The Functional Analysis of Soybean (*Glycine max* L.) Flowering Genes

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Doctor of Philosophy

December 2019

Faculty of Veterinary and Agricultural Sciences

The University of Melbourne

Submitted in total fulfilment of the requirement of the degree of
Doctor of Philosophy

Abstract

Soybean (*Glycine max* L.) is one of the major crop plants since its seeds contain high levels of oil and protein. In addition, soybean's unique ability in fixing nitrogen makes it a key plant for sustainable agriculture due to the increasing cost of nitrogen fertilizers caused by continuing depletion of petroleum. The demand for improvement of soybean yield has been rapidly growing due to the plant's high nutritional values and its significance in sustainable agriculture. Flowering is a major step in plant life cycles as it plays crucial roles in reproductive success. Late flowering under favourable conditions allow plants to maximize their vegetative growth, which ultimately leads to improved seed production, whilst early flowering allows plants to secure their progenies under adverse growth environments. Comparative analysis of flowering genes between Arabidopsis and soybean has revealed conservation of most flowering genes. However, functions of most flowering genes in soybean are still unknown. Therefore, investigation of soybean flowering genes is expected to provide insight into flowering mechanisms of soybean.

Plant genetic transformation is an important tool to improve agricultural traits and investigate functions of genes. Since reported success in soybean genetic transformation has been limited to inferior-breeding cultivars, development of stable transformation systems for commercial soybean cultivars will provide a new solution to meet the ever-increasing demand for soybean. In the present study, transformation systems for commercial cultivars of soybean were developed using the *Agrobacterium*-mediated transformation method. Transgenic soybean plants (cv. Bragg) containing yellow fluorescence protein (YFP) and herbicide resistant gene (*bar*) were produced using half-seed transformation method. Shoot elongation efficiency was increased (6 fold) by addition of phenolic compound inhibitors [adenine hemisulfate (40mg/L) and PVP 40,000 (500 mg/L)] during shoot elongation, resulting in improvements in the growth of transgenic shoots. Total 23 independent T0 putative transgenic lines were produced and herbicide resistance was confirmed via basta brush test (100mg/L glufosinate). Total two basta resistance lines exhibited YFP expression in leaves. Stable expressions of transgenes were observed in T1 and T2 generations.

The roles of soybean *LFY* homolog (Glyma.06G163600.1) in flowering initiation was confirmed in this study. *LEAFY* gene (*LFY*) is one of floral meristem identity genes and plays essential roles in flowering. In soybean, two *LFY* homologs

(Glyma.04G202000.1 and Glyma.06G163600.1) are annotated and they show high sequence similarity (Glyma.04G202000.1: 73.4% and Glyma.06G163600.1: 69.5%) with Arabidopsis *LFY (AtLFY).* Furthermore, soybean *LFY* homologs have two conserved DNA-binding domains (N- and C-domains). The expression levels of both soybean *LFY* homologs gradually increased in flowering inductive conditions and Glyma.06G163600.1(*GmLFY1*) showed a higher expression than Glyma.04G202000.1 (*GmLFY2*). These high sequence conservation and expression patterns of soybean *LFY* homologs suggested *GmLFY* genes may have roles in flowering initiation. Ectopic expression of *GmLFY1* in transgenic Arabidopsis and tobacco plants induced early flowering phenotypes. Moreover, up-regulations of genes (*AP1*, *SOC1* and *LFY*) involved in flowering were also detected in transgenic plants. These results suggest that *GmLFY1* may regulate flowering time via the conserved process as in Arabidopsis. Besides, tissue-specific *GUS* expressions on sepals were detected in flowers of transgenic tobacco plants (*GmLFY1::GUS*), indicating that *GmLFY1* is also involved in flower development.

The ageing pathway is one of the identified flowering genetic pathways and the *miR156-miR172* module plays major roles in this process via repression of their target genes. In this study, expression patterns of soybean *miR156* and *miR172* (*gma-miR156a*, *gma-miR172a*) in vegetative and reproductive developments were confirmed. Expression of *gma-miR156a* was higher than that of *gma-miR172a* in the vegetative developmental stage and it decreased with ageing. On the other hand, expression of *gma-miR172a* was elevated under flowering inductive conditions. These expression patterns of soybean *miR156a* and *miR172a* genes suggest that they may be involved in the developmental process. Ectopic expression of *gma-miR156a* in transgenic tobacco plants caused significant delays in flowering initiation with extended juvenile developmental traits (round shape of leaves). In contrast, transgenic tobacco plants overexpressing *gma-miR172a* exhibited early flowering phenotypes with adult traits on leaves (narrow shape). Significant down-regulations of *miR156* target genes (*SPL* transcription factor family) and *miR172* target genes *(AP2-like* genes) were detected in transgenic tobacco plants. These results showed that *gma-miR156a* and *gma-miR172a* may regulate ageing process via repressions of their target genes.

In the present study, transgenic commercial soybean cultivar (cv. Bragg) was produced using the *Agrobacterium*-mediated method. In addition, this study provided evidence of conserved roles of *GmLFY1*, *gma-miR156a* and *gma-miR172a* in flowering and plant developments via heterologous expressions in transgenic Arabidopsis and

tobacco plants. *GmLFY1* is involved in floral meristem development and initiation of flowering. *gma-miR156a* is responsible for juvenile developments via repression of *SPL* transcription factor family. In contrast, *gma-miR172a* plays major roles in adult developmental phase by down-regulations of its target genes. These results will provide new insights on the genetic improvement of soybean.

Declaration

This is to confirm that the thesis comprises my original work towards the PhD. Due acknowledgements have been made in the text to all published or unpublished material used. The thesis is less than 100,000 words in length, exclusive of tables and bibliography.

Sangil Lee
December, 2019

Acknowledgements

I sincerely thank the University of Melbourne for providing me with financial support (MIRS and MIFRS scholarships) during the course of this research. Very special gratitude goes out to everyone at the Faculty of Veterinary and Agricultural Sciences at the University of Melbourne for providing the studentship funding. It would have been impossible to complete my doctorate degree without the financial support of the University of Melbourne’s Faculty of Veterinary and Agricultural Sciences.

I wish to express my sincere appreciation to my supervisor, Prof. Prem Bhalla and Prof. Mohan Singh who have provided me not only with kind advice, guidance and supervision throughout the course of this research but also have inspired me to develop new insight for research and life in general. I would like to pay my special regards to their continuous support throughout my PhD study and related research, for their patience, motivation and immense knowledge. Their advice and guidance helped me during my research and the writing of this thesis. It is hard to imagine having better advisors and mentors for my doctorate degree. It was lucky to study under their supervision and I am very proud to be a member of the Plant Molecular Biology and Biotechnology group.

I would like to thank the laboratory managers for their assistance and to Steve Elefteriadis for his great work involving the maintenance of the glasshouse. I wish to acknowledge the excellence of the biomedical histology facility. I wish to thank all my laboratory members who have provided help, support and a warm atmosphere. It was pleasure to meet and work with all of them. I would like to pay to my special regards to Dr. Lim Chee Liew, Dr. Martin O’Brien, Dr. Etsuko Itabashi, Dr, Noorina Seedat, Dr. Danny Heang, Dr. Edwin Wong, Dr. Paul Knight, Dr. Junji Miyazaki, Dr. Marta Cifuentes, Dr. Agnieszka Golicz, Dr. Wei Li, Dr. Akanksha Sharma and Dr. Aqsa Tabssum for their kind advice on my research. I give sincere thanks to my colleagues and friends, Faliq, Hina, Senhao, Sukanya, Neeta and Divya for their support.

I would like to thank my family for loving and supporting me in all ways during my doctorate degree and my life in general. Most importantly, I am indebted to my wife, Erica, and our precious daughter, Sofia, who have provided me with everlasting support.

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Chapter 1. Introduction

Sustainable agriculture has begun to present itself as a solution to issues of food security that the world now faces. The green evolution of the 1960s led to great improvements and dramatic increases in agricultural productivity. The significant improvements in three major parts of agriculture, development of new variety, use of nitrogen fertilizer and mechanization have lead great improvement on total yield of agriculture (Ramankutty et al., 2018). Advances in plant breeding and genetics have enabled developments of new varieties with desirable characteristics such as high-yield, dwarf and abiotic- or biotic resistance (Evenson and Gollin, 2003). Developments and cultivations of new cultivars in crop plants such as rice, wheat and maize have significantly increased yields in developing countries (Evenson and Gollin, 2003). The invention of techniques to synthesize nitrogen fertilizer from atmosphere (Haber-Bosch process) opened new era in agriculture (Ramankutty et al., 2018). Before advent of this process, nitrogen had been a major limited nutrient in soils (Ramankutty et al., 2018). Since the discovery of process for nitrogen synthesis, the usage of nitrogen fertilizers has been rapidly increased (Ramankutty et al., 2018). In addition, the increase total yields in agriculture by applications of synthetic nitrogen fertilizer have provided foods for more than quarter of world population over the last 100 years (Erisman et al., 2008). Even though total yield of agriculture has gradually increased after the green evolution, around 2 billion people have suffered from imbalanced diets and 800 million people are still in hunger (Tulchinsky, 2010; FAO, IFAD and WPA, 2015). Since yield increase in agriculture have been met by expansion of agricultural lands via clearing of lands such as deforestation, agriculture now has been a major factor for global environmental degradation. In terms of environment and food security, sustainable agriculture provides an unparalleled solution (Foyer et al., 2016).

Importance of legumes in sustainable agriculture

Legumes have become important plants for agricultural sustainability due to it's unique ability to fix biological nitrogen (Foyer et al., 2016). *Leguminosae* (*Fabacea*e), the flowering plant in legume family, is cultivated throughout world in various environmental conditions (Lewis et al., 2005). *Leguminosae* is the third largest family in plant kingdom consisting of three subfamilies, *Caesalpiniodeae, Mimosoideae* and *Papilionoideae.* The legumes are only the second most important food crop after cereal as they are good sources for human diets and feeding animals (Liew et al., 2014). The essential phytochemicals for human health such as protein, oil, fibre and polyphenols are the major components of legume grains (Kouris-Blazos and Belski, 2016). As suitable plants for intercropping and crop rotation practices, legumes also have been widely cultivated in the world. In crop rotation practices, legumes have benefits for intercropping plants. The symbioses of legumes and rhizobia (the group of soil bacteria) have the ability to fix nitrogen from environment. This ability makes legumes as unique plants for sustainable agriculture. Legumes provide fixed nitrogens which are obtained from symbioses of legume-bacterium to intercropping plants resulting in improvements of yield and resistance to disease (Reeves et al., 2016). Annually, about 21 mega ton (Mt) of nitrogen is produced via symbioses of legume-bacterium (rhizobia) and 25-35% of fixed nitrogen (about 5-7 Mt) are returned into soil resulting in saving 8-12 billion USD (Angelsen and Kaimowitz, 2001).

Soybean: a key legume plant

Soybean (*Glycine max* L.) is one of oilseed crops and is widely cultivated in the world as a major legume plant (Foyer et al., 2016). Soybean is originated from China and adopted in east Asia such as Korea and Japan. In 1765s, soybean was introduced into North America and spread into Central and South America in mid 1900s. However, soybean now has been cultivated globally including America, Europe, Oceania and Africa, as soybean has high nutritional values and industrial uses (Foyer et al., 2016). Nowadays, soybean is one of the important crop plants and the leading plant in the world oilseed production (USDA, 2019). Soybean has been used in various fields such as for human health, feeding animals and eco-friendly sources for industrial purposes due to it's high oil and rich protein contents. In the case of oil from soybean, it has been used in versatile forms from cooking oil to a source for biodiesel. In the industrial sector, soy-oil has been used as lubricants and a source for paints and non-toxic crayon (Pratap et al., 2012). In response to the depletion of fossil fuels and global warming by emitting of carbon dioxide, soybean has been used as a source of biodiesel (Primentel and Patzek, 2005; Irwin and Good, 2017). In addition, soybean is used as affordable sources of proteins for feeding animals and for human diet. Soybean-derived protein meal has been used in feeding animals and accounts for 70% in total meal production (FAO, 2019). Furthermore, various types of soy foods such as tofu, miso, tempeh and edamame has been consumed for human as high quality protein sources. Soybean also provide health materials for human well-being (Sugano, 2005). Isoflavone from soybean, a type of phytoestrogen, reduces cancer incidence rate and phytosterol from soybean prevents the absorption of cholesterol by blocking sites of cholesterol and steroid hormones (Matvienko et al., 2002; Sarkar and Li, 2003).

The need to improve yield of soybean

Because of the agronomical and economical significance of soybean, demands for soybean production around the world has been growing rapidly (Hartman et al., 2011, Ray et al., 2013). To increase soybean yield, many studies in agricultural practices and breeding have been conducted (Patahan and Sleper, 2008). The yield of soybean has been improved gradually via developments of elite cultivars (high-yield, lodging resistant) and introductions of new soybean cultivation practices. During 1940 to 2000, soybean yield has been increased by 60% via improvements in quality, yield (lodging resistance) and biotic stress resistance (Wilcox et al., 2001). Considering average yield growth of soybean, Ray et al (2013) reported that increasing rate of soybean production will be increased annually 1.3% by 2050. However, this yield rate may not be sufficient for meeting projected demands of soybean (Ray et al., 2013). The global yield rate of soybean would have to be increased to 2.4% by 2050 to meet demands of soybean considering increasing biodiesel consumption, growing population and changes in diet habits (Ray et al., 2013).

Studies on soybean yield

To increase yield of soybean, studies on breeding and genetics have been conducted (Shoemaker et al., 2008; Patahan and Sleper, 2008). Until now, genetic information on over 900 quantitative traits loci has been identified and applied for breeding (Shoemaker et al., 2008; Patahan and Sleper, 2008). Since soybean has been adopted in various latitudes and environments, times of maturation vary in soybean. Day length and temperature are major factors in soybean maturation as soybean is a short day flowering plants (Garner and Allard, 1930; Hartwig, 1973). To maximize yield at specific regions, soybeans are categorized into different maturity groups according to the time period from sowing to full maturation of seeds (Hartwig, 1973). Since first report on effects of photoperiod and temperature on soybean maturity, total 13 maturity groups

(000: early maturity – X: late maturity) are classified (Garner and Allard, 1930; Hartwig, 1973; Zhang et al., 2007). Soybean cultivars in maturity group 000 can grow at higher geographical latitudes whilst varieties in maturity group X can thrive in lower latitudes (Hartwig, 1973). When a soybean cultivar in a certain maturity group is cultivated in higher latitude than its optimal growing region, it will show delayed flowering and maturity. On the other hand, this cultivar grown lower latitude than optimal region will exhibit early flowering and reductions in yield due to unsufficient vegetative growth period (Miladinovic et al., 2018). Therefore, precise selections of proper cultivars for cultivations at specific geographic locations have allowed improvements of total soybean yield in USA. Nowadays, studies on classifications of maturity groups for domestic soybean cultivars in major soybean production countries have been conducted to maximize soybean yield (Alliprandini et al., 2009; Liu et al., 2017b).

Flowering time: a key factor for soybean productivity

Flowering is the major plant developmental transition from the vegetative state to the reproductive state (Henderson and Dean, 2004). Since flowering is the first step of sexual reproduction, eventually forming seeds, it has huge importance in agriculture. In soybean, correlation between flowering time and total seed yield was reported by Cooper (2003). In this report, early flowering of soybean (14 days earlier than normal) induced by high temperature during vegetative growth allowed soybean plants to produce more seeds under favourable conditions (presented higher light intensity during reproductive developmental phase). Total soybean seed yields of early flowering lines were increased 25 to 37% compared to other lines. This result indicates controlling of flowering time can be a key to increasing the yield of soybean.

Many studies on identifications of soybean quantitative traits related in soybean flowering and maturity have been conducted (Owen 1927; Bernard, 1971; Abe et al., 2003; Cober et al., 1996; Xia et al., 2012; Watanabe et al., 2012). Total 11 *E* loci (*E1*-*E10*, and *J*) have been reported and characterized the functions in flowering and maturation (Samanfar et al., 2017). Among these loci, 8 loci except for *E6*, *E*9 and *J* are related with delay of flowering at different extents by interacting with abiotic factors or other quantitative trait loci (Watanabe et al., 2012). Five *E* loci (*E1*, *E3, E4, E7* and *E8*) have been identified for their roles in perception of photoperiod under nonflowering inductive condition (long day). *E1* plays major roles in flowering by repression of initiation of flowering via suppression of a flowering gene expression (Xia et al., 2012). *E3* and *E4* are involved in controls of flowering initiation via regulations of photoreceptions (Liu et al., 2008a; Watanabe et al., 2009). Recessive alleles of *E7* and *E8* did not confer complete insensitivity on photoperiod under long day condition suggesting that these two *E* loci (*E7* and *E8*) might have a weaker effect on perceptions of light than *E3* and *E4*. *E2* was identified as a soybean *GIGANTEA* homolog which is involved in photoperiod-dependent flowering pathway in Arabidopsis (Watanabe et al., 2012). Effects on flowering time of other *E* loci (*E6, E8, E9* and *J*) also have been investigated (Bonato and Vello, 1999; Kong et al., 2014; Samanfar et al., 2017; Yue et al., 2017. Dominant Allele of *E6* (*E6*/*E6*) promoted flowering initiation (Bonato and Vello, 1999). Recent studies on soybean flowering and maturity revealed that recessive allele of *E9* (*e9*/*e9*) induced delayed flowering initiation whilst recessive allele of *E10* (*e10*/*e10*) promoted flowering (Kong et al., 2014; Samanfar et al., 2017). *J* is involved in controls of juvenile developmental period in tropical soybean as single nucleotide deletion in *J* gene exhibited extended juvenile period (Yue et al., 2017).

In addition to roles of light in flowering, other factors such as temperature, ageing process and endogenous stimulus in plants plays regulator roles in flowering initiation. It is well known that flowering is controlled by multiple environmental stimulus and endogenous signals (Simpson and Dean, 2002). In Arabidopsis, a model plant, flowering induction is initiated by several types of genetic pathways, photoperiod, vernalisation, ambient temperature, autonomous, gibberellin and ageing pathway (Roux et al., 2006; Gupta and Chakrabarty, 2013; Cheng et al., 2017). These pathways promote expression of floral pathway integrator genes and subsequently activate the expression of floral meristem identity genes, thereby initiating flowering (Kim et al., 2009). Specifically, when plants enter the inductive condition for flowering, signals from each flowering pathways stimulate the expression of floral pathway integrator genes including *FT* (*FLOWERING LOCUS T*), *SOC1 (SUPPRESSOR OF OVEREXPRESSION OF CONSTANS)* and *LFY (LEAFY)*. The activation of these integrators up-regulate the expressions of floral meristem identity genes, *LFY, AP1 (APETALA1)* resulting in initiation of flowering (Amasino and Michaels, 2010). *LFY,* a transcription factor, plays master role in evocation of flowering (Moyroud et al., 2009). In Arabidopsis, *LFY* has essential roles in timing and patterns of flowering (Moyround et al., 2009, 2010). The loss-of-function of *LFY* produced abnormal flowers (shoot-like inflorescences), whilst its ectopic expression caused extremely early flowering phenotypes in Arabidopsis (Weigel et al., 1992; Weigel and Nilsson, 1995). MicroRNA is composed of 21-24 nucleotides and has effects on regulation of genes via suppressions of target gene expression. During the developmental process, microRNAs (miRNAs) play important roles in plant growth from vegetative phase to reproductive phase by repressing of expressions of their target genes (Achard et al., 2004; Lauter et al., 2005; Zhang et al., 2006). In Arabidopsis, the interaction of *miR156* and *miR172* plays important role in flowering. *miR156* has pivotal roles in the juvenile stage (Wu et al., 2009) by repressing its target genes, *SPL* (*SQUAMOSA PROMOTER BINDING-LIKE*) family, while *miR172* is involved in regulation of flowering time and floral organ identity by down-regulation of its target genes, *AP2* (*APETALA2*)*, AP2-like* (Aukerman and Sakai, 2003; Chen, 2004). Even though the knowledge gained from model plants such as Arabidopsis can be labelled a dramatic breakthrough in plant biology, it is difficult that such findings from model plants are applied directly into important crop plants such as soybean (Haerizadeh et al., 2009).

Whole genome sequences and comparative analysis of flowering genes of soybean has been recently conducted (Schmutz et al., 2010; Wong et al., 2011; Jung et al., 2012). But the functions of most of these genes are still unknown (Homrich et al., 2012). Investigations on functions of soybean flowering genes will provide crucial information on controls of flowering time in soybean ultimately will accelerate developments of new elite soybean varieties which contain high-yield (approximately up to 37% yield increase; based on Cooper, 2003). In this regard, research projects focus on the functional analysis of soybean genes in flowering initiation and phase transition from vegetative to reproductive [*LFY* (*GmLFY1*), *miR156* and *miR172* (*gma-miR156a* and *gma-miR172a*)]. Establishing *Agrobacterium*-mediated soybean transformation method is a pre-requisite step to such analysis being carried out. Research projects cover both the transformation method as well as the functional analysis of flowering genes of soybeans using transgenic model plants for genetic analysis, Arabidopsis and tobacco.

