).

However, to determine levels of stable transformation, the transformation efficiency must be checked after 11 weeks rather than a few days post inoculation. Therefore, the high levels of transformation observed here are however likely to be the consequence of transient expression rather than stable transformation. This was evidenced by the fact that the putatively transformed basal plates on selection medium started to show browning and necrosis within 5 days, and eventually died after 10 days. This is similar to the situation described by Qiu Hua *et al.,* (2008). It can be note that transformation in *Lilium* is extremely challenging with only a few examples reported (Brand, 2008; Núñez de Caceres *et al*., 2011).

GUS gene expression was observed in leaf *Arabidopsis* plant using the pGWB3:Ubi1 construct (Fig 3.30), demonstrating that the construct was appropriate to drive high levels of gene expression. In summary, this work has demonstrated that these reporter constructs can be used for transformation experiments and that they can drive high levels of expression, nevertheless the transformation

system for lily requires further modification to enable the effective modification of lily.

Biotechnological control of male sterility for Lily was targeted using the *Arabidopsis MYB26* gene that had been previously been shown to be involved in anther dehiscence (Yang et al., 2007b), which is a key trait for horticulturally important plants such as lily. This was proposed as a potential solution to the problem of large amounts of pollen production in *Lilium* flower (Stokes and Forrester, 2004; Fitzgerald 2010 and Thompson 2012). The *pGKGWY:OsERLP:HvMYB26SRDX* construct was prepared with the aim of down-regulating the *Lilium MYB26* ortholog to control anther dehiscence. However, insufficient time was available to test its activity in lily. Further experiment is needed to test the impact of silencing the ortholog of the *MYB26* gene in lily.

Regarding senescence modification in lily plant; seven independent lily putative transformants were obtained using the pGWB5:*ATIPT4*-CDS construct (Fig 3.26). Unfortunately, none of these were not confirmed as transgenic by PCR analysis (Fig 3.27).

Tissue culture and *Agrobacterium-mediated* genetic transformation have become valuable tools for the improvement of a wide variety of horticulture crops (Magori 2011; Gnasekaran *et al.,* 2014). However, the problems encountered in this work regarding transformation of *Lilium* have shown that the methods for manipulation and transformation of *Lilium* are still not straight forward. Nevertheless constructs have been prepared that can be used in future work with *Lilium* and other species, which have particular potential application in Kurdistan and other countries. In the Kurdistan Mountain Region there have been a significant amount of original or-

namental bulb growing, such as lily, tulip and Narcissus. Tissue culture and propagation to improve these ornamental bulbs offers significant opportunities to the horticulture sector, which is essential for increase the economic value of this crop.

6.2 HvDYT1 over-expression in Barley

Controlling the process of pollen development was targeted by over-expression of the *HvDYT1*gene. The *Arabidopsis DYSFUNCTIONAL TAPETUM 1 (DYT1)* gene has been shown to be crucial for tapetum development and viable pollen formation (Zhang *et al.,* 2006 and Feng *et al.,* 2012). The *HvDYT1* gene is a putative ortholog of the *Arabidopsis DYT1* gene that was identified in barley by Dr Jose Fernandez Gomez, (unpublished data), which is thought to be involved in anther and pollen development. This gene was analysed by phenotypic analysis of development by constitutive *HvDYT1* overexpression in transgenic lines under the control of the ubiquitin1 promoter.

HvDYT1 expression pattern in barley optic variety was identified at different stage of flower (B - LEF3) based upon the staging system of Gomez and Wilson (2012). The results presented in Chapter 4 indicate that high levels of *HvDYT1* expression were seen at Stage B (0-1cm) primary sporogenous stage, these were slightly lower at stage D (2-3cm) PMC meiosis and again expressed at a higher level during Stage F (4-5cm) free microspore stage (Fig 4.3). However, this *HvDYT1* expression pattern in barley is slightly different from the *Arabidopsis* expression pattern that is reported by Zhang *et al.,* (2006); *AtDYT1* is highly expressed during stage 5 to 6 in the tapetum and at a lower level in the meiocytes. In addition, the rice ortholog, *UNDEVELOPED TAPETUM1* (*OsUDT1*), was highly expressed in the

anther at the start of meiosis stage and then the expression level became lower during the later stages (Jung *et al.,* 2005). Therefore the *OsUDT1*expression pattern was also different from the *HvDYT1* expression pattern in barley. Nevertheless the data generated indicates a clear role for this bHLH transcription factor, *HvDYT1,* during pollen development. Further work to confirm that this is in fact the ortholog of the *Arabidopsis DYT1* gene could entail complementation of the *Arabidopsis dyt1* mutant with the barley CDS under the control of the native *ATDYT1* promoter. Additional analysis of the tissue specific expression of the *HvDYT1* gene could be obtained via *in situ* hybridisation to anther sections.

Golden promise *HvDYT1* over-expression T_0 and T_1 lines showed a completely sterile phenotype (Fig 4.7). A similar result of complet male sterility was observed by Gomez and Wilson (2011) in transgenic barley lines overexpressing the *HvMS1* tapetum-specific transcription factor. This was also observed in some *Arabidopsis MS1* over-expression lines (Yang et al 2007). Relationships between male sterility with transgenic copy number and *HvDYT1* over expression were linked in this study in the T_0 generation. However in the T_1 generation this correlation was no longer apparent with altered fertility directly related to levels of over-expression of the *HvDYT1* gene rather than the transgenic copy number (Fig 4.9 and 4.10). Copy number cannot be used as a direct indicator of expression level, particularly in subsequent generations. However, analysis and determination of copy number knowledge can be very useful for identifying low-copy number lines for future experimentation and subsequent transgenic lines applications (Shepherd *et al.,* 2009). As demonstrated the relationship between copy number and sterility were variable and this may at least in part be a reflection that the PCR analysis of copy number was based on amplification of the hygromycin gene that

may be related to the amplify Hg gene in the genome of transformed plant rather than the *DYT1* transgene.

We can therefore conclude that male sterile lines can be created by over-expression of *HvDYT1* without affected the development of the whole plant. Therefore, the molecular analysis of the *HvDYT1* gene may provide a tool to analyse anther and pollen development in agronomically important crops and can be facilitated for the future control of crop fertility; to produce low environmental impact genetically engineered plants by control of release of genetically modified (GM) pollen and production hybrid seed.

6.3 Senescence modification by alteration of the levels of cytokinin using over-expression of *AtIPT4* in barley

Over-expression of the *ATIPT4* gene was used to try to change cytokinin levels to try to delay leaf and flower senescence in barley and lily. A key cytokinin biosynthetic enzyme in plants is *IPT* gene family in *Arabidopsis* (Kieber and Schaller, 2014); molecular study of the IPT gene provide a way to manipulated senescence for potential agricultural applications, particularly to improve of agronomic important traits such as delay leaf senescence, which can impact on quality and quality of crops (Gan, and Guo, 2014). Over-expression of the *IPT* gene in transgenic plants can cause an increase in the level of cytokinins and reduce leaf senescence (Can and Amsino, 1996).

Three transgenic barley plants were obtaned from *Agrobacterium*-transformation with the AGLI::pBract214::*ATIPT4*CDS construct (Fig 5.5); for the first trans-

formation a 3% transformation efficiency was observed, based on Harwood *et al.*, (2009). However, a subsequent transformation generated more transgenic lines with a higher transformation efficiency of 24%. All 24 lines genotyped during regeneration medium were shown to be transgenic (Fig 5.7) and 5 plants among them were confirmed by RT-PCR and qRT-PCR as having transgene expression (Fig 5.9). However, unfortunately during the regeneration protocol, roots were not initiated for many of these transgenics and eventually these plantlets died after being transferred to soil.

The lack of root development was proposed as the result of overproduction of cytokinin, which may have caused repression of IAA and auxin levels and has been observed in other systems (Medford et al., 1989; Kuderova et al 2008; Ruzicka et al 2009). Different concentration of IAA (Section 5.3.2.1.2) were used to try to initiate root formation in the transgenic explants; nevertheless no significant root formation was observed. This may be a consequence of the lack of precise levels of cytokinin due to over-expression of the ATIPT*4* gene and the requirement for specific combinations of cytokinin and auxin concentrations at key stages of development for normal root formation (Ruzicka et al 2009). In order to further understand the reason behind this qRT-PCR was performed to check the level of transgene expression for the 3 lines with root (line 1,2,3) and the 5 lines without root initiation (lines1a - 5a) (Fig 5.9E). Nevertheless, no clear correlation could be observed and it is currently not possible to make any clear conclusions about the effect of over production of cytokinin level by over expression of *ATIPT4* gene on root development.

In addition, T_1 segregation populations of ATIPT4 over-expression transgene lines were obtained from 3 T_0 lines that were able to root and grow normally; Genotyping was confirmed (Fig 5.11; A, B and C); qRT-PCR then was performed for *ATIPT4* T_1 segregation population (Fig 5.12).

One choice for improvement of agronomic crops by altering cytokinin levels is regulation expression of the *IPT* gene. However, this process is dependent on the specificity promoter, developmental stage and environmental conditions (Song *et al.,* 2012). Previous research demonstrated that increasing the quality and quantity of a large range of crops have been successfully achieved by manipulated of IPT gene (Gan and Guo, 2014),including; rice (Hildebrand *et al.*,1998;), wheat (Xi *et al.*, 2004;) maize (Young *et al.*, 2004).The impact of this in barley is as yet not fully determined. However, given the apparent delay in whole plant chlorophyll breakdown it will be interesting to conduct further analysis on the cytokinin levels and yield of the *AtIPT4* transgenic lines.

A clear correlation is evident between *IPT4* expression in the *AtIPT4-OEx* lines and chlorophyll content; consequently over-expression of *AtIPT4* gene appeareds to result in the delay of senescence by impacting on senescence progression possibly via manipulation of the cytokinin pathway (Takei *et al.,* 2004). A correlation between *AtIPT4-OEx* lines and prolonged leaf green (Chlorophyll content) was shown (Fig 5.13). Statistical analysis of chlorophyll content was observed to significantly differ over time for all lines ($P<0.001$: see Table (5.1). Significant difference were also detected between lines with respect to chlorophyll content ($P= 0.005$). Fisher's protected L.S.D testing showed all lines were significantly higher that wild type.

Furthermore, to confirm the impact on senescence photosynthesis efficiency was analysed (Fig 5.14). No significant differences were detected between lines with respect to changed Assimilation rate (P= 0.095), but Fisher's unprotected L.S.D showed the significant difference from wt were observed with line 2 and 3 during pre-anthesis stage (see Table 5.2). No significant differences were also detected between lines with respect to change Assimilation rate (P= 0.082), but Fisher's unprotected L.S.D showed significant difference from wt were observed with line 1 and 2 during post-anthesis stage (see Table 5.3) Thus, it appears that chlorophyll content is directly correlated with Photosynthesis efficiency and the leaves remain photosynthetically active for longer and thus during delay leaf senescence and the flowering period will be extended (Gan and Amasino, 1995; Gan and Amasino, 1996; Bu *et al.*, 2007; Fu *et al.*, 2009*b*; Xu *et al.*, 2010*a*). Extending the period of the photosynthetic production is an opportunity to increasing productivity of cereal crops and increased plant biomass, (Verma *et al.*, 2004).

Nevertheless, no significant changes in chlorophyll content during dark-induced senensce were seen between the transgenic lines and the wild type. (P= 0.53). However, chlorophyll content was observed to significantly differ over time for all lines (P<0.001) (See Fig 5.17 and table 5.4). Significant differences were also detected between lines (P= 0.003) during light-induced senecence and were also observed over time for all lines (P<0.001; see Fig 5.19 and table 5.5). The interaction between line and time was also significant (P= 0.024), but Fisher's protected L.S.D showed that line 1 significantly lower than all lines including wt.

The ability to manipulate senescence is an important trait for the agriculture and horticultural industry. Stay-green plants are genotypes, either mutant or transgen-

ic, which possess elongated leaf lifespan relative to wild type (Kusaba et al., 2013). This can be achieved by many means, for example manipulation of cytokinin signalling pathways or by manipulation of Family of SGR genes. Genetic and molecular analysis revealed that stay-green plant have provide significant opportunities to improve crops, in order to extend the period of time they are photosynthetically active, which could ultimately increase crop productivity (Thomas and Howarth 2000).

Stay-green traits can be classified in to five types by Thomas and Howarth (2000) based on chlorophyll behaviour during senescence. Functional stay-green can be classified in two type (A and B); in type A lose pigment and function was seen when senescence initiate late but the processed at a normal rate, but in type B subsequently proceeds more slowly when senescence began on schedule. Non-functional stay green can be classified in to three types. Type (C) is cosmetic stay-green chlorophyll may be retained more or less indefinitely. Type (D) is pseudo stay-green results in leaf death. Type (E) is hyper-greens, a high chlorophyll content is accumulated without the photosynthetic capacity is increased. In addition, increased chlorophyll content and photosynthetic capacity were represented for leaves or whole canopy of plants (Thomas and Howarth 2000; kusaba *et al.*, 2013).

Functional stay-green mutants have the potential to increase plant productivity by delayed initiation of leaf senescence, or a slowed rate of leaf senescence (Thomas and Howarth 2000; Luche *et al.*, 2015). Agronomically these stay-green traits are interesting because prolonged leaf chlorophyll can be achieved by delay senescence and photosynthetic capacity by speed of senescence progression might have

an advantage effect to increase quality and quantity of yield in cereal crops such as barley and wheat (Thomas and Howarth 2000; Luche *et al.,* 2015). However, cosmetic stay-green, in which only chlorophyll degrading is delayed, may be useful for ornamental plants because of degradation of chlorophyll during senescence has major role to determining the quality of harvest food and ornamental plants (Thomas and Howarth, 2000; Barry, 2009; Luche *et al.*, 2015)

In conclusion, this work suggests that production of endogenous cytokinin can be manipulated by expression of *IPT* gene under different promoters. These studies therefore provide an opportunity to manipulate senescence for potential agricultural applications, to improve of agronomic important traits such as delayed leaf senescence, that it is crucial studied type of senescence due to its impact on quality and quality of yield. However, given the secondary impacts observed from ectopic overexpression of *IPT4* through-out the plant, future work would be best targeting specific expression of IPT genes using tissue-specific or late-acting/senescence-associated promoters that particularly target the reproductive tissues.

6.4 General recommendation and future work.

A number of areas of work are needed to confirm the data suggested in this thesis: -

1- Several constructs such as (pGWB3::OsERFLP, PGWB3::Ubi1, PGWB3::CaMV35S, pGWB4::OsERFLp; pGWB4::LCHS and pGKGWY:: OsERLP::*HvMYB26SRDX*) were prepared can be used for future experiments related to anther and pollen development in horticultural agronomical important crops.

2- More analysis could be done on the barley *HvDYT1* over-expression lines regarding the cross to a HvDYT1RNAi silencing (Fernandez Gomez, unpublished) this will enable analysis of whether reducing the DYT1 over-expression allows the plants to recover pollen formation and thus male fertility.

3- Check different conditions to check whether the sterile phenotype depends on growing conditions or not.

4- Progression of senescence; the expression of the *ATIPT4* gene could also be used under the control of different promoter to alter cytokinin levels.

5- In senescence expremnt, could be used other transformation strategy such as silencing using CRISPR, or manipulate of other genes that is realted to cytokinin pathway.