The aims of research projects are

- Development and optimization of soybean transformation system (*Agrobacterium*-mediated method) for commercial cultivar (cv. Bragg)
- The functional analysis of *GmLFY1* gene in transgenic Arabidopsis and tobacco

- Investigations of spatial and temporal expression patterns of *GmLFY1* in soybean shoot apical meristem during phase transition
- Analysis of expression patterns of *gma-miR156a* and *gma-miR172a* in soybean during flowering inductive conditions
- The roles of *gma-miR156a* and *gma-miR172a* in developmental phase transitions

Chapter 2. Review of Literature

2.1 Soybean (*Glycine max* L.)

The *Leguminosae* (or *Fabaceae*) is the third largest family in Angiosperms (flowering plants) and this family consists of approximately 727 genera with more than 19,325 species. Legumes are of agricultural significance due to their harvest area and total yields (Gepts et al., 2005). Grain legumes provide essential products for human diet as its seeds possess a variety of phytochemicals, including protein, carbohydrate, fibre, vitamins, carotenoids and polyphenols (Kouris-Blazos and Belski, 2016). In addition, legumes are cultivated as plants for intercropping and crop rotation with other crops as they have a unique ability, that is symbiotic nitrogen fixation (Tilman et al., 2002; Herridge et al., 2008). Intercropping legumes with non-legume plants has many benefits such as yield enhancement, increased efficiency in nitrogen-use, reduced occurrences of disease in crops when cultivated with or after legume plants (Reeves et al., 2016). Legume-rhizobia symbioses show an annual nitrogen production of 21 Mt (mega tons), 5 to 7 Mt of which is absorbed into soil, saving 8 to 12 billion in US dollars (Reeves et al., 2016). For these reasons, the demand on legume production is continuing to increase.

Soybean (*Glycine max* L. Merr.) is one of the important legume species and is widely cultivated around the world. Plant height of soybean varies from 0.2 to 2.0 m. It has brown or grey hairs on its pods, stems and leaves. The leaves are trifoliate with 3 to 4 leaflets per leaf, which are 6 to 15cm long and 2 to 7cm wide. Soybean is a short-day plant which initiates flowering in short photoperiod and flowering time varies in different maturity groups showing variability according to the environmental cues such as photoperiod and temperature (Xia et al., 2012). Soybean originated from China and was cultivated as a food crop in the 17th to 11th century B.C., widely spreading to other countries such as India, Japan and Korea. In the 19th century, soybean was introduced to America, which is now the largest soybean producing country. Nowadays, soybean is cultivated globally as one of the most important crop plants (Foyer et al., 2016).

2.1.1 The usage of soybean and its position in the world market

Soybean is one of the most important crops in the world since its seeds have high contents of oil and protein (20% oil and 40% protein). Soybean is used for animal feed as a cheap protein source. Besides, its usage for humans vary from processed foods (e.g. tofu, milk, soy flour) to secondary metabolite sources (e.g. tocopherols, isoflavones, lecithin and saponin) (Sugano, 2005). In addition to its usage for humans and animal foods, its by-products have been used in the manufacture of various products such as biocomposites, candles, soy-ink, soy-crayons, soy-based lubricants, pharmaceutical production, plastics, pesticides and cosmetics (Pratap et al., 2012). Moreover, soybean can uptake nitrogen from the atmosphere via symbiosis with soil bacteria which have the ability to synthesize biological nitrogen. The group of soil bacteria in rhizobium genus have the ability to fix nitrogen from the atmosphere. Biological nitrogen fixation occurs when the N-fixing bacteria establishes nodules on soybean roots. Soybean can obtain about 60% of the total nitrogen required for plant growth via symbiosis with rhizobium. This nitrogen fixation ability of soybean plays important roles in sustainable agricultural practice. Nitrogen (N) is one of the most important nutrients for plant growth. Nitrogen is a key component of amino-acids and is a vital substance for photosynthesis process. Since nitrogen is an essential component in major plant developmental process, nitrogen fertilizer is commonly used to increase crop yield. The invention of artificial nitrogen synthesis process allowed mass productions of nitrogen fertilizers. To meet increased demands of crop plants, the use of nitrogen fertilizers has massively increased over the last century (Ahmed et al., 2017). However, excessive use of nitrogen fertilizers has posed threats to environmental and human health (Ahmed et al., 2017; UNEP, 2019). In agriculture, only 20% of applied nitrogen fertilizers are utilized by crops, whilst the remainder is wasted, causing environmental pollution (Westhoek et al., 2015; UNEP, 2019). Nitrogen waste from agricultural uses creates a nitrogen-rich environment, negatively impacting microorganisms of soil and water, ultimately changing biodiversity (Ward, 2009). In addition, nitrous oxide gas (N_2O) being one of the green house gases, is emitted from nitrogen waste and accelerates global warming (UNEP, 2019). Elevated temperatures caused by global warming will increase drought- and heat-stress, directly affecting plant yield. In this regard, soybean is becoming a crucial plant for sustainable agriculture with respect to serious threats posed by excessive use of nitrogen fertilizers and global warming causing reduction in arable area. Because of the agronomical and economic importance of soybean as sources of oil, protein and other components, soybean production has been growing rapidly (Hartman et al., 2011) (Figure 2.1).

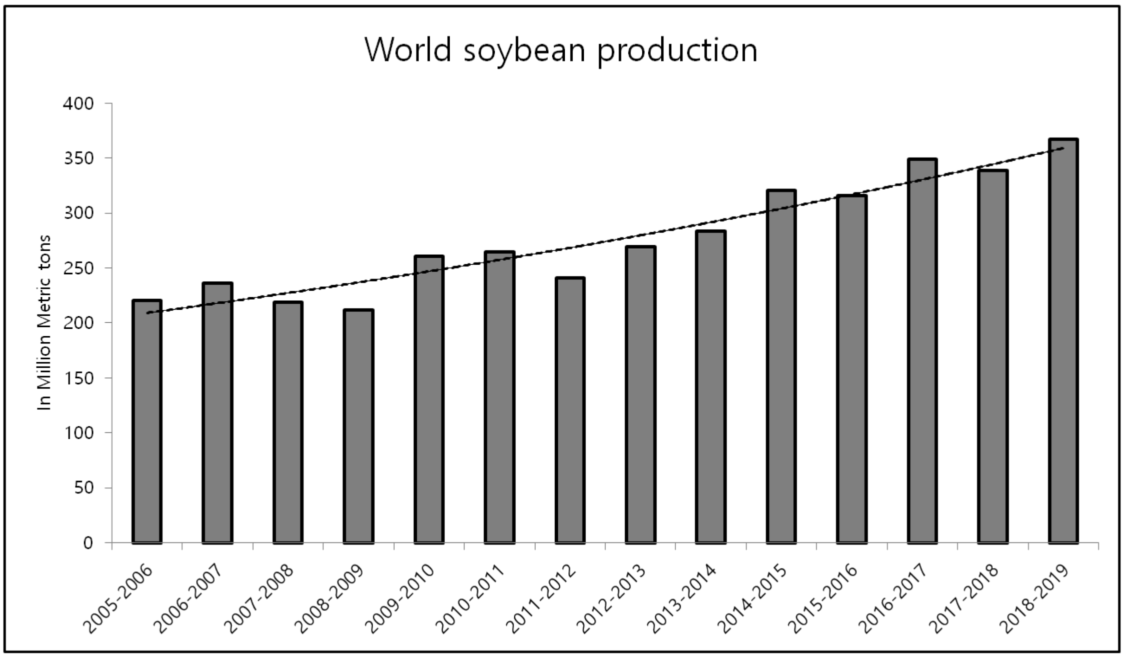


Figure 2. 1. Global soybean production (USDA, 2018).

2.2 Plant genetic transformation

The improvement of plants can be achieved by direct insertion of foreign genes into plant genome via genetic transformation methods. Plant genetic transformation is a powerful tool to improve agricultural traits (e.g. yield improvement and environmental stress resistance). Genetically modified (GM) crop plants (soybean, cotton, corn, canola crops) with important agronomic traits including tolerance to herbicide and insects, have been commercialized (Mall et al., 2019). In soybean, glyphosate-resistant soybean plants (commercial name: RoundUp Ready®) were developed via genetic engineering and were commercialized in the USA (Padget et al., 1995). Since the first introduction of genetically modified soybean cultivar (GM soybean) into agriculture in 1996, GM soybean is now a major GM crop, accounting for about 50% of total global GM crop cultivation area (ISAAA, 2018).

During the last decade, advancement in genome technologies has unveiled genetic information of a number of plants and crops including Arabidopsis, maize rice and soybean (Kaul et al., 2000; Goff et al., 2002; Yu et al., 2002; Schnable et al., 2009; Schmutz et al., 2010). Although extensive information on the genome of major crops have been made available to date, their biological functions and involvements in specific networks remain unclear. To identify roles of genes, two experimental approaches (forward genetics and reverse genetics) are used. These genetic approaches use genetic information and phenotype differences of host plants to identify functions of genes of interest. In forward genetics, mutant plants are firstly isolated by phenotype differences. Subsequently, functions of genes are determined via examinations of genetic information of the mutant plant. Conversely, reverse genetics utilizes mutant plants with ectopically expressed genes of interest or those with suppressed expression in order to identify biological functions of the genes. Biological roles of genes can be identified via examinations of phenotypical differences and expression levels of related genes with target genes in transgenic plants. Gain or loss of functions of genes of interest in plants can be ascertained via transposon-induced mutations or introduction of recombinant plasmids containing overexpression or suppression of genes of interest. Plant genetic transformation methods have been widely used to introduce recombinant plasmids into genomes of host plants to manipulate the expression of target genes. Plant transformation methods can be divided into *Agrobacterium*-mediated gene transfer and direct gene transfer into plant genome (Atif et al., 2011).

Agrobacterium-mediated transformation, which is widely used for plant genetic transformation, utilizes the natural infection ability of *Agrobacterium* to introduce the gene of interest into plant cells (Somers et al., 2003). *Agrobacterium* is a phytopathogenic soil bacterium which causes crown gall disease in dicot plants. *Agrobacterium tumefaciens* that harbours Ti (tumour-inducing) plasmid containing T-DNA (transfer DNA) induces tumour-like growth on the roots of infected plants. The bacterium recognizes phenolic chemicals such as acetosyringone and sugars which are secreted from wounded parts of plants. The *Vir* (virulent) gene family in the Ti plasmid plays important roles in recognition and induction of tumour-like organs (Gelvin, 2003). When the bacterium recognises the phenolic compounds via *VirA* proteins, the bacterium latches onto the wounded parts and introduces T-DNA into plant cells. T-DNA is inserted into the genome of the host plant and the gall is formed on the infected surface of plant tissue

(Gelvin, 2003). Through this gene delivery system, *Agrobacterium* is able to introduce T-DNA into the genome of the host plant. For use in plant genetic transformation without formation of tumours, disarmed Ti-plasmids lacking T-DNA (oncogenes) are constructed (Hood et al., 1986, 1993; Palanichelvam et al., 2000; Kiyokawa et al., 2009). Genes of interest are then inserted into disarmed Ti-plasmids and introduced into *Agrobacterium* for plant genetic transformation.

Particle bombardment (also called biolistics or gene gun) is a method of transformation using high-velocity microprojectile (Sanford, 1988). DNA(exogenous)-coated micro-particles are delivered into cells or tissues using a particle delivery system. The inserted exogenous DNA in plant cells is incorporated into the genome of the host plant (Sanford, 1990; Kohil et al., 2003; Kikkert et al., 2005). Direct DNA transfer method using particle delivery system can be applied to transformation of various types of explants from plant cells to hypocotyls and it is also used for genetic transformation of monocot plants, which do not host *Agrobacterium*. Since genetic transformation using particle bombardment systems has advantages such as direct multiple gene insertions into various plants species and no biological constraints, it has been widely used for plant genetic transformation (Altpeter et al., 2005; Artif et al., 2013).

However, the limitations of each genetic transformation methods have to be considered for successful transformation. One of the limitations of the *Agrobacterium*-mediated method is its lack of efficiency when used for certain varieties of legumes and commercially significant plant types that do not host this bacterium (Artif et al., 2013). Particle bombardment genetic transformation method has a lower rate of transformation efficiency than the *Agrobacterium*-mediated transformation method, as it has the high tendency to induce complex integrations or insertions of multiple copies of exogenous genes. Since these complex insertion patterns increase the possibility of chromosome rearrangement and genomic DNA interspersion, abnormal expression patterns or silence of transgenes are often observed (Kikkert et al., 2005; Wiebker-Strohm et al., 2011).

2.2.1 Soybean transformation

Soybean transformation systems have continued to improve with the assistance of particle bombardment and *Agrobacterium*-mediated transformation methods (Homrich et al., 2012). One of the earliest reports of the development of transgenic soybean plants using plant genetic transformation systems was in the 1980s (Christou et al., 1988; Hinchee et al., 1988). Transgenic plants containing kanamycin resistant gene (*NPT II*) were developed via particle bombardment transformation method (Christou et al., 1988). In addition to a direct gene transfer method, development of transgenic soybean plants using *Agrobacterium tumefaciens*-mediated transformation method was also reported (Hinchee et al., 1988). Since the first soybean transformation was carried out using particle bombardment (Christou et al., 1988), many studies on genetic modification of soybean using biolistics have been conducted (Christou et al., 1988; Christou and McCabe, 1992; Aragão et al., 2000). Meristems from shoot apex have been used as target tissue for the particle bombardment transformation method (Christou et al., 1988; Finer and McMulle, 1991; Christou and McCabe, 1992; Finer et al., 1992; Stewart et al., 1996; Aragão et al., 2000; Droste et al., 2002; Homrich et al., 2008; Wu et al., 2008; Hernandez-Garcia et al., 2009). However, chimeric phenotypes were reported in transgenic lines as apical meristem is made up of three layers (L1, L2 and L3) and meristems are a place where lateral plant organs such as shoots and flowers are formed (Christou and McCabe, 1992; Bhalla and Singh, 2006). Establishment of somatic embryogenesis systems of soybean provided alternative target tissues for soybean transformation using particle bombardment (Christianson et al., 1983). Studies were conducted on developments of transgenic soybean plants via particle bombardment using single cell origin somatic embryos as target tissues (Finer and McMulle, 1991; Finer et al., 1992; Stewart et al., 1996; Droste et al., 2002; Homrich et al., 2008; Wu et al., 2008; Hernandez-Garcia et al., 2009). However, cytological variations have been observed in transgenic soybean plants produced by embryogenic cells with particle bombardment (Singh et al., 1998). Phenotype aberrations such as production of leathery leaves, stunted growth and partial/total seed sterility were observed in two different Asgrow soybean genotypes (Singh et al., 1998). In addition, genotype-dependent chromosomal aberrations such as deletion, duplication and tetraploidy were detected in long culture period indicating that susceptibility to somaclonal variations induced by chromosomal instability differs in soybean genotypes (Trick et al., 1997; Singh et al. 1998).

Compared to particle bombardment transformation systems, *Agrobacterium*-mediated transformation has advantages such as transfer of low copy numbers of genes (mostly single transgene insertions) into the genome of host plants, low frequency of transgene rearrangements and stable transgene expression (Yamada et al., 2012). Because of its advantages, *Agrobacterium*-mediated transformation systems have been widely applied in development of transgenic soybean plants. Advances in genetic transformation systems for soybean brought developments in transgenic soybean plants. Herbicide-tolerant soybean plant is one of the most successfully commercialized GM (genetically modified) crops in the world. Glyphosate resistance was achieved via introduction of *5-enol-pyruvyl-shikimate-3-phosphate synthase* (*EPSPS*) gene using *Agrobacterium*-mediated transformation (Padgette et al., 1995; Lundry et al., 2008). The glyphosate resistant soybean cultivar (Roundup Ready®) was produced using this method and it has now become the most widely cultivated biotech crop in the USA (ISAAA., 2017). In addition to herbicide tolerance, development of insect resistant-transgenic soybean plants has been reported (Parrot et al., 1994; Stewart et al., 1996; Walker et al., 2000; Macrae et al., 2005; Miklos et al., 2007; Homrich et al., 2008; McPherson and Macrae, 2009). *Bacillus thuringiensis* (*Bt*), an entomopathogenic bacterium, produces proteins named δ-endotoxins popularly called *Bt* proteins, which act as a biological pesticide (Hongyu et al., 2000). Transgenic soybean plants which contain *Bacillus thuringiensis insecticidal crystal protein gene* (*Bt cryIAc*) exhibited tolerance to leidoperan pests including *Anticarsia gemmatalis, Helicoverpa zea* and *Pseudoplusia includes* (Parrot et al., 1994; Stewart et al., 1996; McPherson and Macrae, 2009). Although the herbicide-tolerant transgenic soybean cultivar has been traded and cultivated in agricultural markets, there are still limitations for stable soybean transformations. Several factors involved in infection and co-cultivation such as the addition of phenolic compounds, physical treatments (sonication and vacuum treatment) and culture conditions (light, cultivation period and temperature) are considered to be major elements in optimizing soybean transformation systems (Liu et al., 2008b; Artif et al., 2013). Therefore, factors involved in *Agrobacterium*-mediated transformation have been investigated to increase transformation efficiency.

2.2.2 Factors for successful soybean transformation

To optimize stable soybean transformation systems, many studies on the improvement of transformation efficiency have been conducted. The type of explant, proper selection regimes and conditions of infection and co-cultivation are identified as factors that improve efficiency of soybean transformation. For successful regeneration of transgenic plants from explants, the ability of multiple shoot formation plays an important role in transformation efficiency (Liu et al, 2008b; Jeon and Chung, 2009; Raza et al., 2017). Cotyledonary node and imbibed seeds have been widely used for genetic transformation of soybean (Hinchee et al., 1998; Olhoft et al., 2003, Paz et al., 2006; Kim et al., 2012b; Li et al., 2017). In these types of explants, axillary meristems at the junction between cotyledon and hypocotyl which have the ability to form multiple shoots were selected as target cells for soybean transformation (Olhoft et al., 2003; Paz et al., 2006). Since the ability of regeneration into plant varies among cultivars, successful transformation often relies on the selection of cultivars that are highly efficient in multiple shoot formation. In addition, the use of appropriate selection reagents is one of the key factors for successful transformation (Zeng et al., 2004). Kanamycin was used as a selection reagent in the early soybean transformation experiments without notable success (Christou et al., 1988; Hinchee et al., 1988). In later experiments, the uses of hygromycin and herbicide (phosphinothricin and glyphosate) reported success in their use as selection reagents (Olhoft et al, 2003; Paz et al., 2006; Kim et al., 2012b; Li et al., 2017). The use of hygromycin as a selection reagent reduced the occurrence of generations of non-transgenic plants during selection (Olhoft et al., 2003). Different types of herbicides (imidazolinone, phosphinothricin and glyphosate) have been used for soybean transformation (Zhang et al., 1999; Zeng et al., 2004; Paz et al., 2006; Rech et al., 2008; Kim et al., 2012b; Li et al., 2017). There have been reports of establishment of efficient transformation systems for soybean using different concentrations of glufosinate during selection of transgenic shoots regenerated from cotyledonary node explants (Zhang et al., 1999; Zeng et al., 2004). In contrast, the use of glufosinate in the same concentration during selection exhibited maximized transformation efficiency when using imbibed explants, suggesting cultivar-specific consideration should be given when choosing appropriate selective reagents and selection regime in order to optimize soybean transformation systems (Paz et al., 2006). Due to soybean's susceptibility to *Agrobacterium* (Pedersen et al., 1982; Droste et al., 1994; Mauro et al., 1995), there have been studies conducted on the factors affecting infection and co-cultivation (Olhoft and Somers, 2001; Olhoft et al., 2001). The cotyledon of soybean responds vigorously to pathogen attack, resulting in tissue browning and necrosis (Boué et al., 2000; Olhoft and Somers, 2001). Polyphenol oxidases (PPOs) and peroxidases (PODs) cause the accumulations of tannins and phytoalexins at wounded sites, resulting in tissue browning

(Vámos-Vigyázó L and Haard, 1981). Oxidative burst occurs as the plant's first reactions to wounding and pathogen attacks as it produces reactive oxygen species (Wojtaszek, 1997). Oxidative burst is considered to be one of the activators of cell death program and one of the hypersensitive responses to pathogen attack, which creates a barrier of dead cells (Olhoft et al., 2001). This highly sensitive wound and pathogen reaction of soybean's cotyledonary-node explants seems to inhibit T-DNA delivery into soybean and regeneration of transgenic shoots into transgenic plants (Olhoft et al., 2001). To reduce severe responses of cotyledonary node explants to *Agrobacterium*-mediated transformation, the effect of antioxidants such as thiol compounds (L-cysteine, Sodium thiosulfate, dithiothreitol) on tissue necrosis were investigated (Olhoft et al 2001; Olhoft and Somers, 2001). L-cysteine is known as one of the inhibitors of PPOs and PODs (Mayer and Harel, 1979). In soybean transformation, addition of L-cysteine to the co-cultivation step increased the T-DNA transfer to explants by 5 fold and reduced tissue browning during selection (Olhoft and Somers, 2001). In addition, when other thiol compounds (sodium thiosulfate, dithiothreitol) were added with L-cysteine in solid co-cultivation medium, transient GUS expression were significantly increased (3 fold), indicating that thiol groups increase transformation efficiency by reduction of wound and pathogen response of explants during infection with *Agrobacterium* (Olhoft et al., 2001).

Despite investigations on various factors involved in transformation efficiency and examinations carried out on the effects of these factors on soybean transformation to date, the need for a stable and routine soybean transformation system still remains. Reports of successful genetic transformation systems for soybean have been limited to those relating to inferior-breeding lines and these systems are cultivar-specific (Lundry et al., 2008; Levy-Booth et al., 2009). In addition, the entire genome of soybean has been sequenced and many genes of soybean have been identified (Schmutz et al., 2010; Jung et al., 2012). However, the functions of such genes still remain largely undemonstrated (Homrich et al., 2012). To improve the quality and productivity of soybean cultivars, further investigation and successful identification of the functions of soybeans genes are necessary. Development of transgenic soybean plants exhibiting overexpression or suppression of target genes via plant transformation methods can elucidate the functions of soybean genes. In this regard, development of a universal soybean transformation system which is cultivar-independent is a prerequisite step to meaningful genetic analysis and improvement of soybean.

2.3 Flowering: A key event for reproductive success

Flowering is one of the crucial events in plant life cycle as it plays important roles in reproductive success. During the vegetative developmental phase, apical meristems produce aerial parts of plants including leaves and stems. Under favourable conditions for flowering, the environmental and endogenous signals activate phase transition of plants from vegetative to reproductive development. Meristematic cells at apical meristems are changed from vegetative meristems to floral meristems which subsequently generate reproductive organs such as flowers. The seeds are formed after fertilization and this process is crucial as formation and dispersal of seeds has a huge impact on the preservation of its progeny. As such, elaborated pathways for timing of flowering have evolved in order to ensure their reproductive success. Late flowering allows plants to produce and accumulate more resources that are ultimately used for reproductive development, including seed formation. On the other hand, early flowering enables plants to adapt and secure their progeny in unfavourable flowering conditions (Simpson and Dean, 2002; Komeda, 2004; Weinig and Schmitt, 2004). Therefore, the timing of flowering plays crucial roles in plant fitness and crop yield (Huijser and Schmid, 2011; Srikanth and Schmid, 2011). Since flowering stands at the centre of importance in plant life, extensive studies have been conducted to uncover various flowering pathways. *Arabidopsis thaliana* is frequently used as a model plant for genetic research since it has advantages such as relatively small sizes of genome (135 mega base pair) and rapid life cycles. The main genetic mechanisms of flowering in Arabidopsis have well been characterized (Blazquez 2005; Parcy, 2005). Photoperiod, vernalisation, ambient temperature, autonomous, presence of gibberellin acids (GA) and ageing pathways have been identified as the major factors of genetic pathways for flowering evocation in Arabidopsis (Gupta and Chakrabarty, 2013; Cheng et al., 2017).

In soybean, studies on important agronomic traits (e.g. flowering time, stem growth) have been conducted to improve the yield of soybean. Soybean is a short-day plant which initiates flowering in short photoperiod conditions and its flowering time varies in different maturity groups (Xia et al., 2012). Photoperiod has been considered a major factor regulating soybean's flowering time. Although soybean is cultivated over various latitudes between 50 degrees north and 35 degrees south, the cultivation area of each cultivar is latitudinally distributed (Watanabe et al., 2012). These latitudinal distribution of soybean cultivation is strongly associated with its photoperiod sensitivity

(Watanabe et al., 2012; Xia et al., 2012). In this regard, the genetic locations and information on traits involved in photoperiod and flowering time controls were investigated via genetic and quantitative analysis. Studies on the flowering quantitative trait, loci, (*E* loci) revealed that eleven *E* loci (*E1 to E10* and *J*) were involved in the regulation of flowering time (Cao et al., 2015) (Table 2.1). Genetic locations of six *E* loci (*E1, E2, E3, E4, E7* and *E9*) have been mapped in soybean chromosomes into Gm06, Gm10, Gm19, Gm20, Gm01 and Gm16 respectively (Watanabe et al., 2012; Kong et al., 2014). To date, molecular characteristics of four *E* loci (*E1, E2, E3 and E4)* have been identified (Abe et al., 2003; Cober et al., 1996; Liu et al., 2008a; Watanabe et al., 2009, 2011; Xia et al., 2012). The functions of these four *E* loci have been proven to be relevant to photoperiod responses in flowering pathway (Abe et al., 2003; Cober et al., 1996). The maturity locus *E1* acts as a flowering repressor by suppressing expressions of two soybean *FT* homologs *GmFT2a* and *GmFT5a* under long-day conditions (Xia et al., 2012). Recessive alleles in *E1* (*e1/e1*) promote early flowering (Bernard, 1971; Abe et al., 2003; Xia et al., 2012). Two natural mutants (*e1-fs*, *e1-nl*) and three EMS-derived mutants of *E1* showed early flowering time. Transcriptional profiling analysis revealed the bimodal expression patterns of *E1* (Xia et al., 2012). *E1* transcriptions were reset at dawn and dusk. The strongest expression was detected in fully expanded leaves and lowest expression was detected before daybreak. The level of expression gradually decreased during the dark (Xia et al., 2012). Overexpressing *E1* in soybean exhibited delayed flowering time with decreased expression levels of *FT*, suggesting *E1* regulates flowering time via suppression of *GmFT* transcriptions under LD (Xia et al., 2012). *E2* is identified as a soybean *GIGANTEA* (*GmGIa*), a homolog of Arabidopsis *GI*, via map-based cloning strategy (Watanabe et al., 2011). In Arabidopsis, *GI* gene encodes a nuclear-localized membrane protein, thereby acting as an upstream of *CONSTANS* (*CO*) and *FT* (*FLOWERING LOCUS T*) (Fowler et al., 1999; Mizoguchi et al., 2005). *GI* protein and F-box ubiquitin ligases up-regulate the expression of *CO* via repression of *CYCLING DOF FACTORs* (*CO* suppressors) (Nelson et al., 2000; Sawa et al., 2007; Fornara et al., 2009). In the recessive analysis of soybean, mutation of *E2* locus (*e2/e2*) cause early flowering under natural day-length (Watanabe et al., 2011). In addition, elevated expression of *GmFT2a* was detected in the *e2/e2* genotypes. These results from positional cloning and phenotype analysis on mutants indicate that *E2* loci is a soybean homolog *GI* (*GmGIa*) (Watanabe et al., 2011). Among environmental factors for flowering evocation, photoperiod is one of the major elements enabling recognition of seasonal changes. The light signals are received by several photoreceptors on leaves, including phytochromes absorbing red light (R) and far-red light (FR) and the cryptochromes and phototropins absorbing blue light and UV-A, respectively (Chen et al., 2004). Studies on the role of phytochromes in flowering have been carried out in LD plants, such as Arabidopsis (Goto et al., 1991; Johnson et al., 1994; Devlin et al., 1996; Aukerman et al., 1997; Neff and Chory 1998; Devlin et al., 1999; Mockler et al., 1999) and pea (Weller et al., 1997, 2001). Analysis of loss-of-function mutants has revealed that each phytochrome has a distinct role in flowering. In Arabidopsis, the loss-of-function *phyA* mutant (*PHYTOCHROME A*) exhibited delayed flowering initiation under LD and SD conditions with a night break (Johnson et al. 1994; Reed et al., 1994). In one of the legume species, pea (*Pisum sativum*), mutations on *PHYA* homolog caused late flowering phenotypes under both short day and long day (extended with extra light provided by incandescent lamps) (Weller et al., 1997, 2001). On the other hand, *PHYTOCHROME B* (*PHYB*) has an inhibitory role in flowering evocation (Lin, 2000). In a monocot plant, rice, double mutants of phytochromes (*phyA phyB*) flowered significantly earlier than wild-type under natural-day-length conditions, whilst monogenic mutation of *PHYA* exhibited a weaker effect on flowering time regulation (Takano et al., 2005). Similarly, a mutation in the *PHYB* gene in sorghum (*Sorghum bicolor*) also induced early flowering under flowering inductive conditions (short day) (Childs et al., 1995, 1997). The roles of *PHY* genes in photo-response indicate that they play important roles in crop adaptation to a variety of environmental conditions. Two individual studies revealed that *E3* and *E4* encode *GmPHYA3* and *GmPHYA2* respectively, which are homologs of the photoreceptor *PHYA* (Liu et al., 2008a; Watanabe et al., 2009). Recessive homozygote (*e3/3e*) can promote flowering under LD conditions where day length was extended to 20 hours, using high R:FR (High Red: Low Far-Red) ratio fluorescent lamps (Buzzell, 1971). Using the map-based cloning strategy, the responsible genes in this QTL were identified as a *PHYA* gene (*GmPHYA3*) (Watanabe et al., 2009). The *E4* locus was identified as another *PHYA* homolog in soybean, *GmPHYA2* (Liu et al. 2008). An *e4/e4* (recessive allele at *E4* locus) mutant in the *e3/e3* homozygous background can confer the insensitivity at natural day length extended to 20 hours using high R:FR ratio incandescent lamps, (Cober et al., 1996).

Table 2. 1. Genetic information and functions of identified soybean maturity loci.

Maturity loci	Genetic location in soybean genome	Identified genes in soybean	Phenotypes	references
E1	Ch06 (Glyma.06G23040)	B3 superfamily	Recessive alleles (*e1/e1*): early flowering, Overexpression of *E1* in soybean: delayed flowering under non-flowering inductive condition (LD)	Bernard, (1971); Abe et al. (2003); Xia et al. (2012)
E2	Ch10 (Glyma.10G36600)	*GIGANTEA* (*GmGIa*)	Recessive alleles (*e2/e2*): early flowering	Watanabe et al. (2011)
E3	Ch19 (Glyma.19G41210)	*PHYTOCHROME A* (*GmPHYA3*)	Recessive allele (*e3/e3*): flowered under LD (high R:FR; high Red:Low Far-Red ratio)	Buzzell, (1971); Watanabe et al. (2009)
E4	Ch20 (Glyma.20G22160)	*PHYTOCHROME A* (*GmPHYA2*)	Recessive allele (*e4/e4*): flowered under natural day length extended to 20hr using high R:FR ratio	Cober et al. (1996); Liu et al. (2008)
E5	Not determined	Not determined	Recessive alleles(*e5/e5*): early flowering	Watanabe et al. (2012)
E6	Not determined	Not determined	Recessive alleles(*e6/e6*): early flowering	Watanabe et al. (2012)
E7	Ch06	Not determined	Recessive alleles(*e7/e7*): early flowering	Watanabe et al. (2012)
E8	Gm04	Not determined	Recessive alleles (*e8/e8*): early maturity	Cober et al. 2010)
E9	Ch16	Not determined	Recessive allele (*e9/e9*): early maturity	Kong et al. (2010, 2014)
J	Ch04 (Glyma.04G050200)	*EARLY FLOWERING 3* (*GmELF3*)	Recessive alleles (*j/j*): late flowering	Yue et al. (2017)

2.3.1 Genetic analysis of flowering pathways

Seasonal flowering time controls are regulated by synchronized interactions between environmental and endogenous signals (Mouradove et al., 2002). In flowering plants, the complex gene networks involved in the transition from vegetative to reproductive phase have evolved to ensure reproductive success. Investigations in identification of genetic information in major flowering pathways have been carried out in Arabidopsis (Blazquez, 2005; Gupta and Chakrabarty, 2013; Cheng et al., 2017). Arabidopsis has been used as a model plant in several flowering studies, as its genome size is small (135 mega base pair) and its entire genome sequencing has been completed (Kaul et al., 2003). More than 180 genes involved in its flowering genetic pathways have been identified and the roles of flowering genes have been investigated using gain- or loss-of-function mutants and transgenic Arabidopsis plants (Fornara et al., 2010). With the advancement of genome sequencing technique, comprehensive genetic information on the genome of many plants including crops have been obtained (Kaul et al., 2000; Goff et al., 2002; Yu et al., 2002; Schnable et al., 2009; Schmutz et al., 2010). Comparative analyses of flowering genes in Arabidopsis as against other important crops such as soybean have revealed conservation of most flowering genes amongst plants (Hetch et al., 2005; Jung et al., 2012; Taoka et al., 2013).

Photoperiod pathway: a key factor for the wide adaptability of soybean

Changes in day-lengths are one of the consistent indicators of seasonal changes. Plants recognise seasonal photoperiod differences to regulate the timing of flowering. In the 1920s, Garner and Allard (1920) revealed that changes of day lengths were recognized by the leaves and introduced the concept of photoperiod. Genetic photoperiod pathway has been well studied in Arabidopsis, a facultative LD plant. Day-length information with endogenous circadian clock mechanisms regulate two major genes for photoperiodic flowering pathway, *CO* and *FT* (Song et al., 2010). *CO* is a nuclear protein encoding a B-box zinc finger protein and regulates the expression of the *FT* gene (encoding small protein for flowering initiation, florigen) in the day-length-dependent manner (Suarez-Lopez et al., 2001; Song et al., 2012). Loss of function of *co* mutant Arabidopsis plants exhibited delayed flowering with reduced expression of *FT* in flowering inductive conditions (long day) (Samach et al., 2000). On the other hand, overexpression of *CO* in Arabidopsis induced early flowering and it up-regulated *FT* expression (Samach et al., 2001). The *CO* gene is expressed in the phloem companion cells on leaves and activates the expression of *FT* which is one of the floral pathway integrators (Suarez-Lopez et al., 2001; Song et al., 2012). Expression of *CO* is regulated by *GIGANTEA* (*GI*) and F-box ubiquitin ligases encoded from a group of genes including *FLAVIN-BINDING KELCH REPEAT F-BOX 1* (*FKF 1*) and *ZEITLUPE* (*ZTL*) (Somers et al., 2004; Sawa et al., 2007; Song et al., 2014). *GI* and *FKF1* repress expressions of *CYCLING DOF FACTORs* (*CDFs*), which are the suppressors of *CO* and *FT* expressions (Fornara et al., 2009; Song et al., 2012). In post-translation, the stability of *CO* protein is regulated by the ubiquitin ligase *CONSTITUTE PHOTOMORPHOGENIC 1* (*COP1*). In dark, *COP1* degrades *CO* proteins by ubiquitin-proteasome degradation and in light, photoperiod information perceived by *PHYTOCHROME B* (*PHYB*) (the photoreceptor of far red) activates the expression of *COP1* to regulate the stability of *CO* protein (Jang et al., 2008; Zuo et al., 2011). These oscillated expression patterns of *CO* allow it to regulate *FT* expression in flowering inductive conditions (Fornara et al., 2011). When *FT* is activated in the leaf, the protein of the *FT* genes is translocated to the shoot apical meristem and forms a protein complex with basic leucine-zipper transcription factors (bZIP), a *FD* gene, to activate *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) (a floral pathway integrator) and *APETALA 1* (*AP1*) (floral meristem identity gene), thereby initiating transition from vegetative meristems to floral meristems (Truck et al., 2008; Fornara et al., 2010).

The *CO* and *FT* gene families appear to be universally conserved across plant species (Abe et al., 2005; Tamaki et al., 2007; Igasaki et al., 2008; Truck et al., 2008; Pin et al., 2010; Taoka et al., 2011; Wang et al., 2015; Wickalnd and Hanzawa, 2015; Liu et al., 2017a; Takeshima et al., 2019). Similar functions of *CO* in photoperiod flowering have been identified in other plant species. In rice, a short day plant, *HEADING DATE A / Hd1* (*CO* homolog) activates expression of *HEADIN DATE 3 A / Hd3a* (the *FT* homolog) to initiate flowering under flowering inductive conditions (short day) (Izawa et al., 2002; Hayama et al., 2003). Similarly, in Japanese morning glory (*Pharbitis nil*), a short day plant, the *Pharbitis CO* homolog (*PnCO*) induces expression of *PnFT* (*Pharbitis FT* homolog) (Hayama et al., 2007). These *CO-FT* flowering induction modules in photoperiodic flowering have been identified in other plant species including alfafa, barley, grapes, potato, ryegrass and wheat (Martinez-Garcia et al., 2002; Nemeto et al., 2003; Martin et al., 2004; Almada et al., 2009; Herrmann et al., 2010; Campoli et al., 2012; Kikuchi et al., 2012). While conservation of *CO* roles in photoperiodic flowering has been reported in many plants, functional variations of *CO* have also been identified. In rice, *Hd1* represses the expression of Hd3a under non-flowering inductive conditions (long day) (Hayama et al., 2003). Besides, overexpression of two *CO* homologous genes in woody perennial poplar (*Populus Deltoides*, *CO1, CO2*) regulates expressions of a group of genes involved in metabolic processes and shows no effect on flowering time (Hsu et al., 2012). In soybean, 26 *CO* homologs have been identified (Wu et al., 2014), amongst which two pairs of homologous genes (*GmCOL1a/GmCOL1b, GmCOL2a/GmCOL2b*) have shown a high number of amino acid sequences similar to Arabidopsis *CO.* However, diurnal expression patterns were only detected in *GmCOL1a/GmCOL1b.* Expressions of *GmCOL2a/GmCOL2b* were very low under flowering inductive conditions (short day conditions: 10/14h, day/night). In contrast to transcription patterns of two soybean *CO* homologs, Arabidopsis *co*-mutant plants were fully complemented by these four soybean homologs (*GmCOL1a, GmCOL1b, GmCOL2a* and *GmCOL2b*). These differences in transcriptions and functions of soybean *CO* homologs suggested the functional divergences of photoperiodic regulatory mechanisms in soybean during evolution (Wu et al., 2014).

It is likely that the roles of *FT* and *FT-like* genes in flowering time regulation are well conserved in dicots and monocots (Lifschitz et al., 2006; Laurie et al., 2011; Taoka et al., 2011; Wickland and Hanzawa, 2015). In tomato, ectopic expression of the tomato *FT* ortholog (*SINGLE-FLOWER TRUSS / SFT*) caused early flowering in day-neutral tomato (Lifschitz et al., 2006). Likewise, two *FT* homologs of rice, *HEADING DATE 3A* (*Hd3a*) and *RICE FLOWERING LOCUS T 1* (*RFT1*) play regulatory roles in flowering (Tamaki et al., 2007; Komiya et al., 2008; Taoki et el., 2011). Overexpressing *Hd3a* in transgenic rice plants promoted flowering time in flowering inductive conditions (Tamaki et al., 2007). Besides, up-regulation of the downstream gene of *FT* (*AP1* homolog of rice, *OsMADS 15*) were also detected in transgenic rice plants (Tamaki et al., 2007; Komiya et al., 2008). In addition to constitutive expression of *FT* homologs in rice, suppressions of *RFT1* and *Hd3a* by RNA-interference (RNAi) caused delayed flowering time in RNAi-transgenic rice plants than in wild-type rice plants (Komiya et al., 2008). These early or late flowering induced by ectopic- or suppressed-expressions of *FT* homologs in transgenic tomato and rice showed functional conservations of *FT* in tomato and rice. Recent research in soybean has identified 12 *FT* genes and genetic characteristics of 4 *FT* genes are reported (Kong et al., 2011; Liu et al., 2017a; Takeshima et al., 2019) (Table 2.2).

Table 2. 2. Summary of identified genetic characteristics of soybean *FT* homologs (*GmFTs*).