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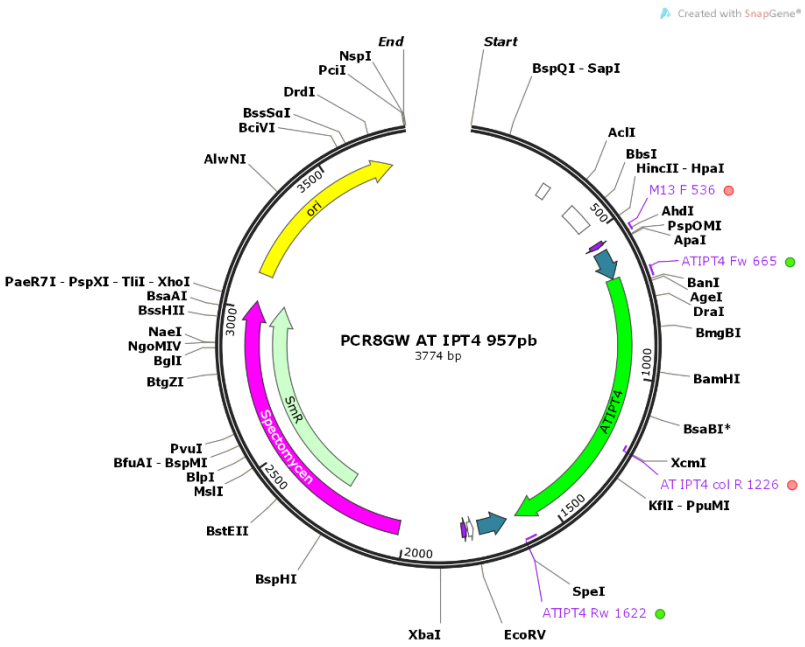
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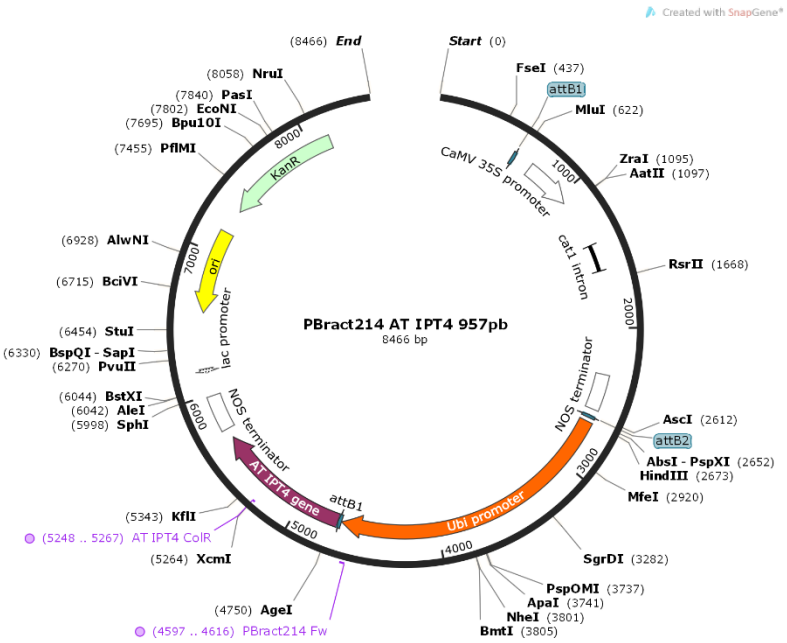
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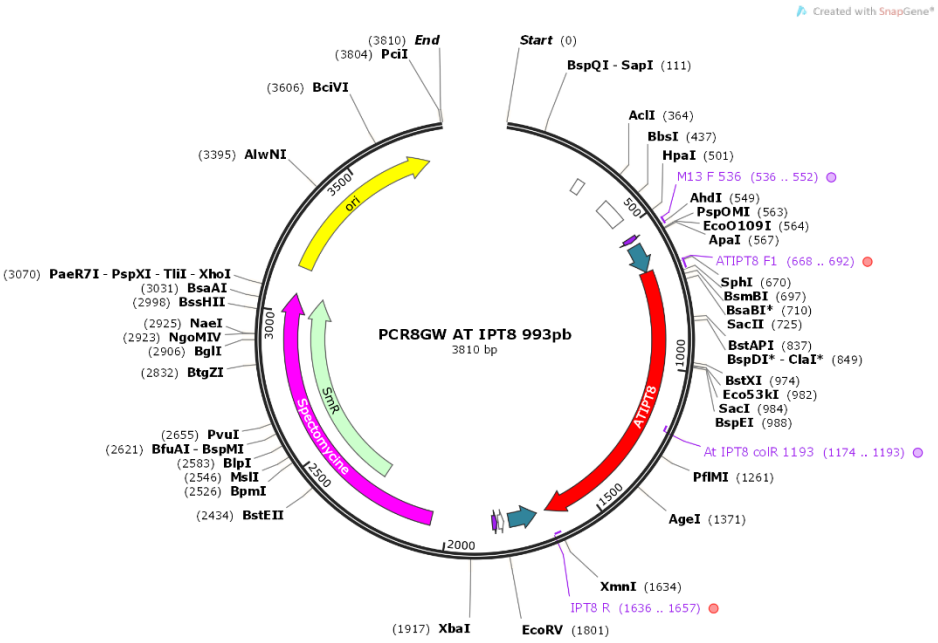
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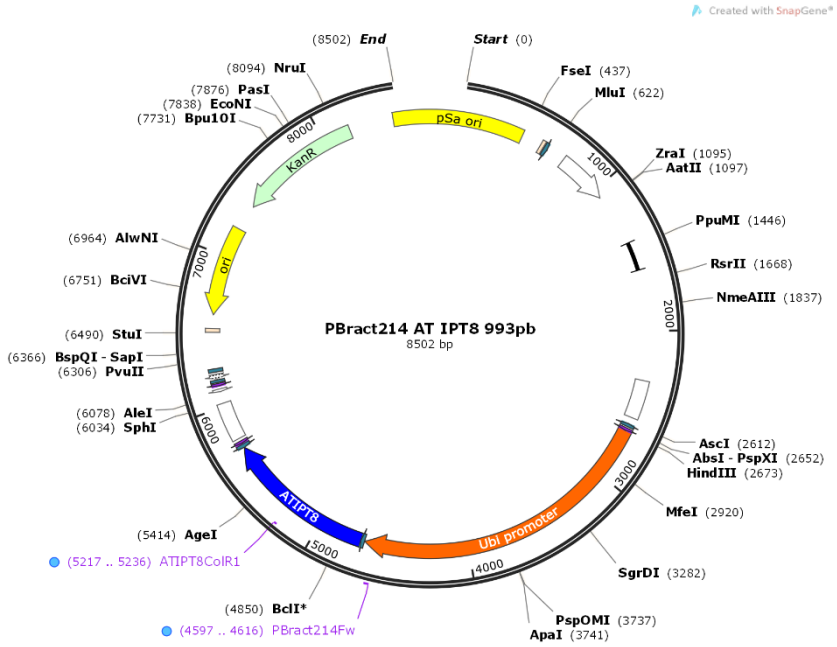
APPENDICES

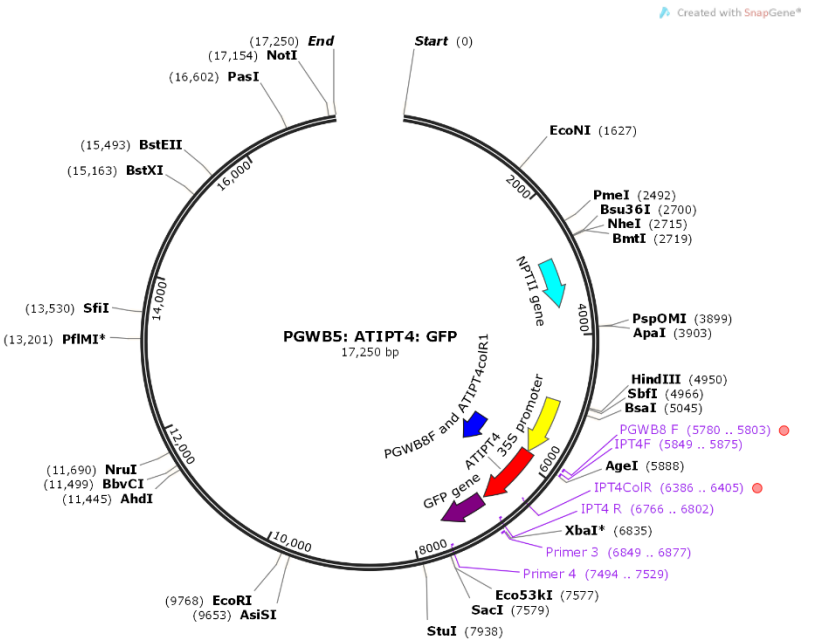
APPENDIX 1 T-DNA MAPS

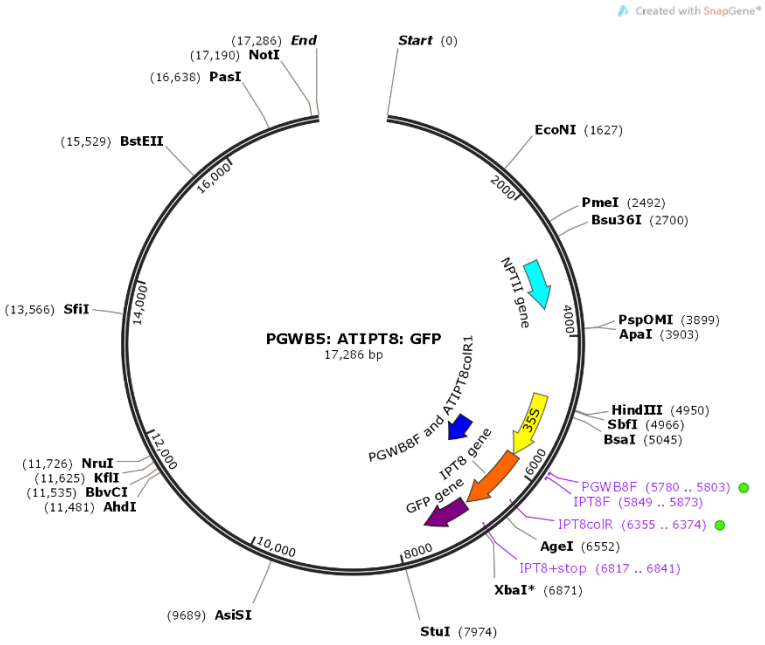


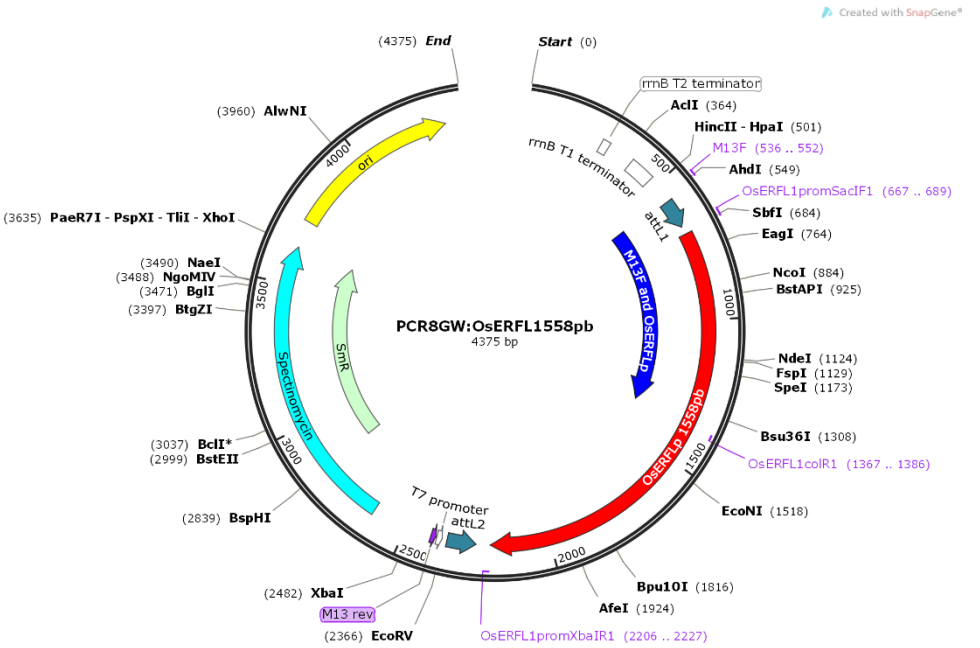


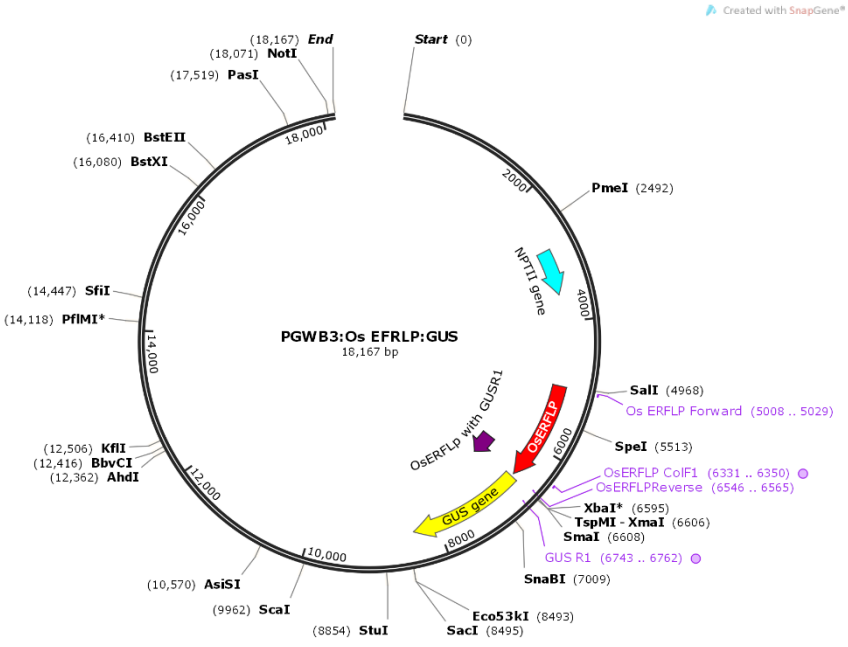

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(3031) BsaAI
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(2546) MslI
(2526) BpmI
(2434) BstEII
(1917) XbaI
EcoRV (1801)
XmnI (1634)
IPT8 R (1636 .. 1657)
PflMI (1261)
AgeI (1371)
At IPT8 colR 1193 (1174 .. 1193)
BstXI (974)
Eco53kI (982)
SacI (984)
BspEI (988)
BstAPI (837)
BspDI* - ClaI* (849)
SacII (725)
BsmBI (697)
BsaBI* (710)
SphI (670)
ATIPT8 F1 (668 .. 692)
AhdI (549)
PspOMI (563)
EcoO109I (564)
ApaI (567)
M13 F 536 (536 .. 552)
HpaI (501)
BbsI (437)
AclI (364)
Start (0)
BspQI - SapI (111)
PCR8GW AT IPT8 993pb
3810 bp
ori
SmR
Spectomycine
ATIPT8
500
1000
1500
2000
2500
3000
3500

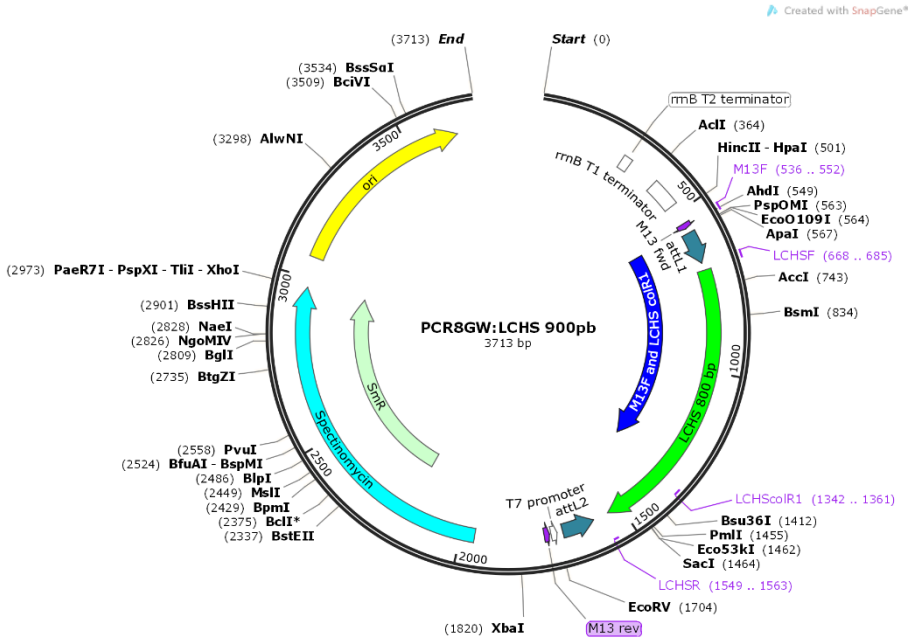

Created with SnapGene®
(8502) End
(8094) NruI
(7876) PasI
(7838) EcoNI
(7731) Bpu10I
(6964) AlwNI
(6751) BciVI
(6490) StuI
(6366) BspQI - SapI
(6306) PvuII
(6078) AleI
(6034) SphI
(5414) AgeI
(5217 .. 5236) ATIPT8ColR1
(4850) BclI*
(4597 .. 4616) PBract214Fw
PspOMI (3737)
ApaI (3741)
SgrDI (3282)
MfeI (2920)
HindIII (2673)
AbsI - PspXI (2652)
AscI (2612)
NmeAIII (1837)
RsrII (1668)
PpuMI (1446)
AatII (1097)
ZraI (1095)
MluI (622)
FseI (437)
Start (0)
PBract214 AT IPT8 993pb
8502 bp
pSa ori
KanR
ori
ATIPT8
Ubi promoter
1000
2000
3000
4000
5000
6000
7000
8000

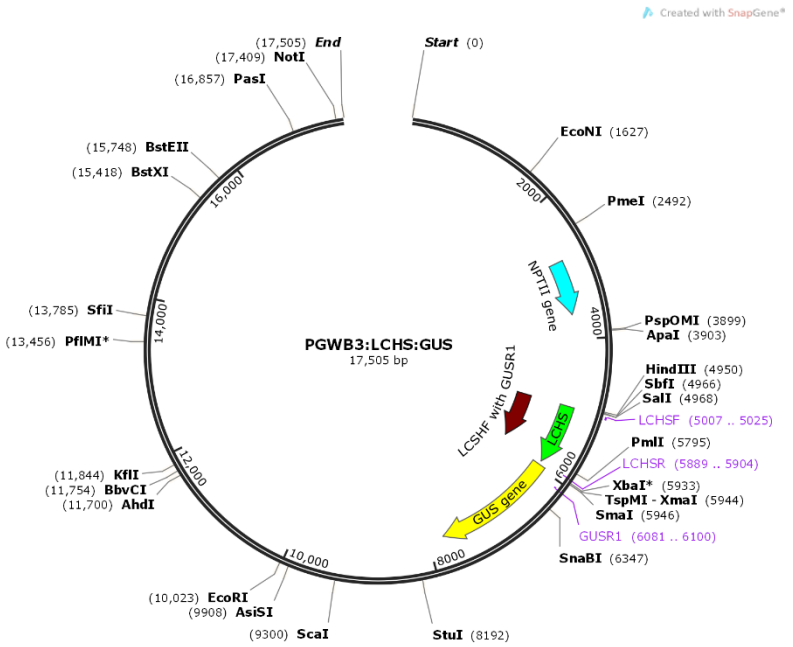

Created with SnapGene®
PGWB5: ATIPT4: GFP
17,250 bp
(17,250) End
Start (0)
(17,154) NotI
(16,602) PasI
(15,493) BstEII
(15,163) BstXI
EcoNI (1627)
PmeI (2492)
Bsu36I (2700)
NheI (2715)
BmtI (2719)
NPTII gene
PspOMI (3899)
ApaI (3903)
(13,530) SfiI
(13,201) PflMI*
HindIII (4950)
SbfI (4966)
BsaI (5045)
PGWB8F and ATIPT4colR1
ATIPT4
35S promoter
GFP gene
PGWB8 F (5780 .. 5803)
IPT4F (5849 .. 5875)
AgeI (5888)
IPT4ColR (6386 .. 6405)
IPT4 R (6766 .. 6802)
XbaI* (6835)
Primer 3 (6849 .. 6877)
Primer 4 (7494 .. 7529)
(11,690) NruI
(11,499) BbvCI
(11,445) AhdI
Eco53kI (7577)
SacI (7579)
(9768) EcoRI
(9653) AsiSI
StuI (7938)