Gene name	Gene ID	Functions in flowering	Expression patterns	Phenotypes	Reference
GmFT1a	Glyma.18G298900.1	Suppression of flowering	Diurnal circadian expression patterns	Overexpression of *GmFT1a* delayed flowering and maturation in Arabidopsis and soybean	Liu et al. (2017a)
GmFT2a	Glyma.16G150700.1	Photoperiod-regulated flowering, shoot apical meristem growth (post-flowering stem growth); flowering maintenance	Diurnal circadian expression patterns	Overexpression of *GmFT2a* caused early flowering in Arabidopsis and soybean, Suppression of *GmFT2a* (targeted mutations by CRISPR/Cas9) mutant plants induced late flowering initiation in soybean	Kong et al. (2010); Sun et al. (2011); Nan et al. (2014); Cai et al. (2018); Takeshima et al. (2019)
GmFT4	Glyma.08G363100.1	Suppression of flowering	Diurnal circadian expression patterns	Overexpression of *GmFT4* delayed flowering initiation in Arabidopsis	Zhai et al. (2014); Liu et al. (2017a)
GmFT5a	Glyma16G044100.1	Photoperiod-regulated flowering	Diurnal circadian expression patterns	Ectopic expression of *GmFT5a* induced early flowering in Arabidopsis and soybean	Kong et al. (2010); Nan et al. (2014)

Since cultivation areas of each soybean cultivar are strictly limited to specific ranges of latitudes, flowering times of soybean cultivars in different maturity groups vary (Watanabe et al., 2012; Xia et al., 2012). Variations in photoperiod sensitivity of soybean play important roles in adaptabilities of soybean at a wide range of photoperiodic environments ranging from 50-degrees north to 35-degrees south, as day-length as an environmental factor is a variable in different latitudes (Nan et al., 2014). Expression patterns of two *FT* homologs of soybean *GmFT2a* and *GmFT5a* exhibited photoperiod-dependence and diurnal-expression patterns in short day conditions (Kong et al., 2010). Ectopic expressions of two soybean *FT* homologs (*GmFT2a/GmFT5a*) promoted flowering initiation with fewer rosette leaves in Arabidopsis (Kong et al., 2010). Moreover, overexpression of *GmFT5a* induced one week earlier flowering initiation than that observed in the case of transgenic Arabidopsis with overexpressed *GmFT2a*, indicating that *GmFT5a* has prominent roles in long day flowering (Kong et al., 2010). The functional analyses of these two soybean *FT* homologs were also conducted in transgenic soybean plants (Nan et al., 2014; Zhai et al., 2014; Cai et al., 2018; Takeshima et al., 2019). Ectopic expressions of *GmFT2a* and *GmFT5a* promoted flowering initiation of soybean in LD (Nan et al., 2014). Otherwise, suppression of *GmFT2a* expression caused delayed flowering in both long-day (16/8h, day/night) and short-day (12/12h, day/night) conditions (Cai et al., 2018). In addition, up-regulations of an *FT*-downstream gene, *GmAP1a* (Arabidopsis *AP1* homolog), were also observed in the transgenic soybean plants indicating that *GmFT2a* and *GmFT5a* regulate flowering time via mechanisms similar to Arabidopsis (Nan et al., 2014). In Arabidopsis, *AP1* expression is regulated by *FT/FD* complex which binds to the promoter region of *AP1* (Truck et al., 2008; Fornara et al., 2010). In soybean, the interaction of *GmFT2a* and *GmFT5a* with a bZIP transcription factor, *GmFDL19* (a FD homolog of soybean) was confirmed using the yeast two-hybrid assay (Nan et al., 2014). The binding of *GmFDL19* to the promoter region of *GmAP1*a was observed via electrophoretic mobility shift assay (EMSA) suggesting conservation of *FT/FD-AP1* flowering pathway in soybean (Nan et al., 2014). There have been reports on the roles of soybean *FT* homologs, other than those in flowering promotion (Zhai et al., 2014; Liu et al., 2017a; Takeshima et al., 2019). *GmFT1a* and *GmFT4* play inhibitory roles in flowering (Zhai et al., 2014; Liu et al., 2017a). The expression of *GmFT1a* and *GmFT4* were primarily detected on leaves similarly to other flowering-inducing *FT* homologs (Zhai et al., 2014; Liu et al., 2017a). However, *GmFT1a* and *GmFT4* have antagonistic expression patterns as against *GmFT2a* and *GmFT5a* (Zhai et al., 2014; Liu et al., 2017a). Expressions of *GmFT1a* and *GmFT4* are up-regulated in long day, whilst suppressions of their expressions were observed in short day (Zhai et al., 2014). In addition, ectopic expression of *GmFT4a* in Arabidopsis induced late flowering phenotypes (Zhai et al., 2014). Delayed flowering and maturation times were also observed in *GmFT1a* overexpressing transgenic soybean. (Liu et al., 2017a). Recently, new roles of soybean flowering-inducing *FT* genes have been reported (Takeshima et al., 2019). After floral induction, *GmFT2a* and *GmFT5a* are involved in shoot apical meristem growth determinations, respectively. Under long day conditions, wild-type soybean produced flowers at shoot apical meristem when it had 17 to 18 nodes (cv. Willam82). In contrast, *GmFT2a* and *GmFT5*a overexpressing transgenic soybean plants produced terminal flowers when they had 12 to14 nodes and 3 to 4 nodes, respectively. In early stages of vegetative growth (8 days after emergence), expression levels of *GmAP1* homologs in *GmFT5a* transgenic soybean plants were higher than those in *GmFT2a* transgenic soybean. The expression levels of *GmAP1s* in *GmFT2a* became similar to *GmFT5* in later vegetative growth stages (30 days after emergence) indicating that *GmFT2a* and *GmFT5a* are involved in post-flowering stem growth at different magnitudes (Takeshima et al., 2019). These diverse roles of soybean *FT* homologs suggest that although *GmFT* genes have conserved functions in the photoperiod flowering pathways, subfunctionalisations or functional diversifications may have occurred during evolution (Liew et al., 2014; Takeshima et al., 2019).

Vernalisation and autonomous flowering pathways: new functions or conserved functions in soybean vernalisation genes

The climatic information transmitted by cold temperatures and shorter photoperiod in winter allow plants to distinguish between different seasons. Many plants acquire competency of flowering following exposure to prolonged cold temperatures. This process is known as vernalisation (Kim et al, 2009). In winter-annual Arabidopsis, *FLOWERING LOCUS C* (*FLC*) and *FRIGIDA* (*FRI*) have been identified as key elements in its vernalisation response (Johanson et al., 2000; Edwards et al., 2006). *FLC,* a MADS-domain containing transcription factor, suppresses the initiation of flowering by repression of the expression of flowering pathway integrators and the key genes of flowering such as *FT*, *SOC1* and *FD* (Michaels, 2009; Amasino and Michaels, 2010). *FRI* is a plant specific gene and encodes a coiled-coil domain protein with an unknown protein domain and is responsible for up-regulation of *FLC* expression (Johanson et al., 2000). A series of epigenetic modifications (trimethylation of histone H3 at K9 and K27) at *FLC* chromatin after vernalisation (exposure to cold) cause mitotically stable repression of *FLC*. Activation of a plant-specific part of the Polycomb Repressive Complex2 (PRC2) which is encoded from *VERNALIZATION INSENSITIVE3* (*VIN3*) plays important roles in methylation at H3K27 of *FLC* (Wood et al., 2006; De Lucia et al., 2008). In addition to *VIN3*, other genes such as *VERNALIZATION1* (*VRN1*) and *LIKE HETERCHROMATIN PROTEIN1* (*LHP1*) play essential roles in the methylation of H3K9 in Arabidopsis (Kim et al., 2009).

Autonomous flowering is the pathway to promote flowering independently of photoperiod (day length) by repressing *FLC*, a central flowering repressor (Cheng et al., 2017). The group of genes involved in autonomous flowering pathways has been identified in Arabidopsis. These genes regulate the expression of *FLC* mainly via RNA-based post-transcriptional regulation mechanisms and chromatin modification (Boss et al., 2004; Quesada et al., 2005; Bäurle and Dean, 2008). The RNA-regulation process of expression levels of *FLC* is controlled by six genes, *FLOWERING LOCUS CA* (*FCA*), *FLOWERING LOCUS KH DOMAIN* (*FLK*), *FLOWERING LOCUS PA* (*FPA*), *FLOWERING LOCUS Y* (*FY*), *PCF11P-SIMILAR PROTEIN 4* (*PCFS4*) and *PEPPER* (*PEP*) (Simpson et al., 2003; Quesada et al., 2003; Ripoll et al., 2006; Liu et al., 2007; Veley and Michaels, 2008; Ripoll et al., 2009; Srikanth and Schmid, 2011). The multi-protein complex made up of *FCA*, *PCFS4*, *FPA* and *FY* negatively regulate *FLC* expression through modification of 3'-end and polyadenylation of antisense of *FLC* (Cheng et al., 2017). *FLK* contains 3 KH (K-homology) RNA-binding domains and may act as a repressor of *FLC* via RNA-directed chromatin regulation or regulation of transcription of *FLC* (Veley and Michaels, 2008; Ripoll et al., 2009). *PEP* is a positive regulator of *FLC* via an unidentified pathway (Bäurle et al., 2007; Liu et al., 2007; Bäuler and Dean 2008). Overexpression of *PEP* in Arabidopsis exhibited delayed flowering phenotypes with up-regulation of *FLC* expression (Ripoll et al., 2006). The repression of *FLC* expression via chromatic modification is mediated by a group of genes, *FLOWERING LOCUS D* (*FLD*), *FLOWERING LOCUS VE* (*FVE*), *HISTONE DEACETYLASE 5* (*HDA5*), *HISTONE DEACETYLASE 6* (*HDA6*), *LUMINIDEPENDENS* (*LD*), and *RELATIVE OF EARLY FLOWERING 6* (*REF6*) (He et al., 2003; Domagalska et al., 2007; Yu et al., 2011; Liu et al., 2015). These six genes (*HIDA5, HIDA6, FLD, FVE, LD* and *REF6*) suppress *FLC* expression via histone modification (demethylation at H3K4 and deacetylation at H3 or H4) (He et al., 2003; Domagalska et al., 2007; Yu et al., 2011; Liu et al., 2014).

Soybean homologous genes in vernalisation and autonomous flowering pathways have been identified (Jung et al., 2012). Interestingly, a set of genes in the vernalisation flowering pathway has been annotated although soybean is a plant species which does not require vernalisation for flowering (Jung et al., 2012). However, few studies have been conducted on functional characterizations of genes in vernalisation and autonomous flowering pathways. In Arabidopsis, the *VRN* gene family regulates expressions of *FLC*, epigenetically. In this family, *VRN1* has been identified as a repressor of *FLC* expression (Levy et al., 2002). Ectopic expression of *VRN1* induced early flowering but loss-of-function *vrn1* mutant only showed reductions in vernalisation response (Levy et al., 2002).

Heterologous expression of a soybean *VRN1* homolog (Glyma.11G13330) exhibited similar functions in Arabidopsis (Lu et al., 2015). Overexpressing Glyma.11G13330 in transgenic Arabidopsis plants induced early flowering. Moreover, *FLC* and *FD* (downstream genes of *VRN 1*) were repressed in transgenic plants, suggesting Glyma.11G13330 has similar functions to Arabidopsis *VRN1* in vernalisation flowering pathway (Lu et al., 2015).

Other key factors for flowering: Ambient temperature and Gibberellin

In Arabidopsis, even a small fluctuation of temperature has significant effects on the timing of flowering. In a low temperature (23℃） under flowering inductive day length (LD), Arabidopsis exhibited similar phenotypes with those of plants grown in a high temperature (27℃) under non-flowering inductive day length (SD) (Balasubramania et al., 2006). GA is an endogenous cue for evocation of flowering. GA is a plant growth regulator and plays important roles in plant development and growth processes, including cell elongation, transitions of meristems to growth of shoot, developments of male reproductive organs and grain development (Gupta and Chakarabarty, 2013).

The several candidate genes involved in the genetic pathway of ambient temperatures have been identified through flowering time analysis of Arabidopsis mutants under different temperatures. Arabidopsis mutant (*svp*: *SHORT VEGETATIVE PHASE, SVP*) and ecotype which has a deletion at *FLOWERING LOCUS M* (*FLM*) exhibited loss of ability to respond to temperature fluctuations (Balasubramanian et al., 2006; Lee et al., 2007). *FLM* plays key regulator roles in ambient flowering pathways and is spliced alternatively under different ambient temperatures (Balasubramanian et al., 2006; Posé et al., 2013). Two different spliced forms of *FLM*, being *FLMβ* (produced under lower temperature) and *FLMδ* (spliced under higher temperature), act antagonistically in flowering via interaction with *SVP* (Lee et al., 2013; Posé et al., 2013). Ectopic expression of *FLMβ* showed delayed flowering, whilst overexpression of *FLMδ* exhibited promoted flowering initiation (Lee et al., 2013; Posé et al., 2013). The *FLMβ-SVP* complex is formed at a low temperature and represses flowering initiation via binding to *SOC1* locus (Lee et al., 2013; Posé et al., 2013). On the other hand, the *FLMδ-SVP* complex negatively regulates *FLMβ-SVP* complex to promote flowering initiation

(Lee et al., 2013; Posé et al., 2013). Plant nucleosomes have canonical histones including H2A. The replacement of H2A with H2A.Z caused flowering acceleration in Arabidopsis. The *ACTIN-RELATED PROTEIN 6* (*ARP6*) is a component of SWR1 complex which is involved in H2A.Z accumulation (Deal et al., 2007) and the mutation of this gene (*arp6*) exhibits early flowering phenotypes with abnormal flower formation (four petals) in Arabidopsis (Choi et al., 2005). Under flowering inductive conditions in low temperatures, H2A.Z acts as a gate keeper which inhibits the expressions of flowering genes (Verhage et al., 2014). *PHYTOCHROME INTERACTING FACTOR4* (*PIF4*), a member of the *PIF* subfamily, plays crucial roles in plant elongation by interacting with *PHYTOCHROME B* (*PHY B*) in the light-quality pathway (Cerdan and Chory, 2003; Endo et al., 2013). Although genetic signalling pathways of elongation and flowering time by light quality were different (Botto and Smith, 2002; Cerdan and Chory, 2003), the involvement of *PIF4* in ambient temperature pathways has been reported (Koini et al., 2009). Severe reduction of leaf hyponasty and petiole elongation were observed in *pif4* mutant plants grown under high temperatures (Koini et al., 2009). The mutants exhibited significantly delayed flowering times under SD conditions (Koini et al., 2009). Decreased expression levels of *FT* also observed in the *pif4* mutants indicated that *PIF4* targets *FT* (Koini et al., 2009). In Chromatin immuno-precipitation (ChIP) experiments, a higher enrichment of *PIF4* at the *FT* locus was observed in plants under higher temperatures, indicating thermo-sensitive direct interaction between *PIF4* and *FT* (Kumar et al., 2012). In soybean, total 15 *PIF* homologous genes (*GmPIFs*) have been identified and grouped into 3 major clades, *GmPIF4, GmPIF3* and *GmPIF5*, based on amino acid sequences and conservations (Wong et al., 2013; Arya et al., 2018). RNA-sequencing analysis during floral transition in soybean revealed that soybean *PIF* groups showed tissue-specific expressions during floral transition (Wong et al., 2013). Expressions of *GmPIFs* were detected in different plant tissues in various plant parts, including shoot apical meristems, leaves, young pods and seeds, suggesting soybean *PIFs* may have different roles in reproductive developments (Wong et al., 2013). Molecular characterization of soybean *PIF4* (*GmPIF4*) have recently been reported (Arya et al., 2018). Total seven *PIF4* homologous genes have been identified in the soybean genome and have been grouped into two clades, *GmPIF4 I* and *GmPIF4 II* (Arya et al., 2018). All *GmPIFs* in the *GmPIF4 I* clade showed diurnal expression patterns which were similar to Arabidopsis *PIF4* (Arya et al., 2018). Expression levels of three *GmPIF4* genes in Clade I fluctuated in different ambient temperatures under flowering inductive conditions (short day). Increased expressions were detected at 35℃ compared to 25℃ (Arya et al., 2018). Ectopic expression of *GmPIF4b* partially complemented Arabidopsis *pif4-101* mutant and promoted early flowering in wild-type Arabidopsis (Col-0) (Arya et al., 2018). These results suggested that *GmPIF4s* may not only have conserved functions of Arabidopsis *PIF4* but also play specific roles in soybean development (Arya et al., 2018).

Gibberellin (GA) is a plant growth regulator, the functional role of which includes initiation of flowering (Gupta and Chakarabarty, 2013). In Arabidopsis, GA biosynthesis mutant (*ga1-3*) plants showed delayed flowering under SD (Wilson et al., 1992; Blázquez et al., 1998). GA promotes flowering via activation of floral pathway integrators including *SOC1* and *LFY* (Blázquez and Weigel, 2000; Moon et al., 2003). Constitutive expression of *SOC1* can rescue *ga1-3* mutant plants. In addition, GA sensitivity was decreased in *soc1 null* mutant Arabidopsis plants (Moon et al., 2003). Overexpression of *LFY* can also rescue *ga1-3* Arabidopsis plants and reduced *LFY* expression was detected in *ga1-3* mutant under SD conditions (Blázquez and Weigel, 1999). These results suggest that GA is involved in the regulation of floral pathway integrator genes but the mechanism of how GA controls *SOC1* and *LFY* are still unknown (Parcy, 2005; Liew et al., 2014). Recent studies in soybean reported that a soybean *GAMYB-binding protein 1 (GmGBP1)* gene is involved in photoperiod and GA flowering pathways (Zhao et al., 2013, 2018). *GmGBP1* is expressed in leaves and its expression is significantly increased under short day conditions (Zhao et al., 2013). Overexpressing *GmGBP1* in transgenic tobacco plants (*GmGBP1*-ox) promoted flowering initiation regardless of day lengths in both long day and short day conditions (Zhao et al., 2013). Moreover, GA-induced phenotypes such as increased plant height and length of internode were observed in transgenic tobacco plants (*GmGBP1*-ox). Up-regulations of *NtCO* were detected in transgenic tobacco plants and applications of GA increased the expression of *GmGBP1*, indicating that *GmGBP1* is involved in flowering time regulation via photoperiod and GA pathways (Zhao et al., 2013). Further evidence highlighting involvements of *GmGBP1* in flowering has been reported (Zhao et al., 2018). Suppression of *GmGBP1* expression via RNA interference in transgenic soybean plants (*GmGBP1*-i) exhibited delayed flowering time in both long day and short day conditions (Zhao et al., 2018). In addition to late flowering phenotype, transgenic soybean plants (*GmGBP1*-i) also exhibited other phenotypes such as decreased plant height and numbers and length of internode. These results suggested that *GmGBP1* has a positive effect on photoperiod and GA flowering pathway in initiating flowering (Zhao et al., 2018).

2.3.2 Flowering pathway integrators and floral meristem identity genes

The stimulus from each flowering genetic pathway are integrated together to evocate flowering. A group of genes (*FT, SOC1* and *LFY*) has been identified as floral pathway integrators which incorporate environmental (photoperiod and vernalisation) and endogenous (GA and autonomous) signals to activate floral meristem identity genes (*LFY, AP1* and *TERMINAL FLOWER 1*: *TFL1*) initiating flowering process (Parcy, 2005). One of the main flowering repressors, *FLC*, was down-regulated via autonomous and vernalisation flowering pathways (Amasino and Michaels, 2010). Environmental (light and temperature) and endogenous (GA) signals activate the expression of *SOC1*. In addition, *FT* is expressed via photoperiod and autonomous flowering pathways. *LFY* gene acts as a downstream of *SOC1* and is up-regulated via GA response pathway. After floral pathway integrator genes are activated by the exo- and endogenous stimulus under flowering inductive conditions, the flowering pathway integrators up-regulate the expression of floral meristem identity genes (*LFY, AP1* and *TFL1*) in meristems (Parcy, 2005). Activation of floral meristem identity genes induces transformation of vegetative meristems responsible for producing aerial parts of plants (leaf and stem), into reproductive meristems, which ultimately form reproductive organs, such as flowers. *AP1*, a MADS-box transcription factor, is involved in the formation of sepal and petal (Mandel et al., 1992). In Arabidopsis, the *lfy* and *ap1* mutants exhibited conversion of flowers into shoots (Irish and Sussex, 1990; Weigel et al, 1992). In contrast, *TFL1* acts antagonistically to *LFY* and *AP1*. *TFL1*, as a repressor of *LFY* and *AP1*, prohibits formation of floral meristem (Kobayashi et al., 1999). *TFL1* mutants (*tfl1*) show inverse phenotypes with *lfy* and *ap1* mutants and converts indeterminated meristem into determinated meristem (Mandel et al., 1992). Although homologous genes in floral pathway and floral meristem identity genes in soybean have been identified, only limited information on functional characteristics of these group of genes are available (Champagne et al., 2007, Meng et al., 2007; Liu et al., 2010; Chi et al., 2011; Jung et al., 2012; Na et al., 2013; Liew et al., 2014).

Two soybean *SOC1* homologs (*GmSOC1* and *GmSOC1-like*) were isolated in a Chinese soybean cultivar (Na et al 2013). Highest expressions of two soybean *SOC1* homologs were detected at shoot apex in early flowering stages under flowering inductive conditions (short day), whilst the levels of expressions of two *GmSOC1* genes continuously decreased in long day conditions. Interestingly, *GmSOC1-like* was expressed 96 hours earlier than *GmSOC1* in short day, suggesting that they may be regulated differently in floral transition (Na et al., 2013). Ectopic expression of *GmSOC1-like* in another legume plant (*Lotus corniculatus cv. Superrot*) induced early flowering, indicating *GmSOC1-like* may play positive roles in flowering initiation (Na et al., 2013). *AP1* homolog of soybean (*GmAP1*) also was isolated and characterized in heterologous expression in tobacco (Chi et al., 2011). *GmAP1* consists of 8 exons with 7 introns and encodes *AP1-like* protein. Expressions of *GmAP1* were detected in flowers, specifically in sepals and petals, suggesting the roles of *AP1* in floral organs developments were conserved in a soybean *GmAP1* homolog (Chi et al, 2011). Early flowering with formations of abnormal flowers in overexpressing *GmAP1* in transgenic tobacco plants also supported the conserved roles of *GmAP1* in flowering initiation and floral organ development (Chi et al., 2011). *TFL1* is a floral repressor and plays inhibitory roles in floral meristem formation (Kobayashi et al., 1999). In 1972, the roles of two genes (*Dt1* and *Dt2)* in the termination of apical stem growth were identified (Bernard, 1972). Reduced plant height and number of nodes are the phenotypes of a recessive allele (*dt1/dt1*) and a dominant *Dt2/Dt2*, respectively (Bernard, 1972). Genetic mapping analysis of these two genes revealed that *Dt1* is one of the soybean *TFL1* homologs (Liu et al., 2010; Tian et al., 2010). Two *TFL1* homologs (*GmTFL1a, GmTFL1b*) were isolated in the soybean genome and they showed high similarity in amino acid levels (Liu et al., 2010). Interestingly, differential transcription patterns were observed between *GmTFL1a* and *GmTFL1b.* The expression of *GmTFL1b* was only detected in immature seeds whilst *GmTFL1a* was expressed in shoot and root apical meristems, suggesting neo- or sub-functionalization of soybean *TFL* genes (Liu et al., 2010). In contrast to roles of Arabidopsis *TFL1, GmTFL1b* is likely to have an effect only on stem growth. Suppression of *GmTFL1b* expression by virus-induced gene silencing (VIGS) in soybean induced decreased node production rate during reproductive phase (Liu et al., 2010). *GmTFL1b*-VIGS soybean plants produced fewer nodes than mock-inoculated soybean plants, but no difference was observed in flowering time (Liu et al., 2010).

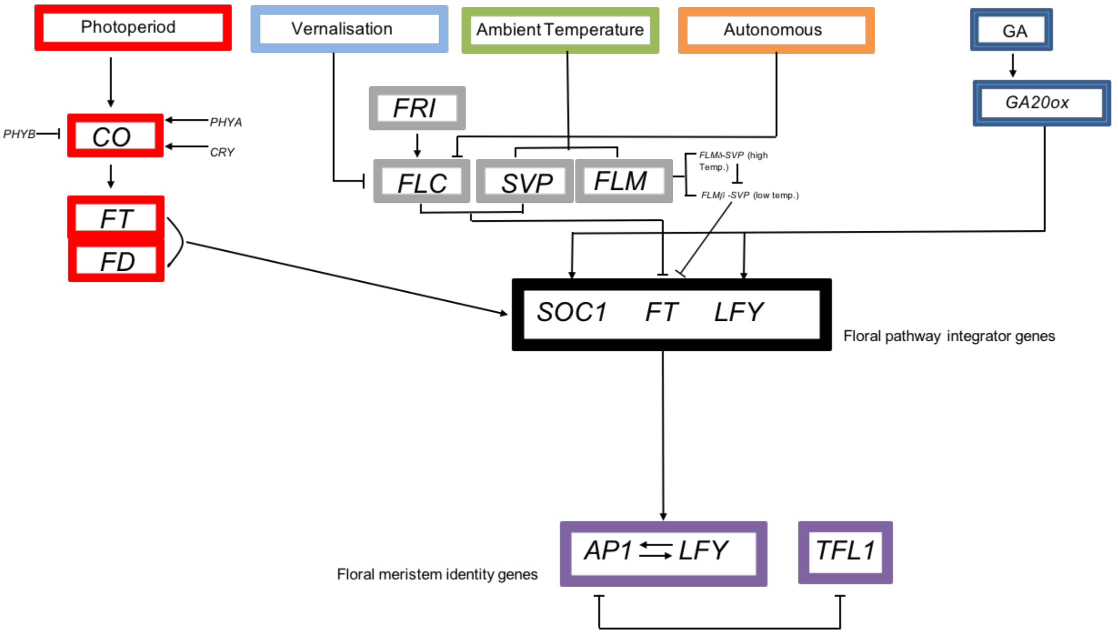


Figure 2. 2. Genetic pathways of flowering.

Genetic control of flowering time in Arabidopsis is illustrated. Environmental and endogenous signals activate initiation of flowering in Arabidopsis. Photoperiod: Different wave-lengths of light are perceived by *PHYA* (*PHYTOCHROME A*, Red) and *CRY* (*CRYPTOCHROME*, Blue). They activate the expression *CO* (*CONSTANS*). In contrast, *PHYB* (*PHYTOCHROME B*) represses *CO* by destabilizing *CO* protein. The *CO* gene encodes protein during daytime and up-regulates the *FT* (*FLOWERING LOCUS T*) expression at leaves. The *FT* is translocated to shoot apical meristem and forms a protein complex with *FD* to activate *SOC1* (*SUPRESSOR OF OVEREXPRESSION OF CONSTANS 1*) and *AP1* (*APETALLA1*). Vernalisation: *FLC* (*FLOWERING LOCUS C*) is a repressor of flowering. *FRI* (*FRIGIDA*) is involved in flowering time regulation by direct expression of *FLC*. After vernalisation, epigenetic modifications at *FLC* chromatin causes repression of *FLC* expression. Ambient temperature: In Arabidopsis, plants grown at high temperature flower earlier than plants grown in low temperature. Flowering time regulation is conducted via direct regulation of the flowering repressor gene, *SVP* (*SHORT VEGETATIVE PHASE*). *SVP* forms a regulatory complex with *FLM* (*FLOWERING LOCUS M*) which directly suppresses the expression of *FT*. *FLMβ-SVP* complex (low temperature) represses *FT* expression, while *FLMδ-SVP* (high temperature) negatively regulates the expression of *FLMβ-SVP* complex to promote flowering initiation. Autonomous: The multi-protein complex in the autonomous flowering pathway represses the expression of *FLC* via RNA-direct chromatin regulation or transcription regulation. GA: *GA20ox* (*GIBBERELLIN 20 OXIDASE enzyme*) promotes biosynthesis of GA and directly activates the expression of *SOC1* and *LFY* (*LEAFY*). The environmental cues (photoperiod, vernalisation, ambient temperature) and endogenous signals (autonomous, GA) activate the expression of floral pathway integrators. These integrators activate the floral meristem identity genes (*AP1, LFY*), thereby initiating flowering. The antagonistic expression of *AP1, LFY* and *TFL*1 (*TERMINAL FLOWER 1*) play important roles in the patterning of flowering.

2.4 *LEAFY* gene

LEAFY gene (*LFY*), one of floral meristem identity genes, acts as a master regulator in flower development and transition of vegetative meristems to floral meristems (Parcy, 2005; Blázquez et al., 2006; Benlloch et al., 2007). *LFY* homologs are widely found in the plant kingdom from algae to land plants including mosses (off-sporing land plants), gymnosperm (non-flowering plants) and angiosperm (flowering plants) (Moyroud et al., 2009, 2010). In Arabidopsis, expression of *LFY* was detected at a very early stage of vegetative development and vegetative shoot apical meristem and the expression gradually increases during plant growth (Blázquez et al., 1997, 2000). When the level of *LFY* expression reaches a critical level, flowering process is initiated via direct up-regulation of *AP1*, a target gene of *LFY* (Parcy et al., 2005; Benlloch et al., 2007). This temporal expression indicates that *LFY* is involved in flowering time regulation (Weigel and Nilsson, 1995; Blázquez et al., 1997). Initial genetic evidences of the relationship between *LFY* and floral meristem identity were obtained by investigation into two *lfy* mutants, *FLORICAULA, Antirrhinum majus* and *LEAFY, Arabidopsis thaliana* (Carpenter and Coen 1990; Coen et al., 1990; Schultz and Haughn 1991; Weigel et al., 1992). Flowers of both mutant plants were converted into shoots. In addition, Arabidopsis *lfy* mutant plants produced flower-shoot intermediate organs and abnormal flowers at apical regions of plants (Schultz and Haughn, 1991; Huala and Sussex, 1992; Weigel et al., 1992). The expression of floral organ identity genes (ABC gene family) were investigated as abnormal flowers were one of the main phenotypes of *lfy* mutants (Hantke et al., 1995; Ruiz-García et al., 1997; Parcy et al., 1998). *AP1* (A class gene) is a direct target of *LFY* but it can be activated via *LFY*-independent manner (Ruiz-García et al., 1997). In addition, it has been identified that *LFY* regulates the expression of B (*APETALA 3: AP3*) and C (*AGAMOUS: AG*) class genes during floral development (Parcy et al., 1998; Liu et al., 2009). In Arabidopsis, *LFY* is expressed consistently in floral bud indicating the requirement of co-regulators to activate target genes of *LFY* (Moyroud et al., 2010). *LFY* co-activates with *UNUSUAL FLORAL ORGANS (UFO)* which encodes an F-BOX protein involving protein ubiquitination through an SCF complex to activate *AP3* in whorls 2 and 3 (Chae et al., 2008). To activate *AG*, *LFY* forms a protein complex with *WUSCHEL* (*WUS*) (Lohmann et al., 2001). *WUS*, a homeodomain transcription factor, is involved in homeostasis of meristems (Laux et al., 1996). In addition to the role of *LFY* in floral development, *LFY* has an effect on the patterning of inflorescences in Arabidopsis (Moyroud et al., 2009, 2010). Arabidopsis is a raceme plant which produces indeterminate inflorescences along its axis. Expression of *LFY* was detected only in floral (lateral) meristems and its expression at shoot apical meristem was repressed by *TERMINAL FLOWER 1* (*TFL1*) (Bradley et al., 1997). Ectopic expression of *LFY* induces transformation of axillary meristems and apical meristem into terminal flowers (Weigel and Nilsson, 1995). Considering the results obtained from Arabidopsis, it is evident that *LFY* plays crucial roles in the time of flowering (flowering time) and the place of flowering (flower development and patterning of inflorescences) (Moyroud et al., 2010). The roles of *LFY* have been confirmed in other dicot and monocot plants. In tomato, *FLASIFLORA* (*FA*), a *LFY* homolog, plays important roles in flowering initiation and formation of flowers (Molinero-Rosales et al., 1999; Kato et al., 2005). The loss-of-function of *FA* (*fa*) exhibited delayed flowering with production of shoot-like inflorescences (Kato et al., 2005). In addition, maize, a monocot plant, possesses two *LFY* homologs (known as *ZFL1* and *ZFL2*) and their loss-of-function displayed late flowering initiation with the loss of floral meristem identity (Bomblies et al., 2003; Bomblies and Doebley, 2006). The roles of *LFY* in the formation of architecture of plants during flowering have also been reported in other plants. *RFL*, a *LFY* homolog of rice, regulates the architecture of inflorescence. The *rfl* mutant plants showed a reduced number of branches, precocious branch termination and failure to tiller (secondary shoots which are generated from the plant base) developments (Kyozuka et al., 1998; Prasad et al., 2003; Rao et al., 2008). However, the roles of *LFY* in regulation of flowering time does not seem to be conserved across all plants. *ABERRANT LEAF AND FLOWER* (*ALF*), a *LFY* homolog of petunia, exhibited its roles only in flower development (Souer et al., 1998; 2008). The loss-of-function of *ALF* (*alf*) induced the formation of leafy shoots instead of flowers. Moreover, constitutive expression of *ALF* in Arabidopsis did not show different phenotypes such as the formation of terminal flowers. A strong *ALF* expression was detected in shoot apical meristem during vegetative developmental phase, long before initiation of flowering. The loss-of-function of *UFO* homologs in petunia (*DOUBLE TOP: DOT*) displayed a similar phenotype with *alf* mutants, however, ectopic expression of *DOT* exhibited formation of precocious flowers and conversion of inflorescences into solitary flowers (Souer et al., 2008). Similar results were reported in tobacco (*Nicotiana tabacum* L.) and poplar (*Populus trichocarpa*). Overexpression of *LFY* homologs of tobacco (*NFL1*) in transgenic tobacco plants did not promote flowering initiation (Ahearn et al., 2001). Although early flowering phenotypes were observed in overexpressing *PtLF* (a *LFY* homolog of poplar), constitutive expression of *PtLF* in transgenic poplar did not alter flowering time (Rottmann et al., 2000). Other roles of *LFY* other than in flowering, were also reported in *legumes*. The *Fabaceae* species including pea (*Pisum sativum*), lotus (*Lotus japonicas*), alfalfa (*Medicago sativa*) produced compound leaves. Mutation of *UNI* (*UNIFOLIATA*, a *LFY* homolog of pea) produced simpler, abnormal leaves, which were without tendrils and had only one leaflet but less number of leaflets (Hofer et al., 1997). Moreover, the expression of *UNI* was detected at margin of leaves during leaf development (Champagne et al., 2007). These results in pea suggested the roles of *LFY* in the formation of compound leaves, being maintenance of cells in their indeterminate state (Hofer et al., 1997; Champagne et al., 2007). Soybean (*Glycine max* L.) is a member of *Leguminosae* and possesses compound leaves. In recent studies on soybean genome, two *LFY* homologs were annotated in soybean, (Schmutz et al., 2010; Jung et al., 2012). However, there is no report on the functional analysis of the *LFY* gene of soybean. Therefore, the investigation of the role of *LFY* in crop plants, such as soybean, is necessary to understand the role of this gene in plant development and flowering. The identification of roles of soybean *LFY* homologs will bring about the improvement of one of the most important crop plants, soybean, by controlling the time of flowering.

2.4.1 The structure of *LFY*

Transcription factors (TFs) regulate gene expression by binding to specific sequences in the promoter or the enhancer of target genes. TFs are involved in the control of the developmental process. To bind to the target genes, TFs have DNA-binding domains (DBDs). *LFY* gene is a transcription factor and plays important roles in flower development and timing of flowering (Moyroud et al., 2009, 2010). In sequence analysis of *LFY* gene, a high level of conservation of two domains (N- and C-domain) were identified (Maizel et al., 2005; Hamès et al., 2008; Sayou et al., 2016) (Figure 2.3). Studies on these conserved structures have revealed that they are involved in DNA binding (Hamès et al., 2008; Sayou et al., 2014, 2016). N-domain is involved in oligomerisation and cooperation with co-regulators (Sayou et al., 2014, 2016). The C-domain is a DNA-binding domain (Hamès et al., 2008). The roles of N-domain in oligomerisation and cooperative binding to target genes were identified via studies on the crystal structure of *Ginkgo biloba LFY* (*GbLFY*) (Sayou et al., 2016). The N-domain is a

Sterile Alpha Motif (SAM) and is made up of five alpha-helices (Sayou et al., 2016). The SAM domain consists of mid-loop (ML) and end-helix (EH) surfaces which play important roles in oligomerization (Kim et al., 2001). The substitutions of amino acids in these surfaces exhibited reduced efficiency of oligomerization and impaired cooperative DNA binding (Sayou et al., 2016). In Arabidopsis, the C-domain was identified as DBDs and as being made up of seven alpha-helices with two beta-strands (Hamès et al., 2008). Out of the seven alpha-helices, one, two and three alpha helices are designated as main grooves and form the helix-turn-helix (HTH) motif. The HTH motif in C-domain plays crucial roles in DNA binding specificity (Hamès et al. 2008). The replacement of amino acids in alpha 2 and alpha 3 (substitutions of amino acids at Asn286 and Lys303 to Alanine) induces a significant reduction of DNA-binding affinity (Busch et al., 1999). In addition, studies on Arabidopsis *lfy* mutants have revealed the key amino acids which are involved in DNA binding in the C-domain (Weigel et al., 1992; Maizel et al., 2005; Hamès et al., 2008). The minor groove of the C-domain is responsible for DNA-based recognition (Hamès et al., 2008). *LFY* recognises the palindrome sequences (T/ANNNCCANT/GT/GNNNNT/A, the centre of the pseudo-palindrome underlined) in the promoter or enhancer regions of target genes (Hamès et al., 2008). The mutation in a minor groove (R234A) causes impairment to the DNA-binding affinity to *AP1*, one of the target genes of *LFY* (Hamès et al., 2008). Furthermore, mutations in the flank region of Arg234 (E235K: lfy-4 mutant and P236L: lfy-5 mutant) also showed reduced DNA-binding affinity within plant mutant phenotypes (Weigel et al., 1992; Hamès et al., 2008). Although investigation on *LFY* structures have been conducted and revealed that *LFY* possesses helix-turn-helix motif and Sterile Alpha Motif, the origin of *LFY* is still elusive. There are no TF groups which carry resemblance with *LFY* in the protein data bank. Hamès et al. (2008) reported that the structure of LFY-C was slightly similar to Tc3 transposase but the similarity was too weak to suggest that *LFY* and Tc3 have a common ancestor. Taken together, *LFY* is a plant-specific transcription factor as there are no similar transcription factors reported to date. The investigation of *LFY* will shed light on the understanding of the evolutionary history of flowering, one of the unique characteristics of plants, since *LFY* plays central roles in the emergence of floral meristems which lead the formation of reproductive organs and flower development by regulation of flower homeodomain gene family (B and C class gene).

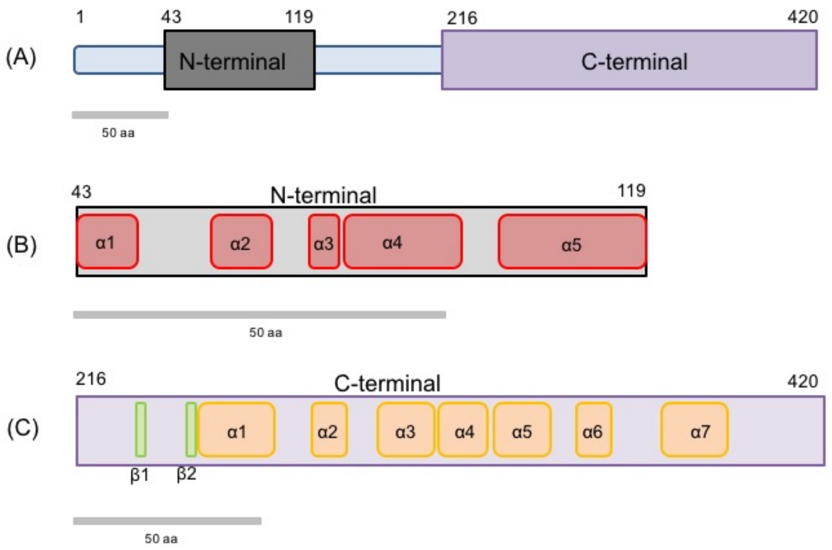


Figure 2. 3. Schematic protein structures of *LFY* gene.

(A) Two highly conserved domain representations of Arabidopsis *LFY* protein (NP_200993.1, 420 amino acids), Black box: N-domain, Purple box: C-domain. (B) N-domain is a Sterile Alpha motif (SAM) consisting of five alpha-helices (Red box). N-domain plays crucial roles in oligomerisation and cooperative binding to target genes. (C) C-domain is a DNA binding-domain. C-domain is made up of two beta-strands (Green box) and seven alpha-helices (Orange box). Scale bar (Grey): 50 amino acids (aa).

2.5 miRNAs: key regulators in plant development and phase transition from vegetative to reproductive development

2.5.1 Studies on microRNAs (miRNAs)

microRNAs (miRNAs) are a small non-coding RNA consisting of 20 to 24 nucleotides (nt), which play pivotal roles in the regulation of genes in both plants and animals (Spanudakis and Jackson, 2014). miRNAs regulate gene expressions by direct or indirect transcriptional and post-transcriptional gene silencing (TGS and PTGS). The first report on miRNA was on animals. In *Caenorhabditis elegans* (*C. elegans*), *linege-4* (*lin-4*) and *lethal-7* (*let-7*) were sequentially expressed and involved in the transitions between stages of larval development (Lee et al., 1993; Wightman et al., 1993). These miRNAs, *lin-4* and *let-7*, have been identified as regulators of temporal development in the worm of *C. elegans* and their expression patterns are coincident with their roles in developmental timing (Lee et al., 1993; Wightman et al., 1993). In addition, other miRNAs in animals also showed their expression patterns in developmentally regulated ways (Bartel, 2004, 2009; Giraldez et al., 2005). Moreover, their tissue and temporal specific expression patterns indicated that miRNAs were regulators of important developmental processes. In plants, the roles of miRNAs in various developmental processes have been identified (Zhang et al., 2006; Chen, 2009).

The miRNA biogenesis process has been well-conserved among animals and plants (Liu et al., 2012; Naqvi et al., 2012). The schematic diagram of biogenesis of miRNAs appears in Figure 2.4. The MIR gene is transcribed by RNA polymerase II in the nucleus. Primary miRNA transcript (Pri-miRNA) containing a 5' cap and 3' ployanenylated tail is produced and introns are spliced (Xie et al., 2005; Liu et al., 2012). Pri-miRNAs are imperfect double-stranded fold-back structure, and the stability of Pri-miRNAs is built by interaction with an RNA-binding protein, DAWDLE (DDL) protein (Yu et al., 2008; Voinnet, 2009, Liu et al, 2012). Pri-miRNAs are processed to precursor-miRNAs (pre-miRNAs) by a protein complex, D/SmD3bodies (Vaucheret, 2006; Liu et al., 2012). This protein complex contains a RNase III family protein, DICER-LIKE 1 (DCL1), which acts as an endonuclease to cleavage of the primary transcript and stem-loop region (Papp et al., 2003). The accessory proteins including HYL1 (the double-stranded RNA (dsRNA)-binding protein HYPONASTIC LEAVES 1), SE (the C2H2-zinc finger protein SERRATE), CBP20 and CBP 80 (the cap-binding CBC complex) facilitate the interaction between miRNA and the protein complex (Fang and Spector, 2007; Laubinger et al., 2008; Voinnet, 2009; Khraiwesh et al., 2012). The mutation of these proteins cause reduction in the levels of mature miRNAs and result in an embryonic lethal, or severe defects in development (Han et al., 2004; Liu et al., 2012). The miRNA/miRNA* duplex which consists of the guide strand miRNA and the passenger strand (miRNA*) and is processed from pre-miRNA by DCL1. This duplex has two additional nucleotides at their 3' end (Voinnet, 2009; Naqvi et al., 2012). S-adenosylmethionine-dependent methyltransferase, HUA ENHANCER 1 (HEN1), methylates the 2'hydroxul groups on the overhanged nucleotides at 3' end of miRNA duplex. This protein adds a poly(U) tail, which acts as an inhibitor of miRNA degradation by SMALL RNA DEGRADING NUCLEASE-1 (SDN-1) (Ramachandran and Chen, 2008; Liu et al., 2012). The miRNA duplex is then transferred into cytoplasm by way of a dependent- and independent-HASTY1 protein transportation. The miRNA duplex is separated into the guide strand and passenger strand (Guleria et al., 2011). Finally, the guide miRNA is combined with miRNA-induced silencing complex (RISC), which consists of the ARGONAUTE 1 (AGO1) complex, the cyclophilin 40-homologue

SQUINT (SQN) and the HEAT SHOCK PROTEIN 90 (HSP90) as an activator of AGO1 activity (Khraiwesh et al., 2012; Yamaguchi and Abe, 2012).

miRNAs regulate the expression of target genes via transcriptional and post-transcriptional gene silencing. These modes of regulations are determined by the similarity of sequences between the miRNA and its target mRNA, which have a perfect or near perfect complementarity with the mRNA of target genes, inhibiting gene expression by RNA cleavage. In contrast, post-transcription gene silencing occurs when miRNAs have a significant number of mismatches with their target sequence or target sites located in 3' UTR regions. In plants, it was recently reported that plant miRNAs have a high sequence complementarity with their target genes, suggesting that miRNAs in plants regulate the expression of target genes via transcriptional cleavages (Broderson et al., 2008).