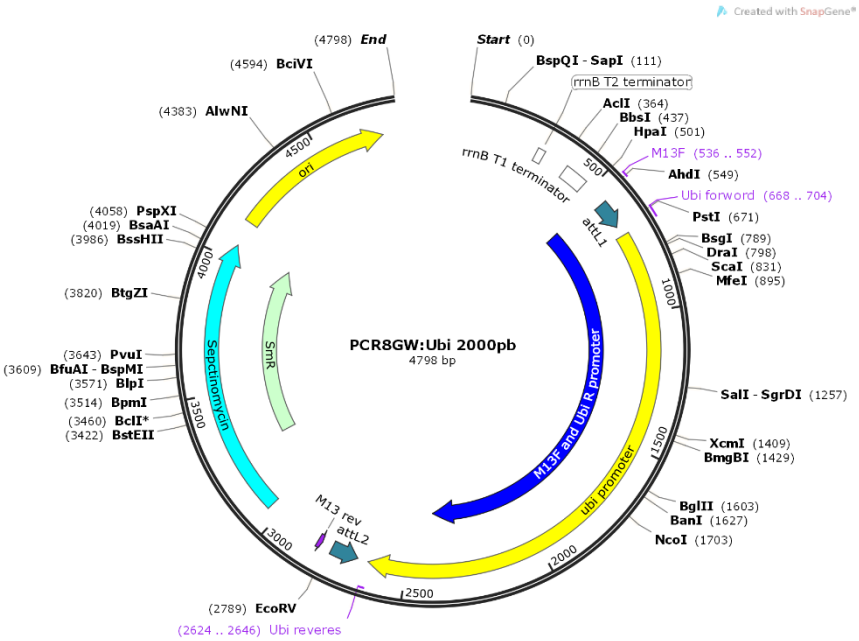

Created with SnapGene®
PGWB5: ATIPT8: GFP
17,286 bp
(17,286) End
Start (0)
(17,190) NotI
(16,638) PasI
(15,529) BstEII
EcoNI (1627)
PmeI (2492)
Bsu36I (2700)
NPTII gene
PspOMI (3899)
ApaI (3903)
(13,566) SfiI
HindIII (4950)
SbfI (4966)
BsaI (5045)
PGWB8F and ATIPT8colR1
IPT8 gene
35S
GFP gene
PGWB8F (5780 .. 5803)
IPT8F (5849 .. 5873)
IPT8colR (6355 .. 6374)
AgeI (6552)
IPT8+stop (6817 .. 6841)
XbaI* (6871)
(11,726) NruI
(11,625) KflI
(11,535) BbvCI
(11,481) AhdI
(9689) AsiSI
StuI (7974)

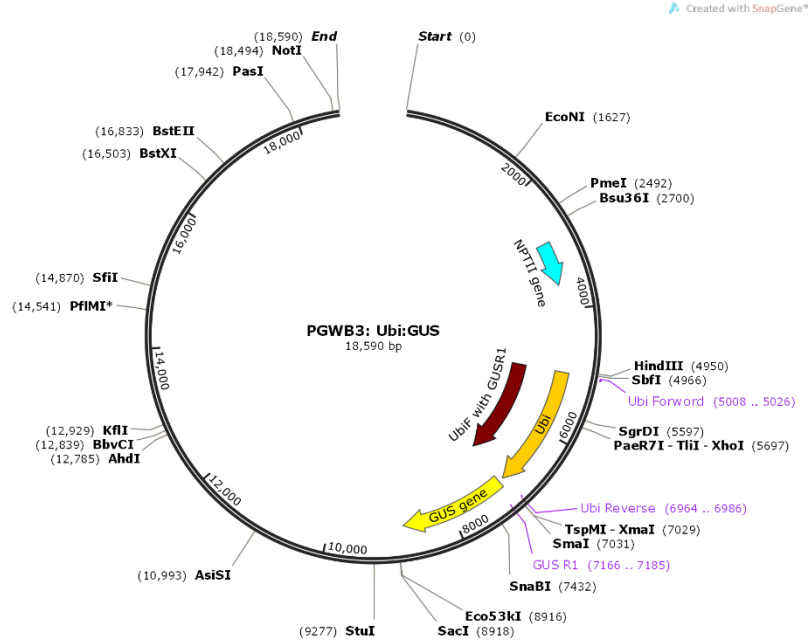

Created with SnapGene®
(4375) End
Start (0)
rrnB T2 terminator
AclI (364)
HincII - HpaI (501)
M13F (536 .. 552)
AhdI (549)
OsERFL1promSacIF1 (667 .. 689)
SbfI (684)
EagI (764)
NcoI (884)
BstAPI (925)
NdeI (1124)
FspI (1129)
SpeI (1173)
Bsu36I (1308)
OsERFL1colR1 (1367 .. 1386)
EcoNI (1518)
Bpu10I (1816)
AfeI (1924)
OsERFL1promXbaIR1 (2206 .. 2227)
(2366) EcoRV
M13 rev
(2482) XbaI
(2839) BspHI
T7 promoter
attL2
(2999) BstEII
(3037) BclI*
Spectinomycin
SmR
(3397) BtgZI
(3471) BglI
(3488) NgoMIV
(3490) NaeI
(3635) PaeR7I - PspXI - TliI - XhoI
(3960) AlwNI
ori
rrnB T1 terminator
attL1
M13F and OsERFLP
OsERFLP 1558pb
PCR8GW:OsERFL1558pb
4375 bp
4000
500
1000
1500
2000
2500
3000
3500

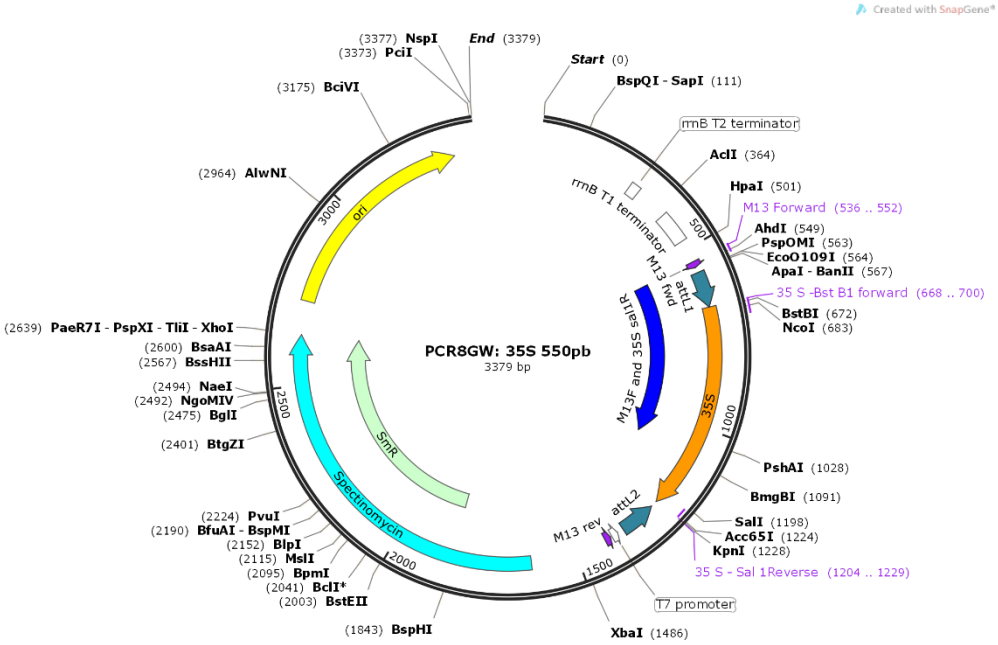

Created with SnapGene®
(18,167) End
Start (0)
(18,071) NotI
(17,519) PasI
(16,410) BstEII
(16,080) BstXI
(14,447) SfiI
(14,118) PflMI*
(12,506) KflI
(12,416) BbvCI
(12,362) AhdI
(10,570) AsiSI
(9962) ScaI
(8854) StuI
Eco53kI (8493)
SacI (8495)
SnaBI (7009)
GUS R1 (6743 .. 6762)
SmaI (6608)
TspMI - XmaI (6606)
XbaI* (6595)
OsERFLPReverse (6546 .. 6565)
OsERFLP ColF1 (6331 .. 6350)
SpeI (5513)
Os ERFLP Forward (5008 .. 5029)
SalI (4968)
PmeI (2492)
NPTII gene
OsERFLp with GUSR1
OsERFLP
GUS gene
PGWB3:Os EFRLP:GUS
18,167 bp
2000
4000
6000
8000
10,000
12,000
14,000
16,000
18,000

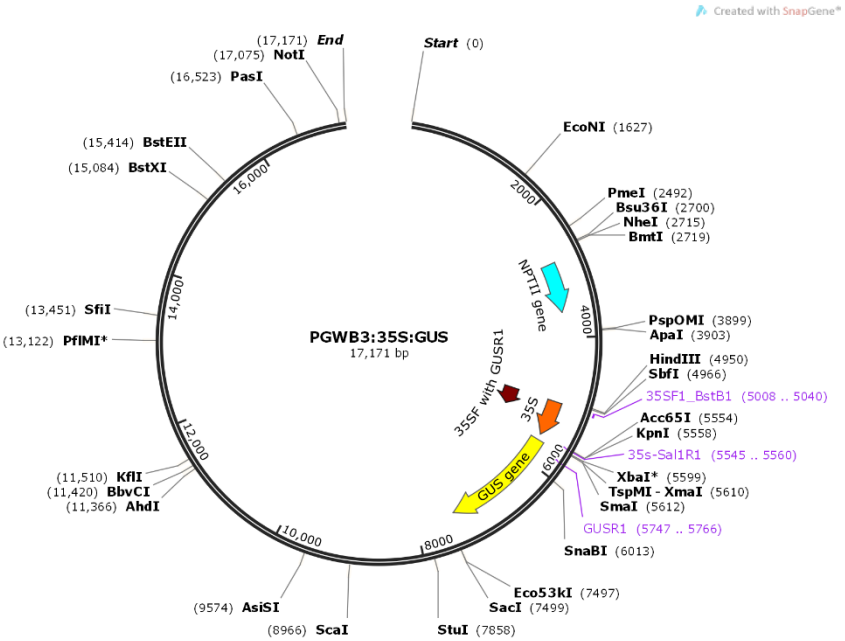

Created with SnapGene®
PCR8GW:LCHS 900pb
3713 bp
(3713) End
Start (0)
(3534) BssSαI
(3509) BciVI
(3298) AlwNI
(2973) PaeR7I - PspXI - TliI - XhoI
(2901) BssHII
(2828) NaeI
(2826) NgoMIV
(2809) BglI
(2735) BtgZI
(2558) PvuI
(2524) BfuAI - BspMI
(2486) BlpI
(2449) MslI
(2429) BpmI
(2375) BclI*
(2337) BstEII
(1820) XbaI
rrnB T2 terminator
AclI (364)
HincII - HpaI (501)
M13F (536 .. 552)
AhdI (549)
PspOMI (563)
EcoO109I (564)
ApaI (567)
LCHSF (668 .. 685)
AccI (743)
BsmI (834)
rrnB T1 terminator
M13 fwd
attL1
M13F and LCHS coding
LCHS 900 bp
T7 promoter
attL2
LCHScolR1 (1342 .. 1361)
Bsu36I (1412)
PmlI (1455)
Eco53kI (1462)
SacI (1464)
LCHSR (1549 .. 1563)
EcoRV (1704)
M13 rev
ori
SmR
Spectinomycin
500
1000
1500
2000
2500
3000
3500

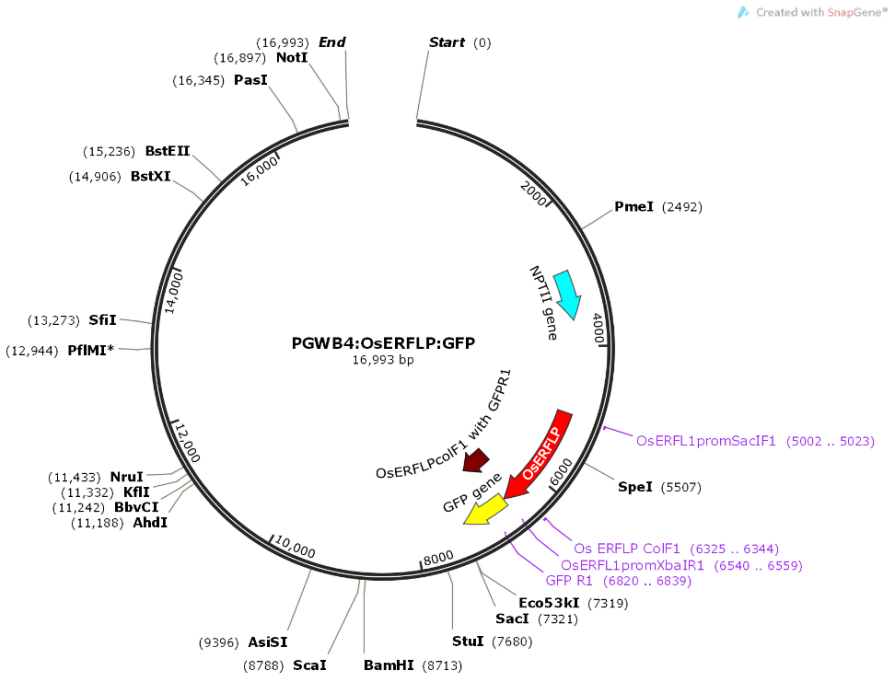

Created with SnapGene®
PGWB3:LCHS:GUS
17,505 bp
(17,505) End
Start (0)
(17,409) NotI
(16,857) PasI
(15,748) BstEII
(15,418) BstXI
(13,785) SfiI
(13,456) PflMI*
(11,844) KflI
(11,754) BbvCI
(11,700) AhdI
(10,023) EcoRI
(9908) AsiSI
(9300) ScaI
StuI (8192)
EcoNI (1627)
PmeI (2492)
PspOMI (3899)
ApaI (3903)
HindIII (4950)
SbfI (4966)
SalI (4968)
LCHSF (5007 .. 5025)
PmlI (5795)
LCHSR (5889 .. 5904)
XbaI* (5933)
TspMI - XmaI (5944)
SmaI (5946)
GUSR1 (6081 .. 6100)
SnaBI (6347)
NPTII gene
LCSHF with GUSR1
LCHS
GUS gene
2,000
4,000
6,000
8,000
10,000
12,000
14,000
16,000

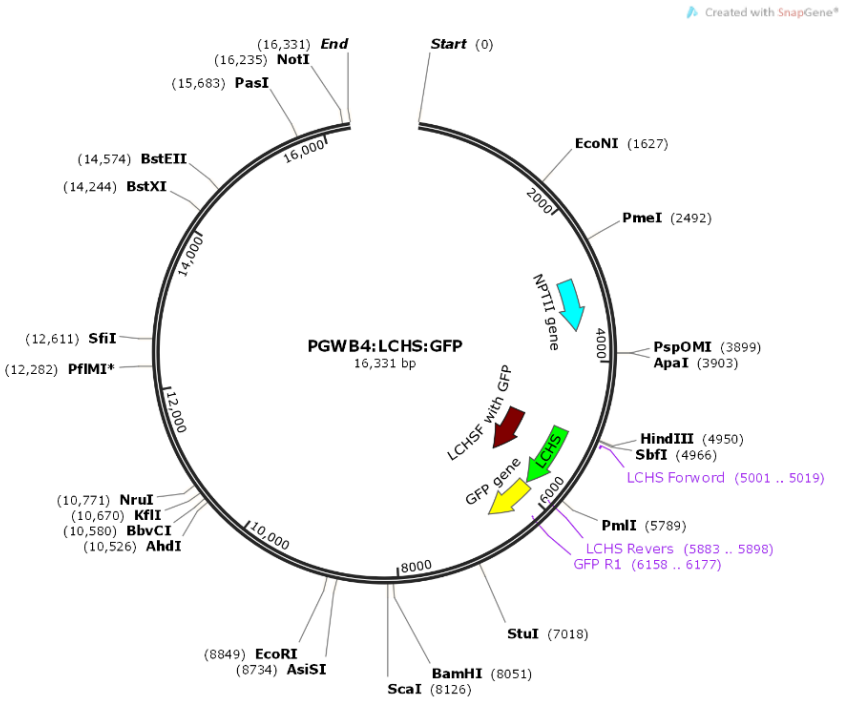

Created with SnapGene®
PCR8GW:Ubi 2000pb
4798 bp
(4798) End
Start (0)
(4594) BciVI
BspQI - SapI (111)
(4383) AlwNI
rrnB T2 terminator
AclI (364)
BbsI (437)
HpaI (501)
M13F (536 .. 552)
rrnB T1 terminator
AhdI (549)
Ubi forward (668 .. 704)
PstI (671)
attL1
BsgI (789)
DraI (798)
ScaI (831)
MfeI (895)
(4058) PspXI
(4019) BsaAI
(3986) BssHII
(3820) BtgZI
(3643) PvuI
(3609) BfuAI - BspMI
(3571) BlpI
(3514) BpmI
(3460) BclI*
(3422) BstEII
ori
SmR
Spectinomycin
M13F and Ubi R promoter
ubi promoter
SalI - SgrDI (1257)
XcmI (1409)
BmgBI (1429)
BglII (1603)
BanI (1627)
NcoI (1703)
M13 rev
attL2
(2789) EcoRV
(2624 .. 2646) Ubi reveres
4500
4000
500
1000
1500
2000
2500
3000
3500

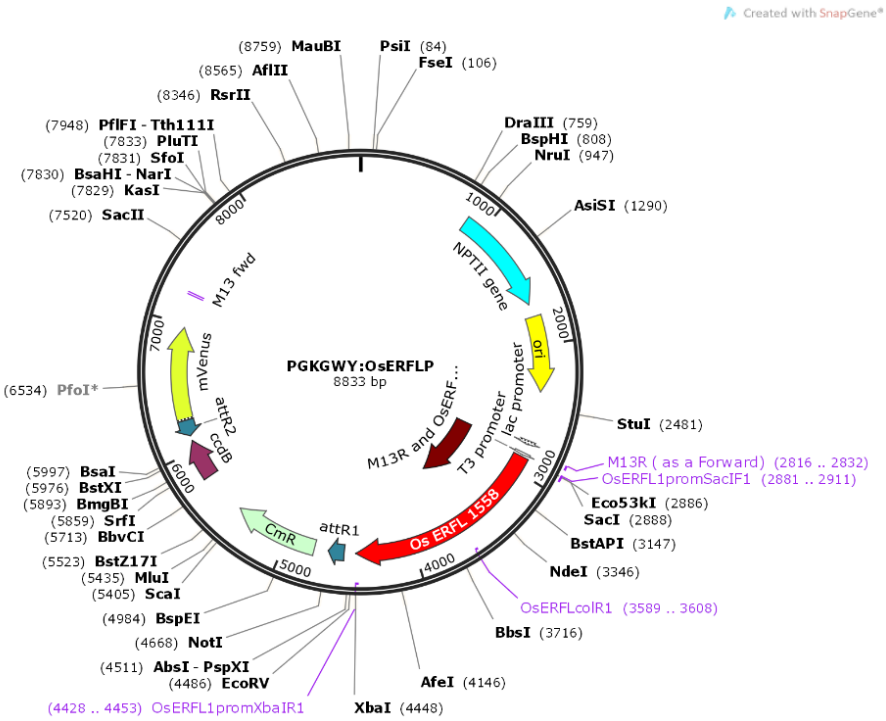

Created with SnapGene®
PGWB3: Ubi:GUS
18,590 bp
(18,590) End
Start (0)
(18,494) NotI
(17,942) PasI
EcoNI (1627)
(16,833) BstEII
(16,503) BstXI
PmeI (2492)
Bsu36I (2700)
NPTII gene
(14,870) SfiI
(14,541) PflMI*
HindIII (4950)
SbfI (4966)
Ubi Forward (5008 .. 5026)
UbiF with GUSR1
Ubi
SgrDI (5597)
PaeR7I - TliI - XhoI (5697)
(12,929) KflI
(12,839) BbvCI
(12,785) AhdI
GUS gene
Ubi Reverse (6964 .. 6986)
TspMI - XmaI (7029)
SmaI (7031)
GUS R1 (7166 .. 7185)
SnaBI (7432)
(10,993) AsiSI
(9277) StuI
Eco53kI (8916)
SacI (8918)
2000
4000
6000
8000
10,000
12,000
14,000
16,000
18,000