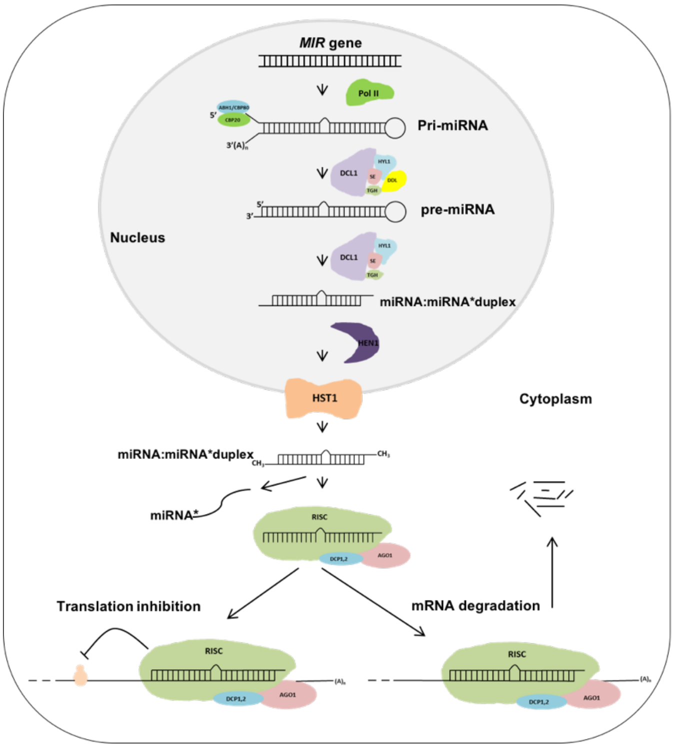


Figure 2. 4. Plant microRNA biogenesis pathway.

The MIR gene is transcribed by RNA polymerase II (Pol II). Primary-miRNA (Pri-miRNA) consists of a hair-pin structure with 5' cap and 3' polyadenylated tail. Pri-miRNA is processed into a stem-loop secondary structure, which is a premature-miRNA (pre-miRNA) by DCL1. Subsequently, the miRNA/miRNA* duplex is cleaved from pre-miRNA by DCL1 with other protein complexes (HYL1, HEN1) and this duplex is then transferred to cytoplasm by HASTY. RISC complex binds to the guide miRNA and attaches to target miRNA in order to inhibit expression via transcription or post-transcription repression. DCL1 (DICER-LIKE PROTEIN 1), RISC (RNA-induced silencing complex), HEN1 (HUA ENHANCER 1), HYL (HYPONASTIC LEAVES 1).

2.5.2 Roles of *miR156* and *miR172* on flowering time regulation

Recently, many studies on miRNAs revealed their crucial roles in plant development and phase transition from vegetative to reproductive (Huijser and Schmid, 2011). The miRNA families which are the involved in flowering initiation have been identified through the gain-of-function and loss-of-function studies (Spanudakis and Jackson, 2014). The roles of *miR156* and *miR172* families on flowering time regulation have been identified and they play important roles in control of flowering time (Yamaguchi and Abe, 2012; Spanudakis and Jackson, 2014) (Figure 2.5). The *miR156* and *miR172* families are the main components of the ageing pathway and are expressed inversely to control the initiation of reproductive development (Wu et al., 2009; Yamaguchi and Abe, 2012). *miR156* is highly expressed in early stages of development such as embryogenic and seedling stages and decreases its expression during ageing process. On the other hand, accumulation of *miR172* is detected on leaves and reproductive organs such as floral buds (Wu et al., 2009; Zhu and Helliwell, 2010). This inverse temporal expression pattern of *miR156* and *miR172* indicates that these miRNA families have an effect on the control of phase transition from vegetative to reproductive development (Yamaguchi and Abe, 2012).

2.5.3 miR156 family: key regulators for vegetative development and phase transition

miR156 family regulates developmental phase transition via repression of its target genes, that is, *SQUAMOSA PROMOTER BINDING-LIKE* (*SPL*) transcription factor families (Wu et al., 2009). In Arabidopsis, 15 mature miR156 are encoded by 10 miR156 loci (miR156a-j) and regulate 11 of 17 *SPLs* via transcript cleavage (Park et al., 2005; Huijser and Schmid, 2011; Yamaguchi and Abe, 2012; Tripathi et al., 2018). The roles of *miR156* on flowering time were firstly identified in *miR156* overexpressing transgenic Arabidopsis plants. Ectopic expression of *miR156* caused delayed flowering time with prolonged juvenile developmental period (Wu and Poethig, 2006; Huijser and Schmid, 2011). Overexpression of *miR156* produced more rosette leaves and leaves of transgenic plants (*35S::miR156*) exhibited delayed appearance of adult phenotypes (abaxial trichomes) than wild-type plants (Wu and Poethig, 2006). There have been reports of extended vegetative developmental phase by constitutive expression of *miR156* in other plant species, such as rice, maize and tobacco, indicating that *miR156* has conserved roles in flowering (Xie et al., 2006; Chuck et al., 2007; Feng et al., 2016). The upstream factors mediating the age-dependent reduction of *miR156* have largely remained unknown (Spanudakis and Jackson, 2014). Recent studies revealed that the nutrition status in plants regulate *miR156* levels (Yang et al., 2013; Yu et al. 2013). High deposition of metabolically active forms of sugars (sucrose and glucose) selectively controls the expression of MIR156A and MIR156C (Yang et al., 2013; Yu et al., 2013). They reported that a high accumulation of sugar in shoot apex exhibited a reduction of *miR156* expression, whilst the expression of *miR156* increased in sugar deprivation conditions, resulting in down-regulation of *SPL* genes (Yang et al., 2013; Yu et al., 2013). Sugar may regulate the level of *miR156* by activation of sugar-specific *cis*-acting regulatory elements or by direct destabilization of the primary-miRNAs in a post-transcription level (Yang et al., 2013).

In Arabidopsis, *SPL* transcription factors are target genes of miR156 and play essential roles in development and timing of flowering (Park et al., 2005; Wu et al., 2009; Huijser and Schmid et al., 2011; Yamaguchi and Abe, 2012; Tripathi et al., 2018). Arabidopsis *SPL* genes act redundantly in plant development and flowering, as loss-of-function of a single *SPL* gene exhibited insignificant effects on flowering phenotypes (Yamaguchi and Abe, 2012). The *SPL* transcription factor family in Arabidopsis is grouped into four different clades (*SPL3/4/5*, *SPL2/10/11*, *SPL9/15*, *SPL6/13*) by phylogenetic analysis and two clades (*SPL3/4/5* and *SPL9/15*) have a significant effect on phase transition (Guo et al., 2008; Spanudakis and Jackson, 2014). Ectopic expression of *rSPL3* (a resistant form of *SPL3* to miR156) in Arabidopsis exhibited extremely early flowering phenotypes and precocious appearance of adult traits on leaves (Cardon et al., 2002). Similar results were reported in *rSPL4* and *rSPL5* transgenic Arabidopsis plants containing miR156-resistant forms of *SPL4* and *SPL5*, respectively (Wu and Poethig, 2006). However, ectopic expression of *SPL3* transgenic Arabidopsis plants only displayed slightly earlier flowering time than wild-type, indicating the repressing strength of endogenous *miR156* (Wu and Poethig, 2006; Gandikota et al., 2007). *SPL9/15* is another clade involved in flowering time (Spanudakis and Jackson, 2014). *SPL9* and *SPL15* act redundantly, as a single loss-of-function mutant exhibited weak phenotypes (Schwarz et al., 2008). In contrast, double loss-of-function mutant (*spl9 spl15*) exhibited extended vegetative development with delayed flowering initiation, which resembled that of transgenic Arabidopsis plants overexpressing *miR156* (Schwarz et al., 2008). Similar to *SPL3/4/5*, ectopic expressions of *rSPL9* or *rSPL15* in transgenic Arabidopsis plants also showed early flowering and produced leaves with adult traits (Wu and Poethig, 2006; Wu et al., 2009). In addition, *SPL9* accelerated the appearance of adult traits on leaves via direct activation of *miR172b* expression (Wu et al., 2009).

2.5.4 miR172 family: master roles in reproductive developments

MIR172 is one of the earliest identified plant MIRNA genes and plays pivotal roles in promoting adult epidermal identity and flowering time by down-regulation of its target genes (Aukerman and Sakai, 2003). In Arabidopsis, 8 mature miR172 are encoded from 6 miR172 loci (miR172a-e) (Aukerman and Sakai, 2003; Xie et al., 2005; Lu et al., 2006; Jung et al., 2007; Moldovan et al., 2010). The functions of *miR172* was firstly identified in Arabidopsis (Aukerman and Sakai, 2003). Early flowering with abnormal development of flower organs were observed in *miR172b* overexpressing Arabidopsis plants, which were screened from activation-tagging lines. Overexpression of *miR172b* caused early flowering initiation under both LD (inductive) and SD (non-inductive) conditions (Aukerman and Sakai, 2003). In addition, overexpressions of target genes of *miR172* including *AP2* and *AP2-like* genes (*TOE1, TOE2, SMZ* and *SNZ*) caused delayed flowering initiation in Arabidopsis, indicating their repressor roles in flowering (Schmid et al., 2003; Mathieu et al., 2009; Wu et al., 2009; Yamaguchi and Abe, 2012). The functional redundancy of *AP2* and *AP2-like* genes were reported in several studies (Wu et al., 2009; Yant et al., 2010; Yamaguchi and Abe, 2012). The hextuple mutants of six *AP2*-like genes (*smz-2*, *snz-1*, *toe1-2*, *toe2-1*, *toe3-1* and *ap2-6*) exhibited early flowering times similar to that of *miR172b* overexpressing lines (Yant et al., 2010). *miR172* promotes phase transitions (from vegetative to reproductive) and initiation of flowering by repressing its target genes through translation inhibition (Aukerman and Sakai, 2003; Fang and Spector, 2007). The formations of abnormal flowers are one of the main characteristics of *miR172b* overexpressing plants (Aukerman and Sakai, 2003). The defects of flowers from *miR172b* overexpressing plants resembled those of *ap2* (*AP2*, a flower homeotic gene, class A gene) mutants in Arabidopsis, meaning they produced flowers with absence of petals, and carpels instead of sepals (Bowman et al., 1991; Aukerman and Sakai, 2003). *AP2*, a target gene of *miR172*, has diverse roles in plant development including seed development, regulation of stem cells and determinacy of floral organ identity (Bowman et al., 1989; Ohto et al., 2005; Würschum et al., 2006). In flowering, *AP2* acts as a floral repressor and a regulator of floral development (Class A gene) (Bowman et al., 1989; Yant et al., 2010). Yant and colleagues (2010) reported on the target genes of *AP2* genes. In ChIP-Sequencing analysis, a floral pathway integrator gene (*SOC1*) and floral meristem identity genes (*AP1* and *FUL*) have been identified as direct target genes of *AP2*. In addition, the roles of *AP2* in the regulation of *miR156* (positive regulation) and *miR172* (negative regulation) via feedback-loop also were reported (Yant et al., 2010; Huijser and Schmid, 2011). As a class A gene, *AP2* is responsible for formation of sepals and plays a regulator role in developments of petals via combinational expression of class B genes (Coen and Meyerowitz, 1991; Yant et al., 2010). *rAP2* (resistant to *miR172*) transgenic Arabidopsis plants exhibited several floral disrupts, such as indeterminate flowers with numerous reproductive organs and enlarged floral meristems (Chen, 2004).

Since the first study on soybean miRNAs from roots was reported (Subramanian et al., 2008), many conserved and novel miRNA families were identified from different tissues of soybean including developing seeds (Song et al., 2011), shoot apical meristem (Wong et al., 2011), flowers and nodules (Joshi et al., 2011). Recent studies to identify soybean microRNAs and their targets (Zhang et al., 2008; Joshi et al., 2011; Song et al., 2011; Wong et al., 2011; Xu et al., 2013a, b; Cao et al., 2015) were conducted by bioinformatics analysis. However, studies on experimental validations of soybean miRNAs remain limited (Song et al., 2011). In soybean, 28 loci for encoding 28 mature miR156 (MIR156 a-z and aa, ab) and 12 loci for encoding 15 mature miR172 (MIR172 a, b3p, b5p, c, d, e, f, g, h3p, h5p, i3p, i5p, j, k and l) were identified (Subramanian et al., 2008; Kulcheski et al., 2011; Wong et al., 2011; Goettel et al., 2014; Zhao et al., 2015). However, there is no study on soybean miRNA156 and miRNA172 available. Therefore, functions of soybean miRNA156-miRNA172 modules in vegetative and reproductive phases need to be investigated.

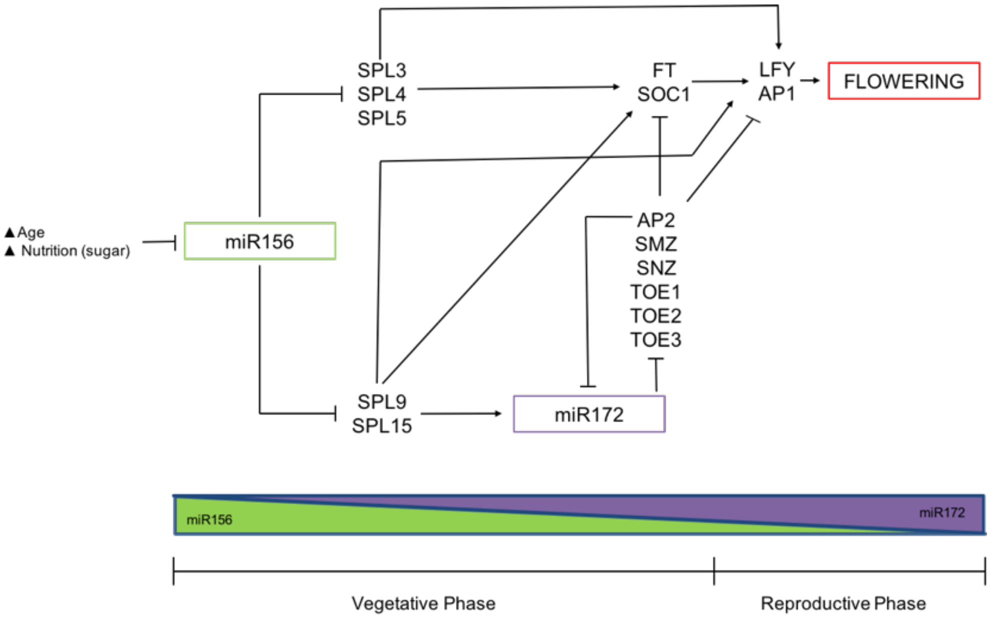


Figure 2. 5. Roles of *miR156* and *miR172* on phase transition from vegetative to reproductive development.

The *miR156-miR172* modules regulate ageing pathway in Arabidopsis. As the plant ages and sugar accumulation levels increase at shoot apex, the expression of *miR156* decreases, resulting in up-regulation of *SPLs*. The *SPL9/15* activates the expression of *miR172*. The expressions of *SPLs* directly activate floral pathway integrators (*FT, SOC1*) and floral meristem identity genes (*LFY, AP1*). *miR172* represses the expression of its target genes (*AP2, TOE1/2/3, SMZ*, and *SNZ*) to promote phase transition to reproductive developmental phase and evocation of flowering.

2.6 The need for further studies on soybean flowering

Even though the knowledge gained from model plants such as Arabidopsis allows basic understanding of plant biology, it is difficult to apply such findings directly to important crop plants such as soybean (Haerizadeh et al., 2009). Moreover, there are major developmental differences between Arabidopsis and soybean. Soybean is a short day plant while Arabidopsis is a long day plant, and soybean has a bigger genome size (1.1-gigabase) than Arabidopsis (135-megabase). Soybean, a paleopolyploid species, has complex genomes compared to Arabidopsis (Shoemaker et al., 2006). Several studies on soybean genome have revealed that soybean is a diplodized tetraploid species (Lackey 1980; Zhu et al., 1994; Shoemaker et al., 1996; Lohnes et al., 1997; Lee et al., 1999; Hymowitz, 2004). Soybean is a member of Papilionoideae subfamily, Leguminosae, to which many important legumes including pea, alfalfa, lotus and medicago belong. Plants in this subfamily, except soybean, contain 6-11 base chromosomes (x=6-11), whilst soybean possesses double-base chromosomes (x=20), suggesting that soybean is a diplodized tetraploid (Lackey 1980; Hymowitz 2004). Analysis of EST collections and the genome sequence analysis from soybean revealed that two major genome duplications [13 million years ago (MYA) and another 59 MYA] have probably occurred in soybean, resulting in gene diversification and chromosome rearrangements (Schlueter et al., 2004; Schmutz et al., 2010). Duplications of soybean genome induced duplication of its genes, resulting nearly 75% of the genes existing therein in multiple copies (Schmutz et al., 2010; Jung et al., 2012). Recently, the whole genome of soybean has been sequenced (Schmutz et al., 2010) and comparative analysis of soybean flowering genes was also conducted by Jung et al (2012). This genetic information of soybean forms the underlying basis for functional analysis of genes involved in its flowering processes. Although the sequencing of soybean genome has identified the number of expressed genes, the functions of most of those genes are still unknown (Homrich et al., 2012). Therefore, investigation of these gene functions is necessary for improvement of soybean.

Chapter 3. Development of transformation systems for commercial cultivars of soybean (*Glycine max* L. cv. Bunya and cv. Bragg)

Abstract

Soybean is one of the most widely planted crops in the world. The popularity of this oil-seed plant is due to its high quality protein and oil provided for human consumption. Furthermore, soybean has been widely used for feeding animals as one of the important sources of protein. In this regard, the strong demand for soybean continues to grow rapidly. The development in plant genetic transformation methods allows direct insertion of exogenous genes into plant genome, resulting in improvements important agronomical traits of crop plants. Since stable soybean transformation systems are only applicable for a few agronomically inferior cultivars such as cv. Jack and William, the development of soybean transformation systems for commercial cultivars is crucial in terms of improving crop capability.

This chapter examined the *Agrobacterium*-mediated transformation system using explants from half-seed of a commercial cultivar (cv. Bunya and Bragg). Firstly, regeneration efficiency of the explants from two commercial cultivars of soybean (Bunya and Bragg) were evaluated. Half-seed explants from two cultivars showed a high regeneration frequency (80% and 83.3% respectively). To develop an *Agrobacterium*-mediated transformation system, half seed explants of Bragg and Bunya were inoculated with the *Agrobacterium tumefaciens* strain EHA105, containing binary vector pUQC10255 (harbouring herbicide tolerant gene: *bar* and yellow fluorescence protein gene: YFP). Two different responses from Bunya and Bragg were observed after co-cultivation. Half-seed explants of Bunya showed a reduction in regeneration efficiency (80.0%→29.2%) and eventually failed to survive on selection medium. In contrast, half-seed explants of Bragg produced putative transgenic shoots and also demonstrated a reduction in regeneration efficiency (83.3%→66.5%). However, putative transgenic shoots failed to elongate in successive culture. To improve shoot elongation, effects of adenine hemisulfate and PVP 40,000 on shoot elongation were evaluated. After 6 weeks of culture, adding phenolic compounds inhibitors resulted in greater numbers of elongated shoots (6-fold) than that of non-treated batches. With additions of adenine hemisulfate and PVP 40,000, a total of twenty-three T0 putative transgenic shoots were produced using half-seed explants of Bragg. Herbicide (Basta$^{®}$) assay was conducted on a total of 13 T0 putative transgenic plants to examine the expression of *bar* gene in transgenic soybean plants. A total of three lines showed herbicide resistance after 5 days of Basta$^{®}$ treatment on the leaves. In yellow fluorescence protein expression analysis, two lines of basta resistant putative transgenic plants showed yellow fluorescence on leaves. The presence and the expression of the *bar* gene in lines T0-8 and T0-14 were confirmed by PCR and RT-PCR analyses. Among twenty-three putative transgenic plants, a line T0-3 showed successful integration and stable expression of the *bar* gene in PCR and southern blot analysis. Overall, total 0.6% of transformation efficiency was obtained (cv. Bragg). The stable inheritance and expression of transgenes were also confirmed in T1 and T2 generations. In T1 generation, 4 out of 5 T1 lines (from T0-3 line) exhibited YFP emissions (YFP positive). Herbicide resistance was confirmed in 3 of 4 YFP positive lines. YFP emission and herbicide resistance were also detected in T2 (total 49 independent T2 lines from four T1 YFP positive lines), indicating stable expression and inheritance of YFP and *bar* genes.

Keyword: Soybean transformation, half-seed explants, Bragg, herbicide tolerance, YFP

3.1. Introduction

Soybean is one of the most important crops in the world. Its seeds, containing 40% protein and 20% oil serve as an affordable source of protein for feeding animals and for human diet (Sugano, 2005). Furthermore, it can be used as a source of biofuel and its by-products have been used in making a variety of products such as biocomposites, candles, soy-ink, soy-crayons, soy-based lubricants, pharmaceuticals, plastics, pesticides and cosmetics (Pratap et al., 2012). In addition to versatile uses of soybean, the ability to fix nitrogen from the atmosphere makes soybean an important plant in sustainable agricultural practice. Rhizobia has an ability to fix nitrogen from the atmosphere. This symbiosis bacteria of soybean infects the roots of soybean and forms nodules where they fix nitrogen. In crop rotations, soybean provides biologically fixed nitrogen to other crop plants cultivated after soybean. Since soybean is one of the most widely planted crops worldwide, this feature has made soybean one of the targets for genetic improvement.