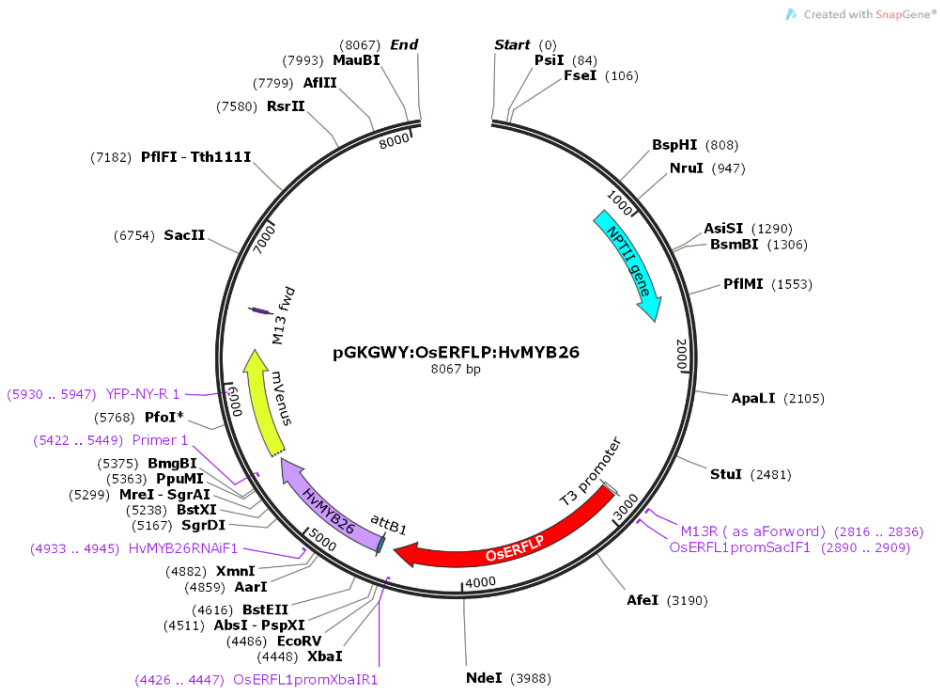

Created with SnapGene®
(3377) NspI
(3373) PciI
End (3379)
Start (0)
(3175) BciVI
BspQI - SapI (111)
rrnB T2 terminator
(2964) AlwNI
AclI (364)
rrnB T1 terminator
HpaI (501)
M13 Forward (536 .. 552)
AhdI (549)
PspOMI (563)
EcoO109I (564)
ApaI - BanII (567)
35 S -Bst B1 forward (668 .. 700)
BstBI (672)
NcoI (683)
(2639) PaeR7I - PspXI - TliI - XhoI
(2600) BsaAI
(2567) BssHII
(2494) NaeI
(2492) NgoMIV
(2475) BglI
(2401) BtgZI
PCR8GW: 35S 550pb
3379 bp
ori
SmR
Spectinomycin
M13F and 35S seq1R
M13 fwd
attL1
35S
PshAI (1028)
BmgBI (1091)
SalI (1198)
Acc65I (1224)
KpnI (1228)
35 S - Sal 1Reverse (1204 .. 1229)
M13 rev
attL2
T7 promoter
XbaI (1486)
(2224) PvuI
(2190) BfuAI - BspMI
(2152) BlpI
(2115) MslI
(2095) BpmI
(2041) BclI*
(2003) BstEII
(1843) BspHI


Created with SnapGene®
(17,171) End
(17,075) NotI
(16,523) PasI
Start (0)
EcoNI (1627)
(15,414) BstEII
(15,084) BstXI
PmeI (2492)
Bsu36I (2700)
NheI (2715)
BmtI (2719)
NPTII gene
(13,451) SfiI
(13,122) PflMI*
PspOMI (3899)
ApaI (3903)
PGWB3:35S:GUS
17,171 bp
HindIII (4950)
SbfI (4966)
35SF1_BstB1 (5008 .. 5040)
35SF with GUSR1
35S
Acc65I (5554)
KpnI (5558)
35s-Sal1R1 (5545 .. 5560)
GUS gene
XbaI* (5599)
TspMI - XmaI (5610)
SmaI (5612)
GUSR1 (5747 .. 5766)
SnaBI (6013)
(11,510) KflI
(11,420) BbvCI
(11,366) AhdI
Eco53kI (7497)
SacI (7499)
(9574) AsiSI
(8966) ScaI
StuI (7858)


Created with SnapGene®
(16,993) End
Start (0)
(16,897) NotI
(16,345) PasI
(15,236) BstEII
(14,906) BstXI
PmeI (2492)
NPTII gene
(13,273) SfiI
(12,944) PflMI*
PGWB4:OsERFLP:GFP
16,993 bp
OsERFLPcolF1 with GFPR1
OsERFLP
OsERFL1promSacIF1 (5002 .. 5023)
GFP gene
SpeI (5507)
(11,433) NruI
(11,332) KflI
(11,242) BbvCI
(11,188) AhdI
Os ERFLP ColF1 (6325 .. 6344)
OsERFL1promXbaIR1 (6540 .. 6559)
GFP R1 (6820 .. 6839)
Eco53kI (7319)
SacI (7321)
(9396) AsiSI
StuI (7680)
(8788) ScaI
BamHI (8713)


Created with SnapGene®
(16,331) End
Start (0)
(16,235) NotI
(15,683) PasI
EcoNI (1627)
(14,574) BstEII
(14,244) BstXI
PmeI (2492)
NPTII gene
(12,611) SfiI
(12,282) PflMI*
PGWB4:LCHS:GFP
16,331 bp
PspOMI (3899)
ApaI (3903)
LCHSF with GFP
LCHS
HindIII (4950)
SbfI (4966)
GFP gene
LCHS Forword (5001 .. 5019)
(10,771) NruI
(10,670) KflI
(10,580) BbvCI
(10,526) AhdI
PmlI (5789)
LCHS Revers (5883 .. 5898)
GFP R1 (6158 .. 6177)
StuI (7018)
(8849) EcoRI
(8734) AsiSI
BamHI (8051)
ScaI (8126)


Created with SnapGene®
PGKGWY:OsERFLP
8833 bp
(8759) MauBI
PsiI (84)
FseI (106)
(8565) AflII
(8346) RsrII
(7948) PflFI - Tth111I
(7833) PluTI
(7831) SfoI
(7830) BsaHI - NarI
(7829) KasI
(7520) SacII
DraIII (759)
BspHI (808)
NruI (947)
AsiSI (1290)
NPTII gene
ori
lac promoter
T3 promoter
M13R and OsERF...
Os ERFL 1558
attR1
CmR
ccdB
attR2
mVenus
M13 fwd
StuI (2481)
M13R (as a Forward) (2816 .. 2832)
OsERFL1promSacIF1 (2881 .. 2911)
Eco53kI (2886)
SacI (2888)
BstAPI (3147)
NdeI (3346)
OsERFLcoIR1 (3589 .. 3608)
BbsI (3716)
AfeI (4146)
XbaI (4448)
(4428 .. 4453) OsERFL1promXbaIR1
(4511) AbsI - PspXI
(4486) EcoRV
(4668) NotI
(4984) BspEI
(5405) ScaI
(5435) MluI
(5523) BstZ17I
(5713) BbvCI
(5859) SrfI
(5893) BmgBI
(5976) BstXI
(5997) BsaI
(6534) PfoI*
1000
2000
3000
4000
5000
6000
7000
8000


Created with SnapGene®
pGKGWY:OsERFLP:HvMYB26
8067 bp
(8067) End
Start (0)
PsiI (84)
FseI (106)
(7993) MauBI
(7799) AflII
(7580) RsrII
(7182) PflFI - Tth111I
(6754) SacII
BspHI (808)
NruI (947)
AsiSI (1290)
BsmBI (1306)
PflMI (1553)
ApaLI (2105)
StuI (2481)
M13R (as aForword) (2816 .. 2836)
OsERFL1promSacIF1 (2890 .. 2909)
AfeI (3190)
NdeI (3988)
(4426 .. 4447) OsERFL1promXbaIR1
(4448) XbaI
(4486) EcoRV
(4511) AbsI - PspXI
(4616) BstEII
(4859) AarI
(4882) XmnI
(4933 .. 4945) HvMYB26RNAiF1
(5167) SgrDI
(5238) BstXI
(5299) MreI - SgrAI
(5363) PpuMI
(5375) BmgBI
(5422 .. 5449) Primer 1
(5768) PfoI*
(5930 .. 5947) YFP-NY-R 1
NPTII gene
T3 promoter
OsERFLP
attB1
HvMYB26
mVenus
M13 fwd
1000
2000
3000
4000
5000
6000
7000
8000

APPENDIX 2 SEQUENCES

M13F with ATIPT4colR1

```
M13F         TGTAAAACGACGGCCAGTCTTAAGCTCGGGCCCCAAATAATGATTTTATTTTGACTGATA
ATIPT4colR1  T----------------------------------------------------TGACTGATA

M13F         GTGACCTGTTCGTTGCAACAAATTGATGAGCAATGCTTTTTTATAATGCCAACTTTGTAC
ATIPT4colR1  GTGACCTGTTCGTTGCAACAAATTGATGAGCAATGCTTTTTTATAATGCCAACTTTGTAC

M13F         AAAAAAGCAGGCT--------------ATGAAGTGTAATGACAAAATGGTTGTGATCATG
ATIPT4colR1  AAAAAAGCAGGCTCCGAATTCGCCCTTATGAAGTGTAATGACAAAATGGTTGTGATCATG

M13F         GGTGCCACCGGTTCTGGCAAGTCATCACTCTCTGTTGATCTCGCTTTACATTTTAAAGCC
ATIPT4colR1  GGTGCCACCGGTTCTGGCAAGTCATCACTCTCTGTTGATCTCGCTTTACATTTTAAAGCC

M13F         GAGATCATCAACTCTGACAAAATGCAGTTCTACGATGGCTTGAAGATCACCACGAATCAA
ATIPT4colR1  GAGATCATCAACTCTGACAAAATGCAGTTCTACGATGGCTTGAAGATCACCACGAATCAA

M13F         TCGACCATTGAAGATCGACGTGGAGTGCCACATCACCTTCTCGGTGAACTAAACCCGGAG
ATIPT4colR1  TCGACCATTGAAGATCGACGTGGAGTGCCACATCACCTTCTCGGTGAACTAAACCCGGAG

M13F         GCTGGAGAAGTCACAGCGGCAGAATTTCGCGTTATGGCGGCTGAAGCCATCTCCGAGATT
ATIPT4colR1  GCTGGAGAAGTCACAGCGGCAGAATTTCGCGTTATGGCGGCTGAAGCCATCTCCGAGATT

M13F         ACTCAACGTAAAAAGCTCCCAATCCTTGCCGGTGGATCCAACTCATACATTCATGCTCTC
ATIPT4colR1  ACTCAACGTAAAAAGCTCCCAATCCTTGCCGGTGGATCCAACTCATACATTCATGCTCTC

M13F         CTTGCAAAATCTTATGACCCTGAAAACTATCCGTTTTCTGATCACAAGGGCTCAATCTGC
ATIPT4colR1  CTTGCAAAATCTTATGACCCTGAAAACTATCCGTTTTCTGATCACAAGGGCTCAATCTGC

M13F         TCCGAGTTGAAATATGATTGTTGTTTCATTTGGATAGATGTGGATCAGTCTGTGTTATTC
ATIPT4colR1  TCCGAGTTGAAATATGATTGTTGTTTCATTTGGATAGATGTGGATCAGTCTGTGTTATTC

M13F         GAGTATCTTTCTTTACGTTTGGATCTTATGATGAAGTCAGGTATGTTCGAGGAGATCGCT
ATIPT4colR1  GAGTATCTTTCTTTACGTTTGGATCTTATGATGAAGTCAGGTATGTTCGAGGAGATCGCT

M13F         GAGTTCCACCGCTCTAAGAAGGCCCCGAAAGAGCCATTGGGGAT
ATIPT4colR1  GAGTTCCACCGCTCTAAGAAGGCCCCGAAAGAGCCATTGGGGATATGGAAGGCTATAGGA


M13F         GTGCAAGAGTTTGATGACTACCTCAAAATGTACAAGTGGGACAATGACATGGATAAATGG
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PBract214F with AtIPT4colR1

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pBract214F TT-----------------------------------------------GTCGATGCTCA
ATIPT4colR1 TTTAGCCCTGCCTTCATACGCTATTTATTTGCTTGGTACTGTTTCTTTTGTCGATGCTCA
pBract214F CCCTGTTGTTTGGTGTTACTTCGCCCATCACAAGTTTGTACAAAAAAGCAGGCTCCGAAT
ATIPT4colR1 CCCTGTTGTTTGGTGTTACTTCGCCCATCACAAGTTTGTACAAAAAAGCAGGCT------

pBract214F TCGCCCTTATGAAGTGTAATGACAAAATGGTTGTGATCATGGGTGCCACCGGTTCTGGCA
ATIPT4colR1 --------ATGAAGTGTAATGACAAAATGGTTGTGATCATGGGTGCCACCGGTTCTGGCA

pBract214F AGTCATCACTCTCTGTTGATCTCGCTTTACATTTTAAAGCCGAGATCATCAACTCTGACA
ATIPT4colR1 AGTCATCACTCTCTGTTGATCTCGCTTTACATTTTAAAGCCGAGATCATCAACTCTGACA

pBract214F AAATGCAGTTCTACGATGGCTTGAAGATCACCACGAATCAATCGACCATTGAAGATCGAC
ATIPT4colR1 AAATGCAGTTCTACGATGGCTTGAAGATCACCACGAATCAATCGACCATTGAAGATCGAC

pBract214F GTGGAGTGCCACATCACCTTCTCGGTGAACTAAACCCGGAGGCTGGAGAAGTCACAGCGG
ATIPT4colR1 GTGGAGTGCCACATCACCTTCTCGGTGAACTAAACCCGGAGGCTGGAGAAGTCACAGCGG

pBract214F CAGAATTTCGCGTTATGGCGGCTGAAGCCATCTCCGAGATTACTCAACGTAAAAAGCTCC
ATIPT4colR1 CAGAATTTCGCGTTATGGCGGCTGAAGCCATCTCCGAGATTACTCAACGTAAAAAGCTCC

pBract214F CAATCCTTGCCGGTGGATCCAACTCATACATTCATGCTCTCCTTGCAAAATCTTATGACC
ATIPT4colR1 CAATCCTTGCCGGTGGATCCAACTCATACATTCATGCTCTCCTTGCAAAATCTTATGACC

pBract214F CTGAAAACTATCCGTTTTCTGATCACAAGGGCTCAATCTGCTCCGAGTTGAAATATGATT
ATIPT4colR1 CTGAAAACTATCCGTTTTCTGATCACAAGGGCTCAATCTGCTCCGAGTTGAAATATGATT

pBract214F GTTGTTTCATTTGGATAGATGTGGATCAGTCTGTGTTATTCGAGTATCTTTCTTTACGTT
ATIPT4colR1 GTTGTTTCATTTGGATAGATGTGGATCAGTCTGTGTTATTCGAGTATCTTTCTTTACGTT

pBract214F TGGATCTTATGATGAAGTCAGGTATGTTCGAGGAGATCGCTGAGTTCCACCGCTCTAAGA
ATIPT4colR1 TGGATCTTATGATGAAGTCAGGTATGTTCGAGGAGATCGCTGAGTTCCACCGCTCTAAGA

pBract214F AGGCCCCGAAAGAGCCATTGGGGATATGGAAGGCTATAGGAGTGCAAGAGTTTGATGACT
ATIPT4colR1 AGGCCCCGAAAGAGCCATTGGGGAT

pBract214F ACCTCAAAATGTACAAGTGGGACAATGACATGGATAAATGGGACCCTATGAGAAAGGAGG
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M13F with ATIPT8colR1

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M13F         TGTAAAACGACGGCCAGTCTTAAGCTCGGGCCCCAAATAATGATTTTATTTTGACTGATA
ATIPT8colR1                                      ATGATTTTATTTTGACTGATA

M13F         GTGACCTGTTCGTTGCAACAAATTGATGAGCAATGCTTTTTTATAATGCCAACTTTGTAC
ATIPT8colR1 GTGACCTGTTCGTTGCAACAAATTGATGAGCAATGCTTTTTTATAATGCCAACTTTGTAC

M13F         AAAAAAGCAGGC---------------ATGCAAAATCTTACGTCCACATTCGTCTCTCCT
ATIPT8colR1 AAAAAAGCAGGCTCCGAATTCGCCCTTATGCAAAATCTTACGTCCACATTCGTCTCTCCT

M13F         TCCATGATCCCGATCACTTCTCCGCGGCTGCGACTGCCACCACCACGATCAGTAGTTCCC
ATIPT8colR1 TCCATGATCCCGATCACTTCTCCGCGGCTGCGACTGCCACCACCACGATCAGTAGTTCCC

M13F         ATGACTACCGTTTGCATGGAACAATCATACAAGCAAAAAGTGGTTGTGATCATGGGAGCC
ATIPT8colR1 ATGACTACCGTTTGCATGGAACAATCATACAAGCAAAAAGTGGTTGTGATCATGGGAGCC

M13F         ACCGGATCAGGCAAGTCATGCCTCTCAATCGATCTAGCAACTCGTTTCTCTGGCGAGATC
ATIPT8colR1 ACCGGATCAGGCAAGTCATGCCTCTCAATCGATCTAGCAACTCGTTTCTCTGGCGAGATC

M13F         GTCAATTCCGACAAGATTCAATTCTACGATGGATTGAAGGTCACTACGAATCAAATGAGC
ATIPT8colR1 GTCAATTCCGACAAGATTCAATTCTACGATGGATTGAAGGTCACTACGAATCAAATGAGC

M13F         ATCCTTGAGAGATGTGGAGTCCCTCACCATCTCCTTGGTGAGCTCCCTCCGGATGATAGC
ATIPT8colR1 ATCCTTGAGAGATGTGGAGTCCCTCACCATCTCCTTGGTGAGCTCCCTCCGGATGATAGC

M13F         GAACTAACTACCTCCGAATTCCGCTCTTTGGCGTCGCGGTCCATCTCCGAGATTACTGCT
ATIPT8colR1 GAACTAACTACCTCCGAATTCCGCTCTTTGGCGTCGCGGTCCATCTCCGAGATTACTGCT

M13F         CGTGGAAACCTCCCGATTATAGCTGGTGGATCAAACTCCTTCATTCATGCTCTCCTTGTC
ATIPT8colR1 CGTGGAAACCTCCCGATTATAGCTGGTGGATCAAACTCCTTCATTCATGCTCTCCTTGTC

M13F         GACCGTTTTGACCCCAAAACCTATCCATTCTCTTCTGAGACATCCATCTCTTCCGGCTTG
ATIPT8colR1 GACCGTTTTGACCCCAAAACCTATCCATTCTCTTCTGAGACATCCATCTCTTCCGGCTTG

M13F         AGGTACGAGTGTT
ATIPT8colR1 AGGTACGAGTGTTGCTTCCTTTGGGTGGATGTCTCAGTGTCGGTCCTGTTCGAGTACCTC
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pBract21F with ATIPT8colR1

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pBract214F   TTTAGCCCTGCCTTCATACGCTATTTATTTGCTTGGTACTGTTTCTTTTGTCGATGCT-C
ATIPT8colR1  ATGAT-------TT-------TATTT---TGACTGATAGTGAC-CTGTT--CGTTGCAAC

pBract214F ACCCTGTTGTTTGGTGTTACTTCGCCCATCACAAGTTTGTACAAAAAAGCAGGCT-----
ATIPT8colR1 AAATTGATGAGCAATGCTTTTTTATA-ATGCCAACTTTGTACAAAAAAGCAGGCTCCGAA

pBract214F ---------ATGCAAAATCTTACGTCCACATTCGTCTCTCCTTCCATGATCCCGATCACT
ATIPT8colR1 TTCGCCCTTATGCAAAATCTTACGTCCACATTCGTCTCTCCTTCCATGATCCCGATCACT

pBract214F  TCTCCGCGGCTGCGACTGCCACCACCACGATCAGTAGTTCCCATGACTACCGTTTGCATG
ATIPT8colR1 TCTCCGCGGCTGCGACTGCCACCACCACGATCAGTAGTTCCCATGACTACCGTTTGCATG

pBract214F  GAACAATCATACAAGCAAAAAGTGGTTGTGATCATGGGAGCCACCGGATCAGGCAAGTCA
ATIPT8colR1 GAACAATCATACAAGCAAAAAGTGGTTGTGATCATGGGAGCCACCGGATCAGGCAAGTCA

pBract214F  TGCCTCTCAATCGATCTAGCAACTCGTTTCTCTGGCGAGATCGTCAATTCCGACAAGATT
ATIPT8colR1 TGCCTCTCAATCGATCTAGCAACTCGTTTCTCTGGCGAGATCGTCAATTCCGACAAGATT

pBract214F  CAATTCTACGATGGATTGAAGGTCACTACGAATCAAATGAGCATCCTTGAGAGATGTGGA
ATIPT8colR1 CAATTCTACGATGGATTGAAGGTCACTACGAATCAAATGAGCATCCTTGAGAGATGTGGA

pBract214F  GTCCCTCACCATCTCCTTGGTGAGCTCCCTCCGGATGATAGCGAACTAACTACCTCCGAA
ATIPT8colR1 GTCCCTCACCATCTCCTTGGTGAGCTCCCTCCGGATGATAGCGAACTAACTACCTCCGAA

pBract214F  TTCCGCTCTTTGGCGTCGCGGTCCATCTCCGAGATTACTGCTCGTGGAAACCTCCCGATT
ATIPT8colR1 TTCCGCTCTTTGGCGTCGCGGTCCATCTCCGAGATTACTGCTCGTGGAAACCTCCCGATT

pBract214F  ATAGCTGGTGGATCAAACTCCTTCATTCATGCTCTCCTTGTCGACCGTTTTGACCCCAAA
ATIPT8colR1 ATAGCTGGTGGATCAAACTCCTTCATTCATGCTCTCCTTGTCGACCGTTTTGACCCCAAA

pBract214F  ACCTATCCATTCTCTTCTGAGACATCCATCTCTTCCGGCTTGAGGTACGAGTGTT
ATIPT8colR1 ACCTATCCATTCTCTTCTGAGACATCCATCTCTTCCGGCTTGAGGTACGAGTGTTGCTTC

ATIPT8colR1 CTTTGGGTGGATGTCTCAGTGTCGGTCCTGTTCGAGTACCTCTCGAAACGTGTCGACCAG
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pGWB8 with ATIPT4colR1

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PGWB8F       GGAAGTTCATTTCATTTGGAGAGAACACGGGGGACTCTAATCAAACAAGTTTGTACAAAA
AIIPT4colR1 C--AGTT---------TGTACAAAAAAGCAGG--CTC----CGAA----TTCGCCCT---

PGWB8F       AAGCAGGCTATGAAGTGTAATGACAAAATGGTTGTGATCATGGGTGCCACCGGTTCTGGC
AIIPT4colR1 --------TATGAAGTGTAATGACAAAATGGTTGTGATCATGGGTGCCACCGGTTCTGGC

PGWB8F       AAGTCATCACTCTCTGTTGATCTCGCTTTACATTTTAAAGCCGAGATCATCAACTCTGAC
AIIPT4colR1 AAGTCATCACTCTCTGTTGATCTCGCTTTACATTTTAAAGCCGAGATCATCAACTCTGAC

PGWB8F       AAAATGCAGTTCTACGATGGCTTGAAGATCACCACGAATCAATCGACCATTGAAGATCGA
AIIPT4colR1 AAAATGCAGTTCTACGATGGCTTGAAGATCACCACGAATCAATCGACCATTGAAGATCGA

PGWB8F       CGTGGAGTGCCACATCACCTTCTCGGTGAACTAAACCCGGAGGCTGGAGAAGTCACAGCG
AIIPT4colR1 CGTGGAGTGCCACATCACCTTCTCGGTGAACTAAACCCGGAGGCTGGAGAAGTCACAGCG

PGWB8F       GCAGAATTTCGCGTTATGGCGGCTGAAGCCATCTCCGAGATTACTCAACGTAAAAAGCTC
AIIPT4colR1 GCAGAATTTCGCGTTATGGCGGCTGAAGCCATCTCCGAGATTACTCAACGTAAAAAGCTC

PGWB8F       CCAATCCTTGCCGGTGGATCCAACTCATACATTCATGCTCTCCTTGCAAAATCTTATGAC
AIIPT4colR1 CCAATCCTTGCCGGTGGATCCAACTCATACATTCATGCTCTCCTTGCAAAATCTTATGAC

PGWB8F       CCTGAAAACTATCCGTTTTCTGATCACAAGGGCTCAATCTGCTCCGAGTTGAAATATGAT
AIIPT4colR1 CCTGAAAACTATCCGTTTTCTGATCACAAGGGCTCAATCTGCTCCGAGTTGAAATATGAT

PGWB8F       TGTTGTTTCATTTGGATAGATGTGGATCAGTCTGTGTTATTCGAGTATCTTTCTTTACGT
AIIPT4colR1 TGTTGTTTCATTTGGATAGATGTGGATCAGTCTGTGTTATTCGAGTATCTTTCTTTACGT

PGWB8F       TTGGATCTTATGATGAAGTCAGGTATGTTCGAGGAGATCGCTGAGTTCCACCGCTCTAAG
AIIPT4colR1 TTGGATCTTATGATGAAGTCAGGTATGTTCGAGGAGATCGCTGAGTTCCACCGCTCTAAG

PGWB8F       AAGGCCCCGAAAGAGCCATTGGGGAT
AIIPT4colR1 AAGGCCCCGAAAGAGCCATTGGGGATATGGAAGGCTATAGGAGTGCAAGAGTTTGATGAC

AIIPT4colR1 TACCTCAAAATGTACAAGTGGGACAATGACATGGATAAATGGGACCCTATGAGAAAGGAG
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pGWB8 with ATIPT8colR1

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PGWB8F      GGAAGTTCATTTCATTTGGAGAGAACACGGGGGACTCTAATCAAACAAGTTTGTACAAAA
AIIPT8colR1 AAAA---------------AG----CAGG-----CTC----CGAA----TTCGCCCT---

PGWB8F      AAGCAGGCTATGCAAAATCTTACGTCCACATTCGTCTCTCCTTCCATGATCCCGATCACT
AIIPT8colR1 --------TATGCAAAATCTTACGTCCACATTCGTCTCTCCTTCCATGATCCCGATCACT

PGWB8F      TCTCCGCGGCTGCGACTGCCACCACCACGATCAGTAGTTCCCATGACTACCGTTTGCATG
AIIPT8colR1 TCTCCGCGGCTGCGACTGCCACCACCACGATCAGTAGTTCCCATGACTACCGTTTGCATG

PGWB8F      GAACAATCATACAAGCAAAAAGTGGTTGTGATCATGGGAGCCACCGGATCAGGCAAGTCA
AIIPT8colR1 GAACAATCATACAAGCAAAAAGTGGTTGTGATCATGGGAGCCACCGGATCAGGCAAGTCA

PGWB8F      TGCCTCTCAATCGATCTAGCAACTCGTTTCTCTGGCGAGATCGTCAATTCCGACAAGATT
AIIPT8colR1 TGCCTCTCAATCGATCTAGCAACTCGTTTCTCTGGCGAGATCGTCAATTCCGACAAGATT

PGWB8F     CAATTCTACGATGGATTGAAGGTCACTACGAATCAAATGAGCATCCTTGAGAGATGTGGA
AIIPT8colR1 CAATTCTACGATGGATTGAAGGTCACTACGAATCAAATGAGCATCCTTGAGAGATGTGGA

PGWB8F      GTCCCTCACCATCTCCTTGGTGAGCTCCCTCCGGATGATAGCGAACTAACTACCTCCGAA
AIIPT8colR1 GTCCCTCACCATCTCCTTGGTGAGCTCCCTCCGGATGATAGCGAACTAACTACCTCCGAA

PGWB8F      TTCCGCTCTTTGGCGTCGCGGTCCATCTCCGAGATTACTGCTCGTGGAAACCTCCCGATT
AIIPT8colR1 TTCCGCTCTTTGGCGTCGCGGTCCATCTCCGAGATTACTGCTCGTGGAAACCTCCCGATT

PGWB8F      ATAGCTGGTGGATCAAACTCCTTCATTCATGCTCTCCTTGTCGACCGTTTTGACCCCAAA
AIIPT8colR1 ATAGCTGGTGGATCAAACTCCTTCATTCATGCTCTCCTTGTCGACCGTTTTGACCCCAAA

PGWB8F      ACCTATCCATTCTCTTCTGAGACATCCATCTCTTCCGGCTTGAGGTACGAGTGTT
AIIPT8colR1 ACCTATCCATTCTCTTCTGAGACATCCATCTCTTCCGGCTTGAGGTACGAGTGTTGCTTC

AIIPT8colR1 CTTTGGGTGGATGTCTCAGTGTCGGTCCTGTTCGAGTACCTCTCGAAACGTGTCGACCAG

AIIPT8colR1 ATGATGGAGTCAGGGATGTTCGAGGAGCTAGCCGGTTTCTACGACCCGAGATATTCCGGG
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M13F with OsERFLPcolR1

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M13F          TGTAAAACGACGGCCAGTCTTAAGCTCGGGCCCCAAATAATGATTTTATTTTGACTGATA
OsERFLPcolR1 TG-----------------------------------------ATTTTATTTTGACTGATA

V M13F        GTGACCTGTTCGTTGCAACAAATTGATGAGCAATGCTTTTTTATAATGCCAACTTTGTAC
OsERFLPcolR1 GTGACCTGTTCGTTGCAACAAATTGATGAGCAATGCTTTTTTATAATGCCAACTTTGTAC

M13F          AAAAAAGCAGGC---------------------------AAGCTTCCATCCCTGCAGGAA
OsERFLPcolR1 AAAAAAGCAGGCTCCGAATTCGCCCTTTACCTGGAGCTCAAGCTTCCATCCCTGCAGGAA

M13F          CTCACTG-CACACGTATCTGTGTGGAGCCGATGGAACTGTAAAAGCACCCGGACGGAAGC
OsERFLPcolR1 CTCACTGACACACGTATCTGTGTGGAGCCGATGGAACTGTAAAAGCACCCGGACGGAAGC

M13F          CCCATTCAATTCGATCGGCCGGACAAACAATGGCAACGAACTCACTCCGCCATTAACTCC
OsERFLPcolR1 CCCATTCAATTCGATCGGCCGGACAAACAATGGCAACGAACTCACTCCGCCATTAACTCC

M13F          GTTTCACCTTTTTACGGAGAGAAGAGCAAGTTCTTCTGCTGCTGCAATGCTGTGTGCGCC
OsERFLPcolR1 GTTTCACCTTTTTACGGAGAGAAGAGCAAGTTCTTCTGCTGCTGCAATGCTGTGTGCGCC

M13F          TTCCTTGCCGATGCGCCATGGATGGATGGATGATTGGCTGCTTGATTGGGGCAGTTATTG
OsERFLPcolR1 TTCCTTGCCGATGCGCCATGGATGGATGGATGATTGGCTGCTTGATTGGGGCAGTTATTG

M13F          CAGGCCAACTGCTCTGTCGAAAGAGTGTCCCTCGGAAAGTGGCTTTAGAAGTTGGAGTAA
OsERFLPcolR1 CAGGCCAACTGCTCTGTCGAAAGAGTGTCCCTCGGAAAGTGGCTTTAGAAGTTGGAGTAA

M13F          ACCTTGCAGTGCATGGCATACATACAGCATGGAGTGGTATTACCTTGGTTACGAACGCCC
OsERFLPcolR1 ACCTTGCAGTGCATGGCATACATACAGCATGGAGTGGTATTACCTTGGTTACGAACGCCC

M13F          ATCAAGTCCATTTCTGGAGTAGTACACACTGTCCAAGCTTCAGTGTTTGTACCTGGAAAG
OsERFLPcolR1 ATCAAGTCCATTTCTGGAGTAGTACACACTGTCCAAGCTTCAGTGTTTGTACCTGGAAAG

M13F          AAATGCACATTAGCCATATGCGCAAGGCAAGGCAAGGCATTGAGCAAAGTTAGATTCATC
OsERFLPcolR1 AAATGCACATTAGCCATATGCGCAAGGCAAGGCAAGGCATTGAGCAAAGTTAGATTCATC

M13F          AGTTACTAGTTTAGCTAGTTAGCACATACACACTACTGGACAATGACTAGCGAGTGAGCT
OsERFLPcolR1 AGTTACTAGTTTAGCTAGTTAGCACATACACACTACTGGACAATGACTAGCGAGTGAGCT
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M13F          TAGCTAGCTGATCGGCGACAAGTGACAACCAGCTAGTTAGCAATAAGAGCTGGTGATGGT
OsERFLPcolR1 TAGCTAGCTGATCGGCGACAAGTGACAACCAGCTAGTTAGCAATAAGAGCTGGTGATGGT

M13F          TAGCTCATCTCAGTTCATCCTTAGGTTTTCCTCCAGTGAAGGCCATAAATGAGGTCGCCA
OsERFLPcolR1 TAGCTCATCTCAGTTCATCCTTAGGTTTTCCTCCAGTGAAGGCCATAAATGAGGTCG
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PGWB3GUSR with OsERFLPcolF1

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OsERFLPcolF1 AGAGTACACCAACTAGATCTGAACCCTGGTGTGCTACTGTACCAAAAGGCTAAGGCCACT

OsERFLPcolF1 GACAGAGGAGATACGTGACAGACAAGTCCCAGTTGAGTTACTGTCCAACCCCCAAATCTA

OsERFLPcolF1 ACCCTCTTCTACCACCACCCCCACTCGTGCGCCACAGCGCTCTCACACGCGCGCCACACT

PGWB3GUSR                                                 CACATTGAACGCACGC
OsERFLPcolF1 CATGCCATGTATGTCTATATATATGTGCATCTATATATCTTAGCCACATTGAACGCACGC

PGWB3GUSR    TACACTGCAAGAAAGGCAAGTATAGGGGTGGCAGGTAGTTTATATTCACTGGAAACCGAG
Sbjct  481   TACACTGCAAGAAAGGCAAGTATAGGGGTGGCAGGTAGTTTATATTCACTGGAAACCGAG

PGWB3GUSR    AACTTCACTCCTCCCAGCACTCCACACTAGGAGGATGCTCACTGTCTGACTGACTGACTG
OsERFLPcolF1 AACTTCACTCCTCCCAGCACTCCACACTAGGAGGATGCTCACTGTCTGACTGACTGACTG

PGWB3GUSR    GCTGCTGCTGCTGCTCATTCCTTCTCCTACTCTGCCTGCCTCCTCTGCTGTGTCCTTGGG
OsERFLPcolF1 GCTGCTGCTGCTGCTCATTCCTTCTCCTACTCTGCCTGCCTCCTCTGCTGTGTCCTTGGG

PGWB3GUSR    AGAATTCTTCAGGGACAGTATCCCTGCAGAGGTGAGATC----------------------
OsERFLPcolF1 AGAATTCTTCAGGGACAGTATCCCTGCAGAGGTGAGATCTCTAGACAGGTAAAGGGCGAA

PGWB3GUSR    ----ACCCAGCTTTCTTGTACAAAGTGGTTCGATCTAGAGGATCCCCGGGTGGTCAGTCC
OsERFLPcolF1 TTCGACCCAGCTTTCTTGTACAAAGTGGTTCGATCTAGAGGATCCCCGGGTGGTCAGTCC

PGWB3GUSR    CTTATGTTACGTCCTGTAGAAACCCCAACCCGTGAAATCAAAAAACTCGACGGCCTGTGG
OsERFLPcolF1 CTTATGTTACGTCCTGTAGAAACCCCAACCCGTGAAATCAAAAAACTCGACGGCCTGTGG

PGWB3GUSR    GCATTCAGTCTGGATCGCGAAAACTGTGGAATTGATCAGCGTTGGTGGGAAAGCGCGTTA
OsERFLPcolF1 GCATTCAGTCTGGATCGCGAAAACTGTGGAATT

PGWB3GUSR    CAAGAAAGCCGGGCAATTGCT
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M13F with LCHScolR1

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M13F       TGTAAAACGACGGCCAGTCTTAAGCTCGGGCCCCAAATAATGATTTTATTTTGACTGATA
LCHScolR1   T-TA-----------------------------------TGATTTTATTTTGACTGATA

M13F        GTGACCTGTTCGTTGCAACAAATTGATGAGCAATGCTTTTTTATAATGCCAACTTTGTAC
LCHScolR1   GTGACCTGTTCGTTGCAACAAATTGATGAGCAATGCTTTTTTATAATGCCAACTTTGTAC

M13F        AAAAAAGCAGGC----------------------AGTCGTGGTTTGTGAGCTGGATGAAGC
LCHScolR1   AAAAAAGCAGGCTCCGAATTCGCCCTTAAGCTTAGTCGTGGTTTGTGAGCTGGATGAAGC

M13F        CCTAGTCGTGGGCGGCAGCACAGACAAGGACGGTGTGGGAGGGAGAGGTCTACTGTGGAG
LCHScolR1   CCTAGTCGTGGGCGGCAGCGCAGACAAGGACGATGTGGGAGGGAGAGGTCTACTGTGGAG

M13F        ATGGAGAGTG-CACGGCAACGGGGGAGGCCGAGGAGAGTGGGGTTGGGAGGCGGAAGATT
LCHScolR1   ATGGAGAGTGTCACGGCAACGGGAGAGGCCGAGGAGAGTGGGGTCGGGAGGCGGAAGATT

M13F        GCGGGGAGAATAGTGATGCTGCATTCGATGAGTTATCATGAGTGGGACGCCACTTTGGTT
LCHScolR1   ACAGGGAGAATAGTGATGCCGCATTCGATGAGTCATCGCGAGTGGGATGCCACTTTGGTC

M13F        TCGTGACTACTCCGGCTCTTTGAGAAATGTGATGGGTAATTAATAGATTTTTTATTTTCC
LCHScolR1   TCGTGACTACTCCGGCTCTTTGAGAAATGTGGTGGGTAATTAATAGATTTTTTATTTTCC

M13F        AAAAATCCAATAAGGACATGTGCCCCCACTAACTCCTTAATGGCTCCGCCAATGTCTCTA
LCHScolR1   AAAAATCCAATAGGGACATGTGTCCCCACTAGCCCCTTAATGACTCCGCCAATGTCTCTA

M13F        CGTGGTTTGATGATAATAAGAGTGTCATTTTTAATATCCAATGTTTTTTGTCTTCAGGAG
LCHScolR1   CGTGGTTTGATGATAATGAGAGTGTCATTTTCAATATCCGGTGTTTTTGGTCTTCGGGAG

M13F        TTTGAAATATGAATAGCTCAACTCGAAAACTAGGCTCCTACCCACAATGTTTTGCTAGTG
LCHScolR1   TTTGAAATATGAATAGCTCAACTCGAAAACTAGGCTCCTACCTACAATGTTTTGCTAGTG

M13F        TATTTAGTTAGAAAGAACTAAGATAAAAAACATAGATTACCTTAACATTCTCATTTATTG
LCHScolR1   TATTTAGTTAGAAAGAACTAAAATAAAAAACATAGATTACCTTAACATTCTCATTTATTG
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M13F         TTGTACCTTTGTGTTCTATATTATAGACTT-GATTCCATTTACCGTCTCCAACAAAAATA
LCHScolR1    TTGTACCTTTGTGTTCTATATTACAGACTTTGATTCCATTTACCGTCTCCAACAAAAATA

M13F         CAGATTGTATGGTTCTTCGACTGCCGGACACGATAGTCTGGTAAAATGAAACAAAGGAAA
LCHScolR1    CAGATTGAATGGTTCTTCGACTGCCGGACACGATAGTCTGGTAAA-TGAAACAAAGGAAA

M13F         AAAAACAAAAATGTTCTGCTTGCTCCAACTCAGGTGAACCGCTTGTTTTTGTTATTTCCC
LCHScolR1    AAAAACAAAAATGTTCTGCTTGCTCCAACTCAGG
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M13F with Ubi1colR1

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M13F         GTAAAACGACGGCCAGTCTTAAGCTCGGGCCCCAAATAATGATTTTATTTTGACTGATAG
Ubi1colR1                    TCATA------------------TGATTTTATTTTGACTGATAG

M13F         TGACCTGTTCGTTGCAACAAATTGATGAGCAATGCTTTTTTATAATGCCAACTTTGTACA
Ubi1colR1    TGACCTGTTCGTTGCAACAAATTGATGAGCAATGCTTTTTTATAATGCCAACTTTGTACA

M13F         AAAAAGCAGGCT--------------------------------GCAGCGTGACCCGGTCG
Ubi1colR1    AAAAAGCAGGCTCCGAATTCGCCCTTTTGATATCGAATTCCGTGCAGCGTGACCCGGTCG

M13F         TGCCCCTCTCTAGAGATAATGAGCATTGCATGTCTAAGTTATAAAAAATTACCACATATT
Ubi1colR1    TGCCCCTCTCTAGAGATAATGAGCATTGCATGTCTAAGTTATAAAAAATTACCACATATT

M13F         TTTTTTGTCACACTTGTTTGAAGTGCAGTTTATCTATCTTTATACATATATTTAAACTTT
Ubi1colR1    TTTTTTGTCACACTTGTTTGAAGTGCAGTTTATCTATCTTTATACATATATTTAAACTTT

M13F         ACTCTACGAATAATATAATCTATAGTACTACAATAATATCAGTGTTTTAGAGAATCATAT
Ubi1colR1    ACTCTACGAATAATATAATCTATAGTACTACAATAATATCAGTGTTTTAGAGAATCATAT

M13F         AAATGAACAGTTAGACATGGTCTAAAGGACAATTGAGTATTTTGACAACAGGACTCTACA
Ubi1colR1    AAATGAACAGTTAGACATGGTCTAAAGGACAATTGAGTATTTTGACAACAGGACTCTACA

M13F         GTTTTATCTTTTTAGTGTGCATGTGTTCTCCTTTTTTTTTTGCAAATAGCTTCACCTATAT
Ubi1colR1    GTTTTATCTTTTTAGTGTGCATGTGTTCTCCTTTTTTTTTTGCAAATAGCTTCACCTATAT

M13F         AATACTTCATCCATTTTATTAGTACATCCATTTAGGGTTTAGGGTTAATGGTTTTTATAG
Ubi1colR1    AATACTTCATCCATTTTATTAGTACATCCATTTAGGGTTTAGGGTTAATGGTTTTTATAG

M13F         ACTAATTTTTTTAGTACATCTATTTTATTCTATTTTAGCCTCTAAATTAAGAAAACTAAA
Ubi1colR1    ACTAATTTTTTTAGTACATCTATTTTATTCTATTTTAGCCTCTAAATTAAGAAAACTAAA

M13F         ACTCTATTTTAGTTTTTTTATTTAATAATTTAGATATAAAATAGAATAAAATAAAGTGAC
Ubi1colR1    ACTCTATTTTAGTTTTTTTATTTAATAATTTAGATATAAAATAGAATAAAATAAAGTGAC
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M13F          TAAAAATTAAACAAATACCCTTTAAGAAATTAAAAAAACTAAGGAAACATTTTTCTTGTT
Ubi1colR1     TAAAAATTAAACAAATACCCTTTAAGAAATTAAAAAAACTAAGGAAACATTTTTCTTGTT

M13F          TCGAGTAGATAATGCCAGCCTGTTAAACGCCGTCGACGAGTCTAACGGACACCAACCAGC
Ubi1colR1     TCGAGTAGATAATGCCAGCCTGTTAAACGCCGTCGACGAGTCTAACGGACACCAACCAGC

M13F          GAACCAGCAGCGTCGCGTCGGGCCAAGCGAAGCAGACGGCACGGCATCTCTGTCGCTGCC
Ubi1colR1     GAACCAGCAGCGTCGCGTCGGGCCAAGCGAAGCAGACGGCACGGCATCTCTGTCGCTGCC

M13F          TCTGGACCCCTCTCGAGAGTTCCGCTCCACCGTTGGACTTGCTCCGCTGTCGGCATCCAG
Ubi1colR1     TCTGGACCCCTCTCGAGAGTTCCGCTCCACCGTTGGACTTGCTCCGCTGTCGGCATCCAG

M13F         AAATTGCGTGGCGGAGCGGCAGACGTGAGCCGGCACGGCAGGCGGCCTCCTCCTCCTCTC
Ubi1colR1    AAATTGCGTGGCGGAGCGGCAGACGTGAGCCGGCACGGCAGGCGGCCTCCTCCTCCTCTC

M13F          ACGGCAC-GGCAGCTACGGGGGATTCCTTTCCCACCGCTCCTTCGCTTTCCCTTCCTCGC
Ubi1colR1     ACGGCACCGGCAGCTACGGGG-ATTCCTTTCCCACCGCTC

M13F          CCGCCGTAATAAATAGACACCCCCTCCACACCCTCTTTCCCCAACCTCGTGTTGTTCGGA
```