Plant genetic transformation allows researchers to improve important agricultural traits such as resistant to environmental stress and to elucidate functions of genes by genetic analysis using overexpression of genes and target gene silencing or genome editing by RNAi (RNA interference) method or bacterial CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats-associated protein-9 nuclease) (Fire et al., 1998; Barrangou et al., 2007; Zhang et al., 2014). Since the first development of soybean transgenic plants was reported in 1980s (Christou et al., 1988; Hinchee et al., 1988), soybean transformation systems have been continuously developed. Two transformation methods have been widely used for soybean transformation: Particle bombardment and the *Agrobacterium tumefaciens*-mediated transformation (Homrich et al., 2012).

Particle bombardment, which is also called projectile acceleration, biolistics or gene gun, is a method of transformation that uses helium gas pressure to directly introduce DNA-coated gold or tungsten particles into plant cells (Sanford, 1988). The inserted exogenous DNA fragments are integrated into chromosomal DNA in nucleus (Sanford, 1990; Kohil et al., 2003). Since the first soybean transformation was carried out using particle bombardment (Christou et al., 1988), many attempts for genetic modification of soybean have been made using meristematic cells (Christou et al., 1988; Christou and McCabe, 1988; Aragão et al., 2000). Somatic embryos have also been widely used as a target tissue for soybean transformation using particle bombardment (Finer and Mc McMulle, 1991; Finer et al., 1992; Stewart Jr et al., 1996; Droste et al., 2002; Homrich et al., 2008; Wu et al., 2008; Hernandez-Garcia et al., 2009).

Agrobacterium-based transformation is cost-effective and requires minimal equipment (Yamada et al., 2012). Moreover, *Agrobacterium*-mediated transformation methods result in low copy numbers of genes being transferred and mostly single transgene insertions, producing a reduced frequency of gene silencing events caused by multi-copy numbers of transgene. The stable soybean transformation has been established by using cotyledonary node explants and imbibed mature seeds (Hinchee et al., 1998; Olhoft et al., 2003, Paz et al., 2006) through *Agrobacterium*-mediated transformation. One common explants for *Agrobacterium*-mediated soybean transformation is cotyledonary node, which has axillary meristems at the junction between cotyledon and hypocotyl, and these meristems form multiple shoots (Yamada et al., 2012). The ability of multiple shoot formation has a great effect on transformation efficiency (Jeon and Chung, 2009). However, this ability varies among cultivars, so the selection of cultivars that have a high efficiency of multiple shoot formation is important. To improve the soybean transformation rate, factors which are involved in *Agrobacterium* infection such as an addition of acetosyringone, thiol compounds (L-cysteine, Sodium thiosulfate, dithiothreitol) in infection and co-cultivation stages and proper selection agents and schemes should be optimized. Olhoft and Somers (2001) reported that the addition of thiol compounds on co-cultivation media seems to prevent wound- and pathogen-induced responses, because it facilitates *Agrobacterium* to infect explants leading to increase of transformation efficiency. In addition, the use of proper selective agents is crucial for successful transformation (Zeng et al., 2004). Hygromycin and herbicide (phosphinothricin and glyphosate) have been widely used for soybean transformation (Olhoft et al, 2003; Paz et al., 2006; Kim et al., 2012b; Li et al., 2017).

After 30 years since the first report of successful soybean transformation, routine and stable soybean transformation system still remains difficult. Soybean transformation efficiency is still low and cultivars for successful transformation are limited (Table 3.1). Therefore, optimization of soybean transformation methods for commercial cultivars is very important in order to strengthen crop improvement capability. Sequencing of soybean genome has identified numbers of expressed genes but the functions of most of these genes are still unknown (Schmutz et al., 2010; Homrich et al., 2012). Therefore, the investigation into the functions of genes in soybean are necessary. In this regard, plant biotechnology such as plant transformation is a good tool for analysis of functions of genes in that it allows the investigation of gene functions by direct regulation of expression of genes.

This study aims to develop a transformation system of commercial cultivars of soybean via an *Agrobacterium*-mediated transformation method. The objectives of this chapter are as follows:

- Comparison of genetic transformation potentials of commercial cultivars of soybean (*Glycine max* L. cv. Bunya and cv. Bragg)
- Analysis of factors that improve shoot elongation (types of explants, addition of antioxidant)

- Confirmation of integration of genes in transgenic plants by molecular analysis
- Confirmation of expression of the transgenes in the transgenic plants
- Confirmation of stable expression of the transgenes in T1 and T2 generation of transgenic soybean plants.

Table 3. 1. Effects of genotypes of soybean in soybean transformation.

Genotypes	Types of explants	Agrobacterium strain	Selective marker gene	Reporter gene	Transformation efficiency (%)	Reference
Bert	Cotyledonary node	AGL1	*bar*	GUS	0.9	Olhoft and Somers. (2001)
Jack	Cotyledonary node	EHA101	*bar*	*GUS*	2.8	Song et al. (2012)
Jack Purple	Half Seed	EHA101	*bar*	*GUS*	9.2	Li et al. (2017)
Throne	Half seed	EHA101	*bar*	*GUS*	1.0	Paz et al. (2006)
Williams	Half seed	EHA101	*bar*	*GUS*	3.6	Paz et al. (2006)
Wiiliam79	Half seed	EHA101	*bar*	*GUS*	1.8	Paz et al. (2006)
William82	Half seed	EHA101	*bar*	*GUS*	4.5	Paz et al. (2006)
	Cotyledonary node	EHA101	*bar*	*GUS*	4.4	Song et al. (2012)
Kwangan	Half seed	EHA105	*bar*	*PAC (Psy-2A-CrtI gene)*	5.0	Kim et al. (2012)
Heinong 44	Hypocotyl	EHA105	*NPTII*	*FAD2 (Δ-12 fatty acid desaturase gene*)	9.3	Wang and Xu. (2008)
Heinon 37	Cotyledonary node	EHA101	*bar*	*GUS*	0.3	Song et al. (2012)
Yuechun	Cotyledonary node	EHA101	*bar*	*GUS*	2.4	Song et al. (2012)
Tianlong 1	Cotyledonary node	EHA101	*bar*	*GUS*	4.6	Song et al. (2012)
	Half Seed	EHA101	*bar*	*GUS*	10.0	Li et al. (2017)

3.2. Materials and methods

3.2.1 Plant materials and growth conditions

Commercial soybean (*Glycine max* L) cultivars, Bunya and Bragg, were grown in the plant growth room (The University of Melbourne) under a controlled photoperiod condition (light intensity 400 μmol m^{-2} s^{-2}, 10 h/14 h, 25±1℃) to set seeds.

3.2.2 Preparation of plant tissue culture medium

Compositions of medium, antibiotics and hormones used in this study are presented in appendices (Table A1).

3.2.2.1 Seed sterilization

Mature soybean seeds of two commercial cultivars, Bunya and Bragg were sterilized with chlorine gas generated from a mixture of 3.5 ml 12 M HCl and 100 ml commercial bleach (12% Sodium hypochlorite) for 16 h in a sealed desiccator. The sterilized seeds were washed 5 times with sterilized double distilled water. Excessive water was briefly removed with sterilized filter papers.

3.2.2.2 Preparation of explants

For hypocotyl, half-split hypocotyl and cotyledonary node explants preparation, sterilized seeds were placed on germination medium in Petri dishes. After 6 days of culture in a tissue culture room (150 μmol m^{-2} s^{-2}, 16/8 h, 25±1℃), germinated seedlings were used for explants preparation. The different explants used in this study were prepared following the methods described below:

Cotyledonary node explants (Olhoft et al., 2003)

The seed coats were removed with the help of sterilized forceps and surgical blade (#10, Feather, Japan) and then hypocotyls 4 - 5 mm below the cotyledonary node were discarded. The primary shoot was removed from seedlings and explants were produced by longitudinal cut between two cotyledons.

Hypocotyl and half-split hypocotyl explants (Wang and Xu, 2008)

Firstly, seed coats were removed. Cotyledons were removed from seedling using forceps and then epicotyl and majority of hypocotyls were cut off. Finally, 1 to 2 cm long hypocotyl segments were obtained. For preparation of half-split hypocotyl explants, the hypocotyl segments which were produced from previous step were dissected by horizontal cut to separate a hypocotyl into two pieces.

Half-seed explants (Paz et al., 2006)

Sterilized seeds were immersed in sterilized distilled water for 20 h at 25℃ in dark. Half length of embryo axis from root tip (4 to 5 mm long) was cut off and then seed coats were removed. Two identical half-seed explants were obtained from one seed by longitudinal cut along the hilum. Epicotyl at the junction of cotyledon and hypocotyl was removed by #10 surgical blade. For analysis of regeneration efficiency of half-seed method in Bunya and Bragg, 10 explants of each cultivar were cultured on Shoot induction medium (SIM) for 2 weeks in the tissue culture room (150 µmol m^{-2} s^{-2}, 16/8 h, 25±1℃). After 14 days, total regenerated numbers of shoots were recorded. After 2 weeks of shoot induction, cotyledons were removed from explants and transferred to petri plants containing shoot elongation medium (SEM). The petri dishes were cultured for 4 weeks with subculturing every 2 weeks. 4 to 5 cm long elongated shoots were dissected from explants and cultured on rooting medium (RM). Individual experiment was replicated three times.

3.2.3 Soybean transformation

Soybean transformation procedures were followed as described by Paz et al., (2006) with modifications as are described below:

3.2.3.1 Plant transformation vector and *Agrobacterium tumefaciens* strain

The binary vector, pUQC10255 which was kindly provided from Professor Bernard Carroll (The University of Queensland) was used for soybean transformation. pUQC10255 vector carries a Yellow Fluorescent Protein (YFP) cassette under the control of CAMV35S promoter and a herbicide bialaphos resistant gene (*bar*) cassette driven by CAMV35S promoter (Figure 3.1). The binary vector was introduced into *Agrobacterium tumefaciens* strain EHA105 by heat shock method. 300 ng of plasmid was added into

Agrobacterium competent cells and incubated on ice for 30 min. The mixture was incubated for 5 min in a water bath (37℃) to heat shock the cell and placed on ice for 2 min. 1 ml of YEP medium was added into the cell and incubated in the shaker for 4 h at 200 rpm (28℃, dark). The transconjugants were selected on a YEP-agar plate containing 25 mg/L of rifampicin (Sigma, USA) for EHA 105 chromosomal drug marker and 50 mg/L of kanamycin (Sigma, USA) for binary vector drug marker for 2 days in dark at 28℃.

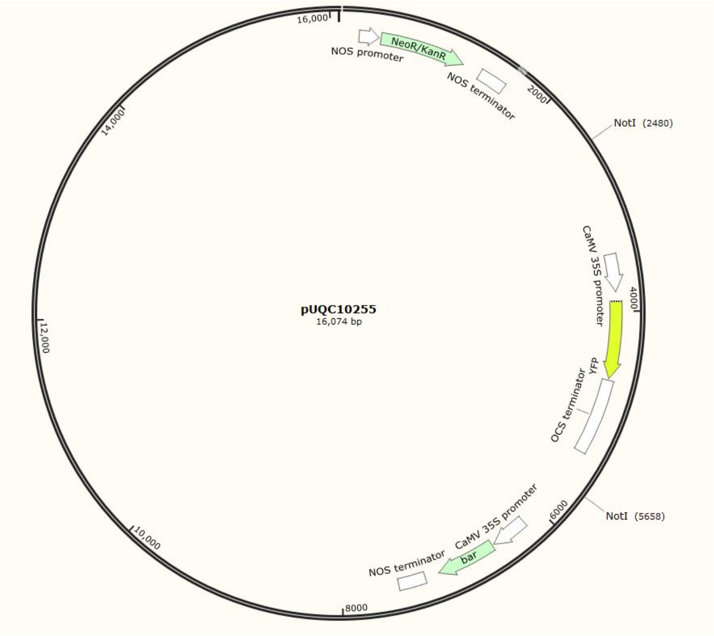


Figure 3. 1. A vector map of pUQC10255.

The binary vector, pUQC10255, was used in this study. The image was generated using a SnapGene® Viewer (www.snapgene.com). The pUQC10255 plasmid harbours KanR (*NPTII*) gene for kanamycin resistance of bacteria, YFP gene for yellow fluorescence and *bar* herbicide bialaphos resistant gene that confers herbicide resistance to transgenic plants.

3.2.3.2 *Agrobacterium* preparation

Three days prior to infection, *Agrobacterium* which contains binary vector pUQC10255 was grown on YEP-agar medium. The developed single colony was obtained by using a sterilized tip and inoculated into 5 ml YEP medium in a 50 ml tube. The inoculated 5 ml YEP medium was cultured for 20 h at 200 rpm (28℃, dark). 250µl of *Agrobacterium* culture from 5 ml YEP medium was transferred into 250 ml of liquid YEP medium in a sterilized 1 L conical flask and cultured for 20 h, 200 rpm (28℃, dark).

Optical density at 600 nm (O.D. $_{600}$) value was measured using UV/Vis Spectrophotometer (Eppendorf, Germany).

3.2.3.3 Infection

250 ml of *Agrobacterium* culture (O.D.$_{600}$ value: 0.8) was separated to four 50 ml tubes. The bacterial culture in 50 ml tubes were centrifuged at 4,500 rpm at 22℃ for 10 min. The pellets from three of four tubes were re-suspended with 25 ml of infection medium (IM) and a pellet in a tube was dissolved in 5 ml of infection medium. The re-suspended *Agrobacterium* cultures were pre-cultured at least for 30 min on a rotator with gentle shaking at room temperature in dark. Three tubes with 25 ml *Agrobacterium* culture were used for inoculation and a tube with 5 ml *Agrobacterium* culture was used for wound treatment.

Before infection, cotyledonary node and half-seed explants were wounded at the junction of cotyledon and hypocotyl by scratching 7 to 8 times with a #11 scalpel blade (Feather, Japan), which was dipped into 5 ml *Agrobacterium* suspension.

For infection, 25 to 30 explants were put into pre-cultured 25 ml of *Agrobacterium* culture in a 50 ml tube. 50 ml tubes containing explants with infection medium were placed in a sonicator (SD-ultra, South Korea) and were sonicated for 20 sec. Subsequently, explants were vacuumed for 30 sec at 666 mbar m^{-2} s^{-2}. After vacuum treatment, explants were incubated for 30 min on a shaker with gentle shake at room temperature. After infection, explants were placed on sterilized paper towels to remove excessive *Agrobacterium* suspension.

3.2.3.4 Co-cultivation of infected soybean explants

Inoculated explants were placed on sterilized filter paper on co-cultivation medium (CCM) in a petri dish (adaxial side down). The infected explants were co-cultivated for 5 days in the tissue culture room (150 µmol m^{-2} s^{-2}, 16/8 h, 25±1℃).

3.2.3.5 Regeneration and selection of putative transgenic explants

Regeneration and selection of transgenic plants were carried out following the procedure below. Additional steps were conducted for cotyledonary node and half-seed method in shoot induction steps. After 5 days of co-cultivation, 25 to 30 explants were washed with 25 ml of washing medium (WM) for 30 min with gentle shaking. Washed explants were then placed on sterilized filter paper to remove excessive WM. The explants were transferred to SIM1 and cultured for 2 weeks in a tissue culture room (150 μmol m^{-2} s^{-2}, 16/8 h, 25±1℃). For cotyledonary node and half-seed explants, after 2 weeks of culture on SIM1, cotyledons and regenerated shoots were removed from explants and then transferred to SIM2 medium. Hypocotyl and split-hypocotyl explants were transferred to SIM2 without removing primary shoots. After 2 weeks of culture on SIM2, explants were embedded in shoot elongation medium 1 (SEM1) for shoot elongation. Explants were subcultured every 2 weeks and incubated until shoots reached 4 to 5 cm long. Well elongated shoots were excised from explants and transferred into rooting medium (RM). After 2 to 4 weeks of culture, plantlets with developed roots were taken out from the tissue culture container and washed with tap water to remove tissue culture medium from roots. The plantlets were then transferred to pots filled with sterilized soil. The pots were covered with plastic covers to retain high humidity for acclimatization and were grown in a plant growth incubator (400 μmol m^{-2} s^{-2}, 16/8 h; 25±1℃). After 5 days, plastic covers were removed from pots and acclimatized putative transgenic plants were grown in the glasshouse (250 μmol m^{-2} s^{-2}, 16/8 h; 25±1℃).

3.2.3.6 Effects of addition of Adenine hemisulfate and polyvinylpyrrolidone (molecular weight 40,000; PVP 40,000) on shoot elongation

Adenine hemisulfate (AD) and PVP 40,000 were added to SEM medium to examine effects on shoot elongation. 40 mg/L of AD and 500 mg/L of PVP 40,000 were added to SEM medium. Shoot clumps (6 weeks after regeneration) were placed on SEM containing AD and PVP 40,000 and incubated in the tissue culture room. Explants were subcultured every 2 weeks. Total numbers of elongated shoots (over 4 cm long) were counted after 8 weeks of culture.

3.2.4 Confirmation of putative soybean transgenic plants

3.2.4.1 Detection of YFP expression from putative transgenic plants

Emission of YFP fluorescence from shoots and plant was examined under blue light with a YFP filter (590-130 nm band pass filter). YFP excitation peak is 514 nm and its emission peak is 527 nm. After 6 weeks of co-cultivation, yellow fluorescence was examined on putative transgenic shoots. Putative transgenic plants and leaves of transgenic plants which were grown in the glasshouse were used to observe YFP expression.

3.2.4.2 Basta-paint assay

The putative transgenic plants growing under glasshouse conditions were used for examination of expression of *bar* gene by application of Basta® (a commercial herbicide containing 200 g/L of ammonium glufosinate, Bayer, Germany) on a leaf. The leaf of 2nd or 3rd trifoliate was randomly selected and marked. 100 mg/L of basta solution was applied on adaxial surface of selected leaves by brushing 5 times. After 5 days, basta resistance was examined.

3.2.4.3 Genomic DNA isolation

Genomic DNA was isolated from leaves using modified CTAB (cetyl trimethylammonium bromide) method (Doyle and Doyle, 1990). About 0.1 g of fully expanded leaves from each plant was put into a sterilized 1.5 ml tube and one tungsten carbide bead (3 mm diameter, Qiagen, Germany) was added into a tube. The tubes were frozen in liquid nitrogen and the samples in tubes were ground using a TissueLyser LT (Qiagen, Germany). Homogenized leaves were mixed with 0.5 ml of pre-warmed (55℃) CTAB solution (2 g of CTAB, 100 mM Tris-HCl pH8.0, 20 mM EDTA, 1.4 M NaCl, 1 g of PVP 40,000 in 100 ml, pH5.0) in 1.5 ml tubes and incubated at 55℃ in water bath for 15 min. The tubes were centrifuged at 12,000g at room temperature for 5 min to precipitate cell debris. Supernatants were transferred to new 1.5 ml tubes. 0.25 ml of chloroform:isoamyl alcohol (24:1) was added into each tube and mixed by inversion The mixtures were centrifuged at 13,000 rpm at room temperature for 1 min. Upper aqueous phase was transferred to new 1.5 ml tubes. To precipitate genomic DNA, 50 µl of 7.5 M ammonium acetate was added followed by ice-cold absolute EtOH (500 µl). The mixtures were inverted carefully 5 to 7 times and stored at -20℃ for 16 h. After overnight incubation, the mixtures were centrifuged at 13,000 rpm for 5 min at 4℃. To wash genomic DNA, the pellet was washed with 70% (v/v) ethanol and was centrifuged at 13,000 rpm for 1 min at 4℃. After washing, the pellets were air dried for 5 min at room temperature. Genomic DNA was dissolved in TE buffer. Concentration and purity of DNA was measured using NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, USA).

3.2.4.4 PCR analysis

Polymerase chain reaction (PCR) was conducted to confirm the presence of a transgene (*bar* gene) in putative transgenic soybean plants. The 439 bp of *bar* coding region was amplified using specific primer pairs (BAR F: 5'-GTACCGGCAGGCTGAAGTCC-3', BAR R: 5'- CGGTCTGCACCATCGTCAAC-3'). Specific primer pairs of *GmACT* was used as an internal control (ACT F: 5'-ATCATGTTTGAGACCTTCAATGTG-3', ACT R: 5'-CTCGAGTTCTTGCTCATAATCTAGG-3'). PCR reaction mixtures contained 2.5 µl of 10X PCR Buffer, 1.5 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dGTP, dTTP, 0.5 µM of each primer and 1 unit *Taq* DNA polymerase (Invitrogen, USA) in a volume of 25 µl with 50 ng of genomic DNA. PCR reactions were performed using a Mastercycler® nexus (Eppendorf, Germany) under the condition: an initial denaturation step at 94℃, for 3 min followed by 30 cycles of 94℃ for 45 sec, 55℃ for 30 sec, 72℃ for 90 sec and one cycle of final extension at 72℃ for 10 min. The amplified PCR products were separated by electrophoresis on a 1.0% agarose gel. Gels were visualized with a ChemiDoc MP imaging system (Biorad, USA).