PGWB3GUSR with Ubi1F

```
GUSR1     GGAATCCTGGGATGGCTCTAGCCGTTCCGCAGACGGGATCGATTTCATGATTTTTTTTGT

GUSR1     TTCGTTGCATAGGGTTTGGTTTGCCCTTTTCCTTTATTTCAATATATGCCGTGCACTTGT

GUSR1     TTGTCGGGTCATCTTTTCATGCTTTTTTTTGTCTTGGTTGTGATGATGTGGTCTGGTTGG
Ubi1F                                                     TGGTCTGGTTGG

GUSR1     GCGGTCGTTCTAGATCGGAGTAGAATT----CTGTTTCAAACTACCTGGTGGATTTATTA
Ubi1F     GCGGTCGTTCTAGATCGGAGTAGAATTAATTCTGTTTCAAACTACCTGGTGGATTTATTA

GUSR1     ATTTTGGATCTGTATGTGTGTGCCATACATATTCATAGTTACGAATTGAAGATGATGGAT
Ubi1F     ATTTTGGATCTGTATGTGTGTGCCATACATATTCATAGTTACGAATTGAAGATGATGGAT

GUSR1     GGAAATATCGATCTAGGATAGGTATACATGTTGATGCGGGTTTTACTGATGCATATACAG
Ubi1F     GGAAATATCGATCTAGGATAGGTATACATGTTGATGCGGGTTTTACTGATGCATATACAG

GUSR1     AGATGCTTTTTGTTCGCTTGGTTGTGATGATGTGGTGTGGTTGGGCGGTCGTTCATTCGT
Ubi1F     AGATGCTTTTTGTTCGCTTGGTTGTGATGATGTGGTGTGGTTGGGCGGTCGTTCATTCGT

GUSR1     TCTAGATCGGAGTAGAATACTGTTTCAAACTACCTGGTGTATTTATTAATTTTGGAACTG
Ubi1F     TCTAGATCGGAGTAGAATACTGTTTCAAACTACCTGGTGTATTTATTAATTTTGGAACTG

GUSR1     TATGTGTGTGTCATACATCTTCATAGTTACGAGTTTAAGATGGATGGAAATATCGATCTA
Ubi1F     TATGTGTGTGTCATACATCTTCATAGTTACGAGTTTAAGATGGATGGAAATATCGATCTA

GUSR1     GGATAGGTATACATGTTGATGTGGGTTTTACTGATGCATATACATGATGGCATATGCAGC
Ubi1F     GGATAGGTATACATGTTGATGTGGGTTTTACTGATGCATATACATGATGGCATATGCAGC

GUSR1     ATCTATTCATATGCTCTAACCTTGAGTACCTATCTATTATAATAAACAAGTATGTTTTAT
Ubi1F     ATCTATTCATATGCTCTAACCTTGAGTACCTATCTATTATAATAAACAAGTATGTTTTAT

GUSR1     AATTATTTTGATCTTGATATACTTGGATGATGGCATATGCAGCAGCTATATGTGGATTTT
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Ubi1F     AATTATTTTGATCTTGATATACTTGGATGATGGCATATGCAGCAGCTATATGTGGATTTT

GUSR1     TTTAGCCCTGCCTTCATACGCTATTTATTTGCTTGGTACTGTTTCTTTTGTCGATGCTCA
Ubi1F     TTTAGCCCTGCCTTCATACGCTATTTATTTGCTTGGTACTGTTTCTTTTGTCGATGCTCA

GUSR1     CCCTGTTGTTTGGTGTTACT----------TC-ACCCAGCTTTCTTGTACAAAGTGGTTC
Ubi1F     CCCTGTTGTTTGGTGTTACTAAGGGCGAATTCGACCCAGCTTTCTTGTACAAAGTGGTTC

GUSR1     GATCTAGAGGATCCCCGGGTGGTCAGTCCCTTATGTTACGTCCTGTAGAAACCCCAACCC
Ubi1F     GATCTAGAGGATCCCCGGGTGGTCAGTCCCTTATGTTACGTCCTGTAGAAACCCCAACCC

GUSR1     GTGAAATCAAAAAACTCGACGGCCTGTGGGCATTCAGTCTGGATCGCGAAAACTGTGGAA
Ubi1F     GTGAAATCAAAAAACTCGACGGCCTGTGGGCATTCAGTCTGGATCGCGAAAACTGTGGAA

GUSR1     TTGATCAGCGTTGGTGGGAAAGCGCGTTACAAGAAAGCCGGGCAATTGCTG
Ubi1F     T
```

M13F with CaMV35colR1

```
M13F          GTAAAACGACGGCCAGTCTTAAGCTCGGGCCCCAAATAATGATTTTATTTTGACTGATAG
CaMV35ScolR1                                          ATGATTTTATTTTGACTGATAG

M13F          TGACCTGTTCGTTGCAACAAATTGATGAGCAATGCTTTTTTATAATGCCAACTTTGTACA
CaMV35ScolR1 TGACCTGTTCGTTGCAACAAATTGATGAGCAATGCTTTTTTATAATGCCAACTTTGTACA

M13F          AAAAAGCAGGC---------------GGGTTCGAAGAATTCCCATGGAGTCAAAAATTCA
CaMV35ScolR1 AAAAAGCAGGCTCCGAATTCGCCCTTGGGTTCGAAGAATTCCCATGGAGTCAAAAATTCA

M13F          GATCGAGGATCTAACAGAACTCGCCGTGAAGACTGGCGAACAGTTCATACAGAGTCTTTT
CaMV35ScolR1 AATAGAGGACCTAACAGAACTCGCCGTAAAGACTGGCGAACAGTTCATACAGAGTCTCTT

M13F          ACGACTCAATGACAAGAAGAAAATCTTCGTCAACATGGTGGAGCACGACACTCTCGTCTA
CaMV35ScolR1 ACGACTCAATGACAAGAAGAAAATCTTCGTCAACATGGTGGAGCACGACACGCTTGTCTA

M13F          CTCCAAGAATATCAAAGATACAGTCTCAGAAGACCAAAGGGCTATTGAGACTTTTCAACA
CaMV35ScolR1 CTCCAAAAATATCAAAGATACAGTCTCAGAAGACCAAAGGGCAATTGAGACTTTTCAACA

M13F          AAGGGTAATATCGGGAAACCTCCTCGGATTCCATTGCCCAGCTATCTGTCACTTCATCAA
CaMV35ScolR1 AAGGGTAATATCCGGAAACCTCCTCGGATTCCATTGCCCAGCTATCTGTCACTTTATTGT

M13F          AAGGACAGTAGAAAAGGAAGGTGGCACCTACAAATGCCATCATTGCGATAAAGGAAAGGC
CaMV35ScolR1 GAAGATAGTGGAAAAGGAAGGTGGCTCCTACAAATGCCATCATTGCGATAAAGGAAAGGC

M13F          TATCGTTCAAGATGCCTCTGCCGACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAG
CaMV35ScolR1 CATCGTTGAAGATGCCTCTGCCGACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAG

M13F          CATCGTGGAAAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATAT
CaMV35ScolR1 CATCGTGGAAAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATAT

M13F          CTCCACTGACGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTAT
CaMV35ScolR1 CTCCACTGACGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTAT

M13F          ATAAGGAAGTTC
CaMV35ScolR1 ATAAGGAAGTTCATTTCATTTGGAGAGGACAGGGTACCGTCGACCCCCAAGGGCGAATTC
```

CaMV35ScolR1 GACCCAGCTTTCTTGTACAAAGTTGGCATTATAAAAAATAATTGCTCATCAATTTGTTGC

CaMV35ScolR1 AACGAACAGGTCACTATCAGTCAAAATAAAATCATTATTTGCCATCCAGCTGATATCCCC

PGWB3GUSR1 with CaMV35SF

```
CaMV35SF    ATTTTCACCCAGGAAACAGCTATGACCATGATTACGCCAAGCTTGCATGCCTGCAGGTCG

GUSR1                                                                G
CaMV35SF   ACTCTAATCAAACAAGTTTGTACAAAAAAGCAGGCTCCGAATTCGCCCTTGGGTTCGAAG

GUSR1       AATTCCCATGGAGTCAAAAATTCAGATCGAGGATCTAACAGAACTCGCCGTGAAGACTGG
CaMV35SF    AATTCCCATGGAGTCAAAAATTCAAATAGAGGACCTAACAGAACTCGCCGTAAAGACTGG

GUSR1       CGAACAGTTCATACAGAGTCTTTTACGACTCAATGACAAGAAGAAAATCTTCGTCAACAT
CaMV35SF    CGAACAGTTCATACAGAGTCTCTTACGACTCAATGACAAGAAGAAAATCTTCGTCAACAT

GUSR1       GGTGGAGCACGACACTCTCGTCTACTCCAAGAATATCAAAGATACAGTCTCAGAAGACCA
CaMV35SF    GGTGGAGCACGACACGCTTGTCTACTCCAAAAATATCAAAGATACAGTCTCAGAAGACCA

GUSR1       AAGGGCTATTGAGACTTTTCAACAAAGGGTAATATCGGGAAACCTCCTCGGATTCCATTG
CaMV35SF    AAGGGCAATTGAGACTTTTCAACAAAGGGTAATATCCGGAAACCTCCTCGGATTCCATTG

GUSR1       CCCAGCTATCTGTCACTTCATCAAAAGGACAGTAGAAAAGGAAGGTGGCACCTACAAATG
CaMV35SF    CCCAGCTATCTGTCACTTTATTGTGAAGATAGTGGAAAAGGAAGGTGGCTCCTACAAATG

GUSR1       CCATCATTGCGATAAAGGAAAGGCTATCGTTCAAGATGCCTCTGCCGACAGTGGTCCCAA
CaMV35SF    CCATCATTGCGATAAAGGAAAGGCCATCGTTGAAGATGCCTCTGCCGACAGTGGTCCCAA

GUSR1       AGATGGACCCCCACCCACGAGGAGCATCGTGGAAAAAGAAGACGTTCCAACCACGTCTTC
CaMV35SF    AGATGGACCCCCACCCACGAGGAGCATCGTGGAAAAAGAAGACGTTCCAACCACGTCTTC

GUSR1       AAAGCAAGTGGATTGATGTGATATCTCCACTGACGTAAGGGATGACGCACAATCCCACTA
CaMV35SF    AAAGCAAGTGGATTGATGTGATATCTCCACTGACGTAAGGGATGACGCACAATCCCACTA

GUSR1       TCCTTCGCAAGACCCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGAGGACAGGGTA
CaMV35SF    TCCTTCGCAAGACCCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGAGGACAGGGTA

GUSR1       CCGGGG---------GTCGAC----ACCCAGCTTTCTTGTACAAAGTGGTTCGATCTAGA
CaMV35SF    CCGTCGACCCCCAAGGGCGAATTCGACCCAGCTTTCTTGTACAAAGTGGTTCGATCTAGA

GUSR1       GGATCCCCGGGTGGTCAGTCCCTTATGTTACGTCCTGTAGAAACCCCAACCCGTGAAATC
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```
CaMV35SF    GGATCCCCGGGTGGTCAGTCCCTTATGTTACGTCCTGTAGAAACCCCAACCCGTGAAATC

GUSR1        AAAAAACTCGACGGCCTGTGGGCATTCAGTCTGGATCGCGAAAA-CTGTGGAATTGATCA
CaMV35SF     AAAAAACTCGACGGCCTGTGGGCATTCAGTCTGGATCGCGAAAAACTGTGG

GUSR1       GCGTTGGTGGGAAAGCGCGTTACAAGAAAGCCGGGCAATTGCT
```

PGWB4GFPR with OsERFLPcolF1

```
OsERFLPcolF1 TACTGTACCAAAAGGCTAAGGCCACTGACAGAGGAGATACGTGACAGACAAGTCCCAGTT

OsERFLPcolF1 GAGTTACTGTCCAACCCCCAAATCTAACCCTCTTCTACCACCACCCCCACTCGTGCGCCA

OsERFLPcolF1 CAGCGCTCTCACACGCGCGCCACACTCATGCCATGTATGTCTATATATATGTGCATCTAT

GFPR1                 CACATTGAACGCACGCTACACTGCAAGAAAGGCAAGTATAGGGGTGGCAG
OsERFLPcolF1 ATATCTTAGCCACATTGAACGCACGCTACACTGCAAGAAAGGCAAGTATAGGGGTGGCAG

GFPR1        GTAGTTTATATTCACTGGAAACCGAGAACTTCACTCCTCCCAGCACTCCACACTAGGAGG
OsERFLPcolF1 GTAGTTTATATTCACTGGAAACCGAGAACTTCACTCCTCCCAGCACTCCACACTAGGAGG

GFPR1        ATGCTCACTGTCTGACTGACTGACTGGCTGCTGCTGCTCATTCCTTCTCCTACTCTG
OsERFLPcolF1 ATGCTCACTGTCTGACTGACTGACTGGCTGCTGCTGCTCATTCCTTCTCCTACTCTG

GFPR1        CCTGCCTCCTCTGCTGTGTCCTTGGGAGAATTCTTCAGGGACAGTATCCCTGCAGAGGTG
OsERFLPcolF1 CCTGCCTCCTCTGCTGTGTCCTTGGGAGAATTCTTCAGGGACAGTATCCCTGCAGAGGTG

GFPR1        AGATC-------------------------ACCCAGCTTTCTTGTACAAAGTGGTGATCA
OsERFLPcolF1 AGATCTCTAGACAGGTAAAGGGCGAATTCGACCCAGCTTTCTTGTACAAAGTGGTGATCA

GFPR1        TGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACG
OsERFLPcolF1 TGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACG

GFPR1        GCGACGTGAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACG
OsERFLPcolF1 GCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACG

GFPR1        GCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCA-CC
OsERFLPcolF1 GCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCAACC

GFPR1        CTCGTG-ACCACCTTCACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAA
OsERFLPcolF1 CTCGTGGACCACCTTCACC

GFPR1        GCAGCACGACTT
```

PGWB4GFP with LCHSF

```
GFPR1    CCTTAATGGCTCCGCCAATGTCTCTACGTGGTTTGATGATAATAAGAGTGTCATTTTT-A
LCHSF                          TCTACGTGGTTTGATGATAATGAGAGTGTCATTTTTCA

GFPR1    ATATCCAATGTTTTTTGTCTTCAGGAGTTTGAAATATGAATAGCTCAA-CTCGAAAACTA
LCHSF    ATATCCGGTGTTTTT-GTCTTCGGGAGTTTGAAATATGAATAGCTCAAACTCGAAAACTA

GFPR1    GGCTCCTACCCACAA-TGTTTTGCTAGTGTATTTAGTTAGAAAGAACTAAGATAAAAAA-
LCHSF    GGCTCCTACCTACAAATGTTTTGCTAGTGTATTTAGGTAGAAAGAACTAAAATAAAAAAA

GFPR1    CATAGATTACCTTAACATTCTCATTTATTGTTGTA-CCTTTGTGTTCTATATTATAGACT
LCHSF    CATAGATTACCTTAACATTCTCATTTATTGTTGTAACCTTTGTGTTCTATATTACAGACT

GFPR1    T--GATTCCATTTACCGTCT-CCAACAAAAATACAGATT-GTATGGTTCTTCGACTGCCG
LCHSF    TTTGATTCCATTTACCGTCTACCAACAAAAATACAGATTTGAATGGTTCTTCGACTGCCG

GFPR1    GACACGATAGTCTGGTAAAATGAAACAAAGGAAAAAAAACAAAAATGTTCTGCTTGCTCC
LCHSF    GACACGATAGTCTGGTAAA-TGAAACAAAGGAAAAAAAACAAAAATGTTCTGCTTGCTCC

GFPR1    AACTCAGGTGAACCGCTTGTTTTTGTTATTTCCCACTCCTCGCAGTCTAACCTTCGTGGT
LCHSF    AACTCAGGTGAACCGCTTGTTTTTGTTATTTCCCACTCCTCGCAGTCTAACCTTCGTGGT

GFPR1    CAGACTTAAAGTAGTGCGTGCCCGCTAGCTACCCTCAGGTGGCAACTACCAATTAACTCG
LCHSF    CAGACTTAAAGAAGTGCGTGCCCGCTAGCTACCCTCAGGTGGCAACTACCAATTAACTCG

GFPR1    GCGGAGACGACGATCACGTGGGAGCTCATCTGCCACTCCCTCACCTATAAATGTCAGTCT
LCHSF    GAGGGGACGACGATCGCGTGGGAGCTCATCTGCCACTCCCTTACCTATAAATGTCGGTCT

GFPR1    CCCTCAACCCCTCCTCACTCATCCCTTCCATCCACCACGCTCTTCCTCACCCTCCCAGTC
LCHSF    CCCTCAACCCCTCCTCACTCATCCCTTCCATCCACCACTCTCTTCCTCACCCTCCCAGTC

GFPR1    CAACCA--------------------CCCAGCTTTCTTGTACAAAGTGGTGATCATGGTG
LCHSF    CAACCAGGATCCAAGGGCGAATTCGACCCAGCTTTCTTGTACAAAGTGGTGATCATGGTG

GFPR1     AGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGAC
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LCHSF     AGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGAC

GFPR1     GTGAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAG
LCHSF     GTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAG

GFPR1     CTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTG
LCHSF     CTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTG

GFPR1     ACCACCTTCACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCAC
LCHSF     ACCACCCTCACCT

GFPR1     GACTTCTT
```

HvMYB26RNAISRDXF1 with YFP-NY-R1

```
YFP-NY-R1    CAAGCTGTGGCCACGCAGTGCCCTCCATCTTCAGTTCACAACTCGGCGGCATGGCTCGGC

YFP-NY-R1    TTCGCCTCTCAGCCGCTGCTCCTCCCCGTCCACGGCGGCGACCTCCAGTACGCCGTCGAC
HvMYB26RNAIF1                                    GCGGCGACCTCCAGTACGCCGTCGAC

YFP-NY-R1    GGGGAGTTCATCAGGCTGTGCCGCGCCGCCGATAACGCCTACCCGGACAACGGCGGCCCC
HvMYB26F1    GGGGAGTTCATCAGGCTGTGCCGCGCCGCCGATAACGCCTACCCGGACAACGGCGGCCCC

YFP-NY-R1    AACCTCTTGGCTCAAGAAGGCGCGGCTGATCGGAGCTGCCTCCCGGCGTTTGCCGAGCCA
HvMYB26F1    AACCTCTTGGCTCAAGAAGGCGCGGCTGATCGGAGCTGCCTCCCGGCGTTTGCCGAGCCA

YFP-NY-R1    AAGGGCGCCGGCGCTTTCGCCGCCGACCCGGGCATGGGTCCGGTGGTGGACTTCATGGAC
HvMYB26F1    AAGGGCGCCGGCGCTTTCGCCGCCGACCCGGGCATGGGTCCGGTGGTGGACTTCATGGAC

YFP-NY-R1    GCCATCCTCGGGTCCTCGTCCACGTCGGCGGCCAGCATTTCGTCCGTCGACAGCTTCTCG
HvMYB26F1    GCCATCCTCGGGTCCTCGTCCACGTCGGCGGCCAGCATTTCGTCCGTCGACAGCTTCTCG

YFP-NY-R1    GCCAACACCGGCATGCAGCTTCACTGGGTTCCC-TG-------------AACCCAGCTT-
HvMYB26F1    GCCAACACCGGCATGCAGCTTCACTGGGTTCCCCTGGATCTGGATCTAGAACTCCGTTTG

YFP-NY-R1    ----TC------------TTGTACC--------TTGTACAAAGTGGTGCTCGAGATGGTG
HvMYB26F1    GGTTTCGCTAAGGGCGAATTCGACCCAGCTTTCTTGTACAAAGTGGTGCTCGAGATGGTG

YFP-NY-R1    AGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGAC
HvMYB26F1    AGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGAC

YFP-NY-R1    GTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAG
HvMYB26F1    GTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAG

YFP-NY-R1    CTGACCCTGAAGCTGATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTG
HvMYB26F1    CTGACCCTGAAGCTGATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTG

YFP-NY-R1    ACCACCCTGGGCTACGGCCTGCAGTGCTTCGCCCGCTACCCCGACCACATGAAGCAGCAC
```

HvMYB26F1 ACCACCCTGGGCTACGGCCTGCAGTGCTTCGCCCGCTACCCCGACC

YFP-NY-R1 GACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAG

FP-NY-R1 GACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAAC

YFP-NY-R1 CGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTG

YFP-NY-R1 GAGTACAACTACAACAGCCACAACGTCTATAT

a) Rice x Brachypodium

```
Rice            ATGCCGCGGCGCGCGAGGGCGAGGGGCGGCGGCGG---CGGCGGCGGGGAGGAGGTGAAG
Brachypodium    ATGCCGCGCCGTCCGAGGGCTCCCCGCGGCGGCGGTAGCGGAGGCGGAGAGGAGGTGAAG

Rice           GTGGAGGATGACTTCATCGACTCGGTCCTGAATTTCGGTGGAGGTGGTGGGGGAGAGGAG
Brachypodium   ATGGAGGA---CTTCGTGGAGTCGATGCTCAACTTCGGTGGCGGCGGCGGCGGTGGAGGG

Rice           GACGGCGACGACGGGGAGGAGGAGCAGCAGCAGCAGGCGGCGGCGGCGGCGATGGGG
Brachypodium   GAGGACGAGAGCGAGGAAGGGGAGCAGTTGC---------CGGCGGGGGAGGAGACC---

Rice           AAGGAGTTCAAGTCCAAGAACCTGGAGGCCGAGCGGCGGAGGAGGGGGAGGCTCAACGGC
Brachypodium   AAGTA---CAAGTCCAAGAACCTGGATGCCGAGAGGCGGCGGAGGGGCAGGCTCAACAAC

Rice           AACATCTTCGCGCTCAGGGCGGTCGTGCCCAAGATCACCAAGATGAGCAAGGAGGCCACC
Brachypodium   AACATCCTCGCGCTCAGGGCCGTCGTGCCCAAGATCACCAAGATGAGCAAGGAGTCCACC

Rice         TTGTCGGACGCGATCGAACACATCAAGAATCTCCAGAACGAGGTTCTTGAGCTGCAGCGC
Brachypodium CTGTCGGACGCAATCGATCACATCAAGAAGCTCCAGAACGAGGTCCTCGAGCTGCAGAGC

Rice         CAGCTCGGCGACTCACCCGGTGAGGCTTGGGAGAAGCAATGCAGCGCGTCCTGCTCTGAA
Brachypodium CAGCTCGCCGACTCCCCCGGCGAGGCTTGGGAGAAGCAAGGCAGCGCGTCGTGCTCCGAA

Rice         TCCTTCGTCCCCACTGAGAACGCCCATTATCAGGGTCAGGTGGAATTGATCTCTCTTGGG
Brachypodium TCCTTCGTCCCCACAGACAACATCCATTATCAGGGTCAGGTGGAGCTGATACCTCTAGGA

Rice         TCATGCAAGTATAACCTGAAGATCTTCTGGACCAAGAGGGCAGGCCTCTTCACCAAGGTG
Brachypodium TCATTTAAGTACAACCTCAAGATCTTTTGGACCAAAAAGGCAGGCCTCTTCACCAAAGTC

Rice         CTGGAAGCACTCTGCAGCTACAAAGTGCAAGTGCTCAGCCTGAACACCATATCCTTCTAC
Brachypodium CTGGAAGCACTCTGCAGCTACAATGTGCAAGTGCTCAGCCTGAACACCATCACCTTCTAT

Rice         GGCTACGCAGAGAGTTTCTTCACCATCGAGGTGAAGGGTGAGCAGGATGTTGTGATGGTG
Brachypodium GGCTATGCAGAGAGTTTCTTCTCCATTGAGGTGAAGGGTGAGCCGGATGTTGTGATGGTG

Rice         GAGCTGAGGAGCCTTCTCTCCAGCATTGTGGAGGTTCCAAGCATCTGA
Brachypodium GAGCTAAGGAACCTCCTGTCCAGTATTGTGGAGGTTCCAAGCATGTGA
```

b) *Brachypodium* x Barley

```
Brachypodium   ATGCCGCGCCGTCCGAGGGCCTCCCGCGGCGGC---AGCGGCGGCG---AGGAGGTGAAG
Barley         ATGCCGCGCCGTCCGAGGGCTCCCCGCGGCGGCGGTAGCGGAGGCGGAGAGGAGGTGAAG

Brachypodium   ATGGAGGACTTCGTCGAGTCGATGCTGAACCTGGGCGGCGGCGGCGGCG----AGGGC--
Barley         ATGGAGGACTTCGTGGAGTCGATGCTCAACTTCGGTGGCGGCGGCGGCGGTGGAGGGGAG

Brachypodium   GAGGAGAGCGAGGAAGGGGAGCAGCTGCCGGCGGCGGACGCGACGCAGTACAAGTCCAAG
Barley         GACGAGAGCGAGGAAGGGGAGCAGTTGCCGGCGGGGGAGGAGACCAAGTACAAGTCCAAG

Brachypodium   AACCTGGACGCCGAGCGCCGGAGGAGGGGCAGGCTCAACGGCAACATCCTCGCGCTCAGG
Barley         AACCTGGATGCCGAGAGGCGGCGGAGGGGCAGGCTCAACAACAACATCCTCGCGCTCAGG

Brachypodium   GCCGTCGTGCCAAACATCACCAAGATGAGCAAGGAGTCCACCCTGGCGGACGCCATCGAT
Barley         GCCGTCGTGCCCAAGATCACCAAGATGAGCAAGGAGTCCACCCTGTCGGACGCAATCGAT

Brachypodium   CACATCAAGAAGCTCCAGAACCAGGTCCTTGAGCTGCAGAGCCAGCTCGCCGACTCGCCC
Barley         CACATCAAGAAGCTCCAGAACGAGGTCCTCGAGCTGCAGAGCCAGCTCGCCGACTCCCCC

Brachypodium   GGTGAGGCCTGGGAGAAGCAAGGCAGCGCGTCCTGTTCTGAATCCTTCGCCCCCACCGAC
Barley         GGCGAGGCTTGGGAGAAGCAAGGCAGCGCGTCGTGCTCCGAATCCTTCGTCCCCACAGAC

Brachypodium   AACATCCATTATCAGGGTCAGGTAGAGCTGATACCTCTAGGGTCTTGTAAGTACAACCTC
Barley         AACATCCATTATCAGGGTCAGGTGGAGCTGATACCTCTAGGATCATTTAAGTACAACCTC

Brachypodium   AAGATCTTCTGGACCAAAAGGGCAGGCCTCTTCACCAAGGTGCTGGAGGCACTCTGCAAC
Barley         AAGATCTTTTGGACCAAAAAGGCAGGCCTCTTCACCAAAGTCCTGGAAGCACTCTGCAGC

Brachypodium   TACAATGTGCAAGTGCTTAGCCTCAACACCATCACCTACTATGGCTATGCAGAGAGTTTC
Barley         TACAATGTGCAAGTGCTCAGCCTGAACACCATCACCTTCTATGGCTATGCAGAGAGTTTC

Brachypodium   TTCTGCATTGAGGTGAAGGGTGAGCAGGATGTTGTGATGGTGGAGCTAAGGGACCTGCTG
Barley         TTCTCCATTGAGGTGAAGGGTGAGCCGGATGTTGTGATGGTGGAGCTAAGGAACCTCCTG

Brachypodium   TCCAGTATTGTAGAGGTTCCAAGCATTTGA
Barley         TCCAGTATTGTGGAGGTTCCAAGCATGTGA
```

c) Multiple alignment

```
AtDYT1          ------------------------------------------------------------
OsUTD1          ATGCCGCGGCGCGCGAGGGCGAGGGGCG---GCGGCGGCGGCGGCGGGGAGGAGGTGAAG
BRADI1G25230    ATGCCGCGCCGTCCGAGGGCTCCCCGCGGCGGCGGTAGCGGAGGCGGAGAGGAGGTGAAG
HvDYT1          ATGCCGCGCCGTCCGAGGGCCTCCCGCGGCGGCAGC------GGCGGCGAGGAGGTGAAG

AtDYT1          ---------------------------------ATGGGTGGAGGAAGCA-----------
OsUTD1          GTGGAGGATGACTTCATCGACTCGGTCCTGAATTTCGGTGGAGGTGGTGGGGGAGAGGAG
BRADI1G25230    ATGG--AGGACTTCGTGGAGTCGATGCTCAACTTCGGTGGCGGCGGCGGCGGTGGAGGG
HvDYT1          ATGG---AGGACTTCGTCGAGTCGATGCTGAACCTGGGCGGCGGCGGCGGCGAGGGCG--

AtDYT1          GATTTCAAGAACCAGTGAGGATGAGCCGTAGG--AAACAAGTAACAAAAGAGAAGGAAGA
OsUTD1          GACGGCGACGA-CGGGGAGGAGGAGCAGCAGCAGCAGGCGGCGGC---GGCGGCGAT
BRADI1G25230    GAGGACGAGAG-CGAGGAAGGGGAGCAG------TTGCCGG------------CGGGGGA
HvDYT1          ----AGGAGAG-CGAGGAAGGGGAGCAG------CTGCCGG------------CGGCGGA

AtDYT1          AGATGAAAACTTCAAATCTCCAAATCTTGAAGCAGAGAGACGTAGAAGAGAGAAGCTT--
OsUTD1          GGGGAAGGAGTTCAAGTCCAAGAACCTGGAGGCCGAGCGGCGGAGGAGGGGGAGGCTCAA
BRADI1G25230    GGAGACCAAGTACAAGTCCAAGAACCTGGATGCCGAGAGGCGGCGGAGGGGCAGGCTCAA
HvDYT1          CGCGACGCAGTACAAGTCCAAGAACCTGGACGCCGAGCGCCGGAGGAGGGGCAGGCTCAA

AtDYT1          CATTGTCGGCTTATGGCTCTGCGATCTCATGTCCCCATTGTCACCAACATGACTAAAGCA
OsUTD1          CGGCAACATCTTCGCGCTCAGGGCG--GTCGTGCCCAAGATCACCAAGATGAGCAAGGAG
BRADI1G25230    CAACAACATCCTCGCGCTCAGGGCC--GTCGTGCCCAAGATCACCAAGATGAGCAAGGAG
HvDYT1          CGGCAACATCCTCGCGCTCAGGGCC--GTCGTGCCAAACATCACCAAGATGAGCAAGGAG

AtDYT1          AGTATTGTTGAAGATGCGATTACTTACATAGGAGAGCTTCAAAACAATGTTAAGAATCTC
OsUTD1          GCCACCTTGTCGGACGCGATCGAACACATCAAGAATCTCCAGAACGAGGTTCTTGAGCTG
BRADI1G25230    TCCACCCTGTCGGACGCAATCGATCACATCAAGAAGCTCCAGAACGAGGTCCTCGAGCTG
HvDYT1          TCCACCCTGGCGGACGCCATCGATCACATCAAGAAGCTCCAGAACCAGGTCCTTGAGCTG
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AtDYT1       TTAGAGACATTTCATGAAATGGAAGAAGCTCCTCCTGAGATTGAT----GA--------A
OsUTD1       CAGCGCCAGCTC----------GGCGACTCACCCGGTGAGGCTTGGGAGAAGCAATGCA
BRADI1G25230 CAGAGCCAGCTC----------GCCGACTCCCCCGGCGAGGCTTGGGAGAAGCAAGGCA
HvDYT1       CAGAGCCAGCTC----------GCCGACTCGCCCGGTGAGGCCTGGGAGAAGCAAGGCA

AtDYT1       G---------------AACAAACGGATCCAATGATAAAACCTG-AAGTTGAAACTAGTGA
OsUTD1       GCGCGTCCTGCTCTGAATCCTTCGTCCCCACTGAGAACGCCCATTATCAGGGTCAGGTG-
BRADI1G25230 GCGCGTCGTGCTCCGAATCCTTCGTCCCCACAGACAACATCCATTATCAGGGTCAGGTG-
HvDYT1       GCGCGTCCTGTTCTGAATCCTTCGCCCCCACCGACAACATCCATTATCAGGGTCAGGTA-

AtDYT1       TCTTAACGAAGAGATGAAGAAACTCGGAATCGAGGAGAATGTGCAATTGTGTAAGATTGG
OsUTD1       ----------GAATTGATCTCTCTT----------------------------------GG
BRADI1G25230 ----------GAGCTGATACCTCTA----------------------------------GG
HvDYT1       ----------GAGCTGATACCTCTA----------------------------------GG

AtDYT1       GGAGAGGAAGTTTTGGTTAAAGATCATAACAGAGAAGAGAGATGGGATCTTTACTAAATT
OsUTD1       GTCATGCAAGTATAACCTGAAGATCTTCTGGACCAAGAGGGCAGGCCTCTTCACCAAGGT
BRADI1G25230 ATCATTTAAGTACAACCTCAAGATCTTTTGGACCAAAAAGGCAGGCCTCTTCACCAAAGT
HvDYT1       GTCTTGTAAGTACAACCTCAAGATCTTCTGGACCAAAAGGGCAGGCCTCTTCACCAAGGT

AtDYT1       CATGGAGGTTATGAGATTTCTCGGATTC---GAGATTATCGATATTAGTC-------TAA
OsUTD1       GCTGGAAGC---------ACTCTGCAGCTACAAAGTGCAAGTGCTCAGCCTGAACACCAT
BRADI1G25230 CCTGGAAGC---------ACTCTGCAGCTACAATGTGCAAGTGCTCAGCCTGAACACCAT
HvDYT1       GCTGGAGGC---------ACTCTGCAACTACAATGTGCAAGTGCTTAGCCTCAACACCAT

AtDYT1       CAACTTCAAATGGAGCAATTCTTATTAGTGCCTCTGTTCAGACACAGGAACTCTGTGATG
OsUTD1       ATCCTTCTAC---GGCTACGCAGAG---AGTTTCTTCAC-----------CATCGAGGTG
BRADI1G25230 CACCTTCTAT---GGCTATGCAGAG---AGTTTCTTCTC-----------CATTGAGGTG
HvDYT1       CACCTACTAT---GGCTATGCAGAG---AGTTTCTTCTG-----------CATTGAGGTG
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AtDYT1       T----TGAACAGACAAAAGATTTTCTTTTGGAAGTTATGAGAAGCAAT----CCATAA--
OsUTD1       AAGGGTGAGCAG------GATGTTGTGATGGTGGAGCTGAGGAGCCTTCTCTCCAGCATT
BRADI1G25230 AAGGGTGAGCCG------GATGTTGTGATGGTGGAGCTAAGGAACCTCCTGTCCAGTATT
HvDYT1       AAGGGTGAGCAG------GATGTTGTGATGGTGGAGCTAAGGGACCTGCTGTCCAGTATT


AtDYT1            ----------------------
OsUTD1            GTGGAGGTTCCAAGCATCTGA
BRADI1G25230      GTGGAGGTTCCAAGCATGTGA
HvDYT1            GTAGAGGTTCCAAGCATTTGA
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APPENDIX 3 PRIMERS

All primers that used for this project

Primer name	Primer sequence 5-3	Tm°C
OsERFL1promSacIF1	TACCTGgagctcAAGCTTCCATCCCTGCAGGAA	59
OsERFL1promXbaIR1	TACCTGtctagaGATCTCACCTCTGCAGGGAT	59
OsERFL1colR1	GCCAGTACGCCGGTAATAAA	59
M13R	CAGGAAACAGCTATGAC	59
M13F	TGTAAAACGACGGCCAG	59
LCHSPF	AAGCTTAGTCGTGGTTTGTGAGCT	61
LCHSPR	GGATCCTGGTTGGACTGGGAGG	65.8
LCHSp colR1	GCGAGGAGTGGGAAATAACA	59
35S F1 Bst B1	GGGTTCGAAGAATTCCCATGGAGTCAAAAATTC	59
35S R1 sal1	GGGGGTCGACGGTACCCTGTCCTCTCCAAATGAAAT	59
PGWB3 GUS F	CCCCAACCCGTGAAATCAAA	59
PGWB3 GUSR1	AGCAATTGCCCGGCTTTCTT	59
GFP_PGWB5_R1	AAGTCGTGCTGCTTCATGTG	59
Ubi F	TTGATATCGAATTCCGTGCAG	59
Ubi R	AGTAACACCAAACAACAGGGTGA	59
UbiProF1	ATGCTCACCCTGTTGTTTGG	59
P BractF	TTTAGCCCTGCCTTCATACG	59
HvAMSqpcrF3	TCACCAGCAGAGCATCATCG	61
HvAMSqpcrR3	CTCGTAGCCCGTGAGGAGAT	61
AtIPT4qpcrF1	CCGGTTCTGGCAAGTCATCA	59
AtIPT4qpcrR2	AACTCGGAGCAGATTGAGCC	59

Primer name	Primer sequence 5-3	Tm°C
ATIPT4ColF1	ACAAGGGCTCAATCTGCTCC	59
AtIPT4colR1	ATCCCCAATGGCTCTTTCGG	59
AtIPT8ColF1	CCCTCCGGATGATAGCGAAC	59
AtIPT8ColR1	AACACTCGTACCTCAAGCCG	59
pGWB8_FOR_Seq	GGAAGTTCATTTCATTTGGAGAGA	59
ATIPT4F	ATGAAGTGTAATGACAAAATGGTTGTG	59
AT IPT4 R1 stop	CTAGTTAAGACTTAAAAATCTTTTTAGTAAATAAAAGTAA	59
ATIPT8F	ATGCAAAATCTTACGTCCACATTCG	59
At IPT8 R1 stop	TCACACTTTGTCTTTCACCAAGAAG	59
HvTubF	AGTGTCCTGTCCACCCACTC	60
HvTubR	AGCATGAAGTGGATCCTTGG	60
HvMYB35qpcrF2	GGGAAGCAGGTGGTCTCTGA	62
HvMYB35qpcrR2	GGTTCACGGGGAGGTAGGAG	62
HygqpcrF2	GCGAGAGCCTGACCTATTGC	61
HygqpcrR2	CGGTGTCGTCCATCACAGTT	61
HvDYT1qpcrF1	TTCGTCGAGTCGATGCTGAA	59
HvDYT1qpcrR4	TGCAGCTCAAGGACCTGGTT	59

APPENDIX 3 MEDIA RECIPES AND CHEMICAL COMPONENT

Chemicals component for Regeneration medium MS-SI (MS-Shoot Induction).

Material required	Amount 1L	0.5 L
MS-basal salts	4.4 g/L	2.2 g/L
Sucrose	60 g/L	30 g/L
Activated charcoal	2.5 g/L	1.25 g/L
Phytogel	2.6 g/L	1.3 g/L
Picloram	2.0 mg/L	(1 ml) = 1000 µl
BAP	1.0 mg/L	(0.5ml) = 500 µl

TBE Buffer 10X

minerals	concentration
Tris-HCl (PH 7.5)	10mM
Na_2- EDTA	1mM
NaCl	0.89M

Luria-Bertani (LB) medium

components	Amount for 1 litre
Yeast Extract	0.5%
Bacto-agar	1.5%
Sodium chloride	10g
Tryptone	1%
Water distillate	Complete to 1liter

Chemicals component for selection Arabidopsis seed medium

Material	Gram for 1L
Murashinge and skoog basal medium MS	2.46g
Agar	7g
Sucrose	5g
Phytagel	2.8g

Barley transformation materials:

Stock solution are prepared for barley transformation,

Components	Stock concentration	Quantity added to 50ml
Myo-inositol	35g/l	1.75g
Proline	69g/l	3.45g
Thiamine	10g/l	0.5g
Dicamba	2.5g/l	0.125g
$CuSO_4.5H_2O$	1.25g/l	0.0625g
2,4,D	2.5mg/ml	0.125g
BAP	1mg/ml	0.050g
Timentin	160mg/ml	2.4g
Hygromycin	50mg/ml	2.5g

*All stock solution diluted in distilled water, except 2,4,D that is prepared with absolute ethanol.

*Timentin was prepared in total volume of 15ml.

First: Callus Induction Medium:

The callus induction medium was prepared at two times the required concentration the following components:

Components	Quantity for 500ml
Murashinge and skoog plant salt base (Duchefa M0221)	4.3g
maltose	30g
Casein hydrolysate	1g

After preparation, this medium the pH was adjusted to 5.8 and then filter sterilised in an autoclaved bottle.

*In advance, phytagel was prepared at two time the required concentration (2x phytagel), 7g Phytagel was added into 1000ml of distilled water, and then vigorously by hand with autoclaved.

Callus induction plates was prepared for transformation by warm up of phaytagel and callus induction medium in water bath at 60°C. The following stock solution then was added into the callus induction medium before adding phytagel.

Components	Stock concentration	Quantity	Total volume for 500ml
Myo-inositol	35g/l	5ml	250ml of callus induction medium and 250ml of phaytagel
Proline	69g/l	5ml	
Thiamine	10g/l	50µl	
Dicamba	2.5g/l	500 µl	
$CuSO_4.5H_2O$	1.25g/l	500 µl	
Timentin	160mg/ml	500 µl	
Hygromycin	50mg/ml	500 µl	

Second: Transition Medium:

The Transition medium was prepared at two times the required concentration the following components

Components	Quantity for 500ml
Murashinge and skoog modified plant salt base without NH_4NO_3(Duchefa M0238)	2.7g
maltose	20g
NH_4NO_3	165mg
Glutamine	750mg

*After preparation, this medium the pH was adjusted to 5.8 and then filter sterilised in an autoclaved bottle.

*In advance, phytagel was prepared at two time the required concentration (2x phytagel), 7g Phytagel was added into 1000ml of distilled water, and then vigorously by hand with autoclaved.

Transition induction plates was prepared for transformation by warm up of phaytagel and callus induction medium in water bath at 60°C. The following stock solution then was added into the transition induction medium before adding phytagel.

Components	Stock concentration	Quantity	Total volume for 500ml
Myo-inositol	35g/l	1.43ml	250ml of callus induction medium and 250ml of phaytagel
Thiamine	10g/l	20µl	
2, 4, D	2.5mg/ml	500 µl	
BAP	1mg/ml	50 µl	
$CuSO_4.5H_2O$	1.25g/l	500 µl	
Timentin	160mg/ml	500 µl	
Hygromycin	50mg/ml	500 µl	

Third: Regeneration medium

The Regeneration medium was prepared at two times the required concentration the following components

Components	Quantity for 500ml
Murashinge and skoog modified plant salt base without NH_4NO_3(Duchefa M0238)	2.7g
maltose	20g
NH_4NO_3	165mg
Glutamine	750mg

After preparation, this medium the pH was adjusted to 5.8 and then filter sterilised in an autoclaved bottle.

*In advance, phytagel was prepared at two time the required concentration (2x phytagel), 7g Phytagel was added into 1000ml of distilled water, and then vigorously by hand with autoclaved.

Regeneration medium was prepared for transformation by warm up of phaytagel and callus induction medium in water bath at 60°C. The following stock solution then was added into the regeneration medium before adding phytagel.

Components	Stock concentration	Quantity	Total volume for 500ml
Myo-inositol	35g/l	1.43ml	250ml of callus induction
Thiamine	10g/l	20µl	medium and 250ml of phaytagel
Timentin	160mg/ml	500 µl	
Hygromycin	50mg/ml	500 µl	

Fourth: Callus Induction Medium (without growth regulator):

The callus induction medium was prepared at two times the required concentration the following components:

Components	Quantity for 500ml
Murashinge and skoog plant salt base (Duchefa M0221)	4.3g
maltose	30g
Casein hydrolysate	1g

After preparation, this medium the pH was adjusted to 5.8 and then filter sterilised in an autoclaved bottle.

*In advance, phytagel was prepared at two time the required concentration (2x phytagel), 7g Phytagel was added into 1000ml of distilled water, and then vigorously by hand with autoclaved.

Callus induction plates was prepared for transformation by warm up of phaytagel and callus induction medium in water bath at 60°C. The following stock solution then was added into the callus induction medium before adding phytagel.

Components	Stock concentration	Quantity	Total volume for 500ml
Myo-inositol	35g/l	5ml	250ml of callus induction medium
Proline	69g/l	5ml	and 250ml of phaytagel
Thiamine	10g/l	50µl	
Timentin	160mg/ml	500 µl	
Hygromycin	50mg/ml	500 µl	

Antibiotics and growth regulator Stocks solution

Material	Stock solution	Final concentration Working concentration	µl per 1000ml
Zeatin	2mg/ml	1 mg/L	500 µl
Kinetin	2mg/ml	1 mg/L	500 µl
Picloram	4mg/ml	2 mg/L	500 µl
Lipoic acid	0.5M	50 µM	100 µl
Lipoic acid	0.5M	100 µM	200 µl
BAP	2mg/ml	1 mg/L	500 µl
Timentine	100mg/ml	250 mg/l	2500 µl
Timentine	100mg/ml	300 mg/l	3000 µl
kanamycin	50mg/ml	50 µg/l	1000 µl
Refampicin	25 mg/ml	50µg/l	2000 µl
Acetosyrin-gone	1M	100 µM	100 µl
Cefotaxime	400mg/ml	400 mg/L	1000 µl
Silwet			