3.2.4.5 Southern blot analysis

The copy numbers of *bar* gene in putative transgenic lines were examined using Southern blot hybridisation (Southern, 2006). A 439 bp of DIG-labelled fragment of *bar* gene was used as the probe for Southern blot analysis (Figure 3.2). The probe was amplified from a pUQC10255 plasmid using DNA DIG-labelling kit (Roche, USA). DIG-labelled *bar* gene probe was synthesized using the same conditions and primer pairs as described in PCR section. 10 µg of genomic DNA from putative transgenic plants were digested with 10 units of restriction enzyme, *BamHI* (single-cutter of cassette) for 16 h at 37℃. The digested DNA was concentrated to 20 µl using DNA concentrator. The concentrated DNA was separated on a 0.8% agarose gel in 1X TAE buffer. The DNA samples were transferred to a positively charged nitrocellulose membrane (Roche, USA) by capillary transfer. The membranes were hybridised with a DIG-labelled *bar* probe at 60℃ for 16 h followed by wash at low stringency washing buffer (2 x SSC, 0.1% SDS), at room temperature, 5 min for 2 times, and then wash with high stringency washing buffer (0.1 x SSC, 0.1% SDS) at 60℃, 15 min for 2 times. The washed membrane was incubated with anti-DIG solution (2 µl of Anti-Digoxygenin-AP in 20 ml of 1% blocking solution) at room temperature for 1 h. The detection buffer [10 µl of CDP-star (Roche, USA), 0.1 ml of 1 M Tri-HCl pH 9.5, 0.1 M NaCl in 1 ml] was applied to the membrane and incubated for 5 min at room temperature. Chemiluminescent was examined in a ChemiDoc MP imaging system (Biorad, USA).

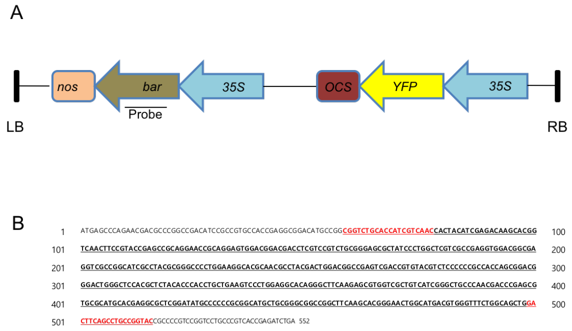


Figure 3. 2. Schematic diagram of T-DNA region in pUQC10255 plasmid and sequence information of *bar* gene.

(A) Structural organization of T-DNA region in pUQC10255 plasmid. RB: right border, *35S:* CaMV35S promoter, *YFP*: yellow fluorescence protein gene, *OCS: OCS* terminator, *bar*: bialaphos resistant gene, *nos: nos* terminator, LB: left border. (B) Full nucleotide sequences of *bar* gene. Red: primer annealing regions for probe synthesis. underline: the sequence of probe for southern blot analysis.

3.2.4.6 Total RNA isolation

Total RNA was extracted using TRIzol reagent following manufacturer's instruction (Invitrogen, USA). 0.1 g of leaves was placed in a RNase-free 1.5 ml tube. Sterilized RNase-free tungsten beads were added into a tube and were frozen in liquid nitrogen. The samples were ground using a TissueLyser LT. 1 ml of TRIzol was added to each homogenized sample and mixed by inversion. The samples in tubes were incubated for 5 min at room temperature and were centrifuged for 10 min at 4℃. Supernatant was transferred carefully to a new 1.5 ml tube. 500 μl of chloroform was added into a tube and mixed immediately by inverting. The samples were incubated for 5 min at room temperature and were centrifuged for 10 min at 4℃. Upper aqueous phase was carefully transferred into a new tube and added 750 μl of ice-cold isopropanol. The mixture was incubated for 10 min at room temperature for precipitation of total RNA. The samples were centrifuged at 13,200 rpm for 25 min at 4℃. Supernatant was discarded and the pellet was washed with 750 μl of 70% (v/v) EtOH. After centrifugation for 5 min at 13,200 rpm at 4℃, pellets were air dried for 2 min at room temperature. The pellets were dissolved in TE buffer and were treated with TURBO^{TM} DNase (Thermo Fisher Scientific, USA) following the manufacturer's instructions to remove possible DNA contamination.

3.2.4.7 RT PCR analysis

For RT-PCR analysis, 1μg of total RNA was used as template for cDNA synthesis. cDNA was synthesized using Superscript^{TM}III Reverse Transcriptase (Invitrogen, USA) with oligo(dT) primer. Total RNA (1 μg) was mixed with 1μl of 50 μM oligo(dT) and 1 μl of 10 mM dNTP mix. RNase-free water was added to a total volume of 13 μl. The mixture was incubated at 65℃ for 5 min and placed on ice. Reverse transcript reaction mixtures contained 4 μl of 5X First-strand Buffer, 1μl of 0.1 M DTT, 1 μl of RNaseOUT^{TM} Recombinant RNase Inhibitor (40 units/ μl) and 1 μl of Reverse Transcriptase (200 units/μl) in a volume of 20 μl was added into total RNA (1 μg) mixture. The tube was incubated at 50℃ for 30 min. After synthesis of cDNA, 80ul of RNase-free water was added to each cDNA template. *GmACT* was used as an internal control. RT-PCR analysis was conducted with 2 μl of cDNA template in a 25 μl reaction volume using

Taq DNA Polymerase (Invitrogen, USA). PCR reactions for detections of *bar* and *GmACT* gene expressions were conducted under the condition: an initial denaturation step at 94℃, for 3 min followed by 30 cycles of 94℃ for 45 sec, 55℃ for 30 sec, 72℃ for 90 sec and one cycle of final extension at 72℃ for 10 min.

3.3. Results

3.3.1 Evaluation of regeneration efficiency of half-seed explants from commercial cultivars of soybean (Bunya and Bragg)

For the development of soybean transformation system for Bunya and Bragg, firstly, regeneration efficiency of half-seed explants from two commercial cultivars of soybean (Bunya and Bragg) were evaluated. Half-seed explants of Bunya and Bragg regenerated shoots within a week on SIM. Multiple shoots were produced at the junction of cotyledonary node and hypocotyl (Figure 3.3). In this experiment, both cultivars showed a high percentage of regeneration efficiency (Table 3.2). Bunya cultivars showed 80% of regeneration frequency and on average, 4.1 shoots/explants were produced. Bragg variety also had high regeneration efficiency (83.3%) with average 4.8 shoots per explant. This result indicated that two commercial cultivars have a potential to be suitable cultivars for genetic transformation.

Table 3. 2. Shoot regeneration of half-seed explants from two commercial cultivars of soybean.

Cultivars	Maturity group	Total number of explants	Regeneration efficiency (%)	Total number of regenerated shoots	Range per explant	Average shoots per explant	Total number of elongated shoots (>3 cm)[a]
Bunya	VI	30	80.0	98	1-10	4.1	11
Bragg	VII	30	83.3	121	1-9	4.8	10

a: Elongated shoots over 3 cm long were counted after 6 weeks of culture.

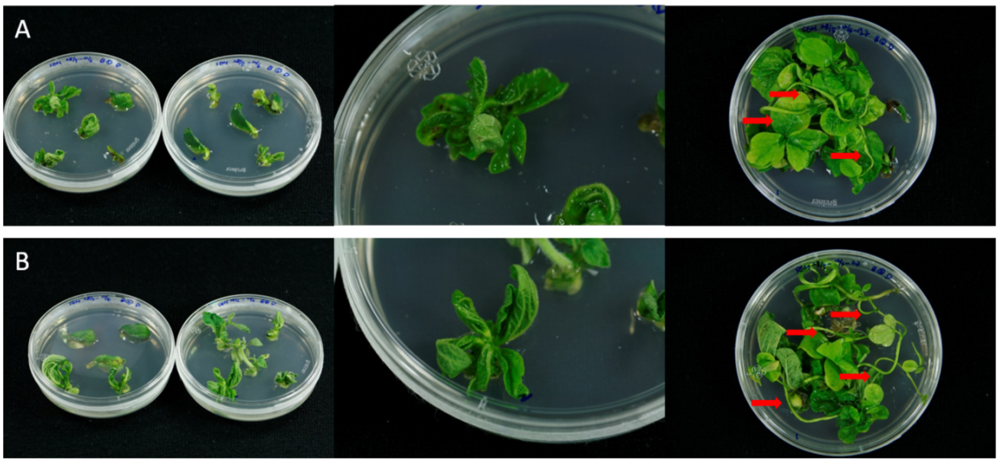


Figure 3. 3. Regeneration from half-seed explants of two commercial cultivars of soybean (Bunya and Bragg).

Half-seed explants of Bunya and Bragg successfully regenerated. (A) Bunya and (B) Bragg regenerated and produced elongated shoots. Left: explants started regeneration after 1 week of culture on SIM. Middle: close-up views of 1-week old explants showed multiple shoots were formed. Right: multiple shoots after 6 weeks of culture (red arrows).

3.3.2. Development of transformation systems for commercial cultivars of soybean

3.3.2.1. Analysis of transformation efficiency of different types of explants

Different types of explants were tested to identify types that were suitable for development of transformation system for commercial soybean cultivars. Since Bunya showed high regeneration efficiency in a previous study (Raza et al., 2017), three different types of explants, hypocotyl, split-hypocotyl and cotyledonary node from Bunya were used in this experiment. Three independent experiments were carried out to compare transformation efficiency. After infection and co-cultivation, explants produced multiple shoots with high regeneration efficiency (hypocotyl; 75±5%, split-hypocotyl; 76.7±7.64% and cotyledonary node; 76.7±2.89%) during shoot induction steps on SIM1 (Figure 3.3A and Table 3.3). However, all explants failed to produce any putative transgenic shoots during selection and eventually died on SEM1 (Figure 3.4B and C).

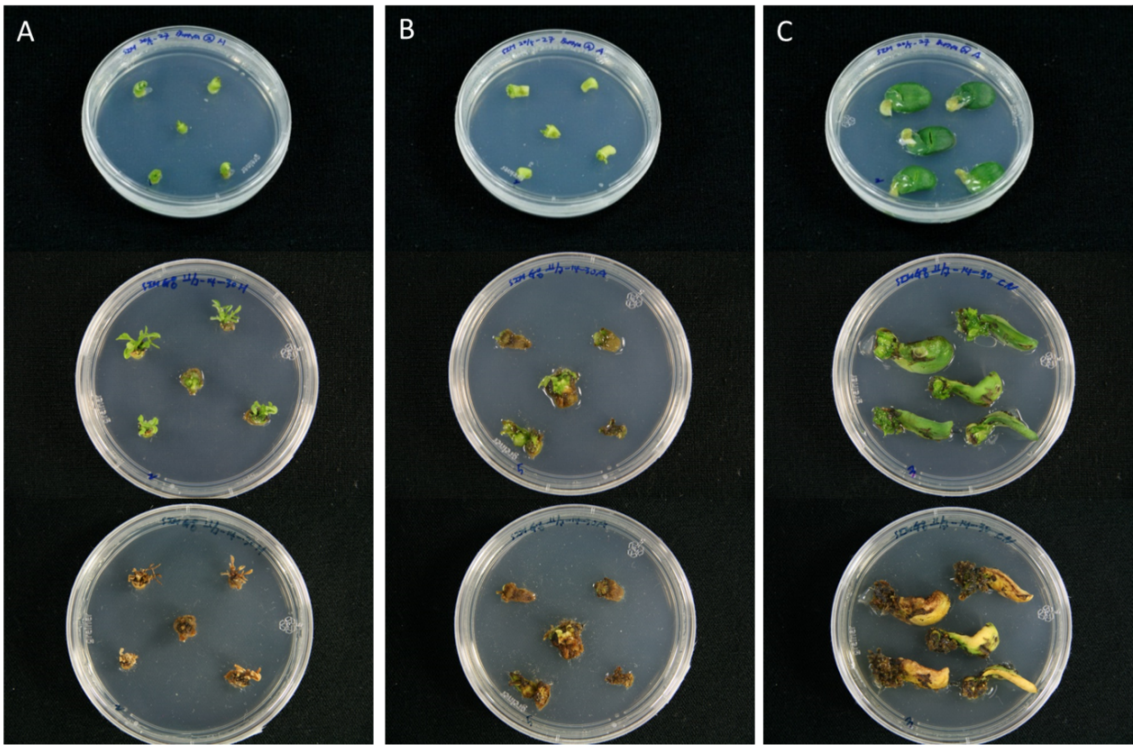


Figure 3. 4. Comparisons of soybean transformation efficiency in three different types of explants, hypocotyl, split-hypocotyl and cotyledonary node from Bunya.

Three different explants, (A) hypocotyl, (B) split-hypocotyl, and (C) cotyledonary node were infected with *Agrobacterium* harbouring herbicide bialaphos resistant gene (*bar*). Top: explants were placed on SIM1 after 5 days of co-cultivation. Middle: multiple shoots regenerated from explants after 2 weeks on SIM1. Bottom: all explants died on SIM2 containing 8 mg/L of glufosinate. No explants survived on selection medium

Table 3. 3. Comparisons of regeneration and transformation efficiency in three different types of explants (Bunya).

Type of explants	Number of infected explants^{A}	Total number of regenerated explants	Total number of survived explants^{B}	Regeneration efficiency (%)	Transformation efficiency (%)
Hypocotyl	60	45	0	75±5	0.0
Split-hypocotyl	60	46	0	76.7±7.64	0.0
Cotyledonary node	60	46	0	76.7±2.89	0.0

Three different types of explants regenerated after infection with *Agrobacterium*. All explants failed to produce transgenic shoots and died on selection medium. Transformation efficiency=B/A*100.

3.3.2.2 Soybean transformation using half-seed explants

In the experiments of transformation efficiency of different types of explants, none of the explants produced transgenic shoots and failed to survive on selection medium. To establish soybean transformation system for commercial cultivars, *Agrobacterium*-mediated transformation using half-seed explants was evaluated. The explants of Bunya and Bragg were prepared and infected with *Agrobacterium* harbouring *ba*r and YFP genes. After co-cultivation, different responses of explants were observed. In Bunya, about 70% of explants remained without colour changes, whilst most of the non-infected explants turned green (Figure 3.5A). Shoots were regenerated mostly from half-seed explants with greenish colour during shoot induction periods (Figure 3.5B). Reduction of regeneration frequency was observed in both cultivars. Bunya showed a significant reduction of regeneration efficiency (29.2±5.16%). In addition, an overgrowth of bacteria from explants was detected during culture resulting in failure to recover and produce multiple shoots (Figure 3.6). In contrast, the regeneration frequency of Bragg was slightly reduced (66.5±12.75%) and successfully produced multiple shoots during selection (Figure 3.7). After 4 weeks of selection, expression of yellow fluorescence from explants in selection medium was examined under blue light and the fluorescence on shoots primordia was detected on 4 putative transgenic shoots (Figure 3.8 and Table 3.4). However, these YFP positive shoots were not able to grow further (poor shoot elongation) and survived on selection medium. Hereafter, Bragg was used for development of transformation system.

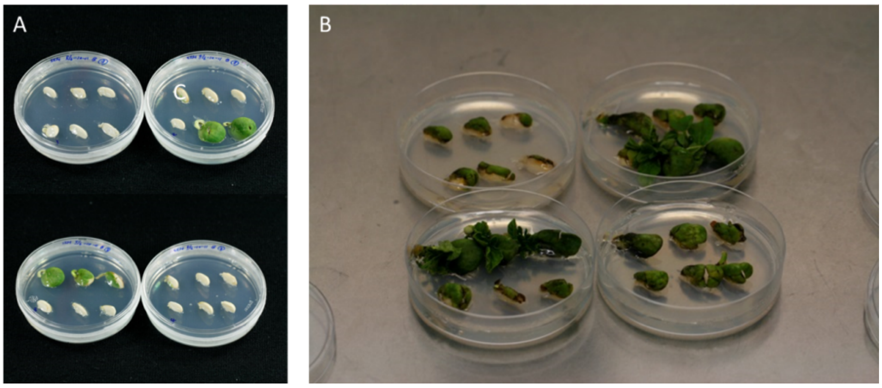


Figure 3. 5. Relationship between colour and regeneration in explants.

The explants which did not change their colour to green mostly failed to regenerate during culture. (A) Half-seed explants of Bunya had few explants which turned green after co-cultivation. (B) Only the green explants successfully regenerated after 2 weeks of culture on SIM1.

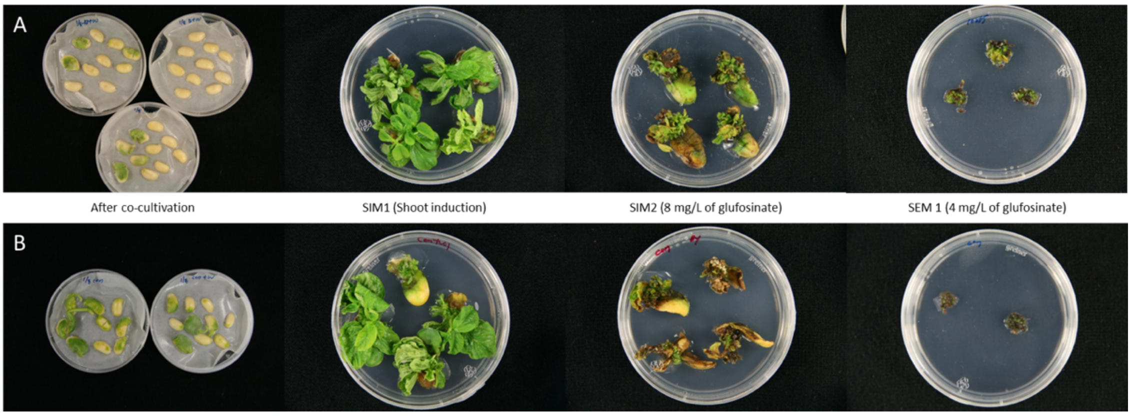


Figure 3. 6. Reduction of regeneration efficiency of half-seed explants (Bunya) after infection with *Agrobacterium.*

Regeneration frequency of half-seed explants decreased after infection with *Agrobacterium.* (A) Many infected explants (about 70%) of Bunya failed to turn green after co-cultivation (left photo). Shoot were regenerated on shoot induction medium (photos second and third from left). After 4 weeks of selection, half-seed explants of Bunya did not survive (right photo). (B) Non-infected half-seed explants of Bunya. Most explants changed colour to green after 5 days on co-cultivation medium (left photo). Non-infected explants regenerated on SIM medium (photos second and third from left). All explants failed to survive on selection medium (right photo).

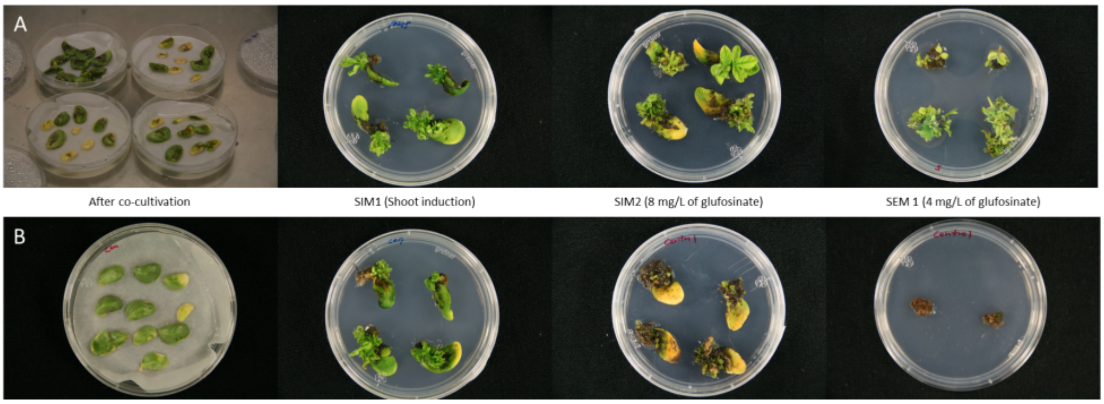


Figure 3. 7. Regeneration frequency of half-seed explants (Bragg) after co-cultivation.

(A) Infected half-seed explants of Bragg successfully regenerated after infection with *Agrobacterium* (left photo) and regenerated on SIM1 (photos second and third from left), putative shoots were produced on SEM medium (right photo). (B) Non-infected explants of Bragg had multiple shoots on SIM1. All explants failed to survive on selection medium.

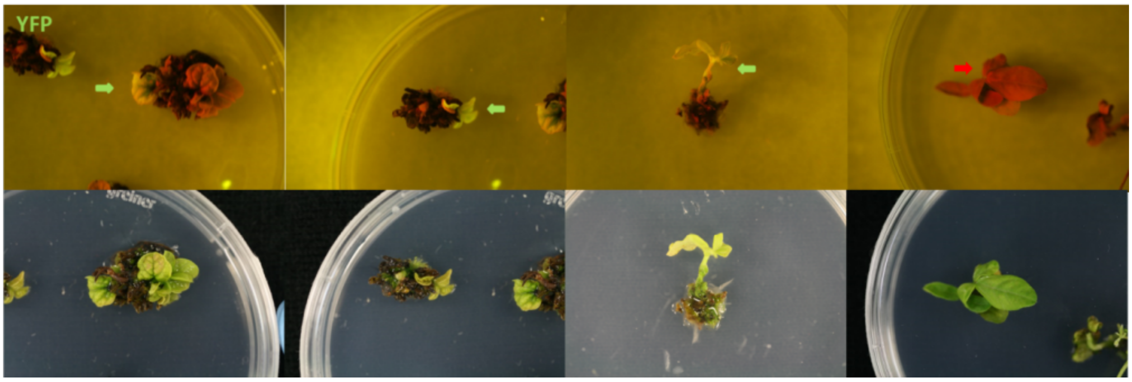


Figure 3. 8. Unelongated putative transgenic shoots (YFP positive) on shoot elongation medium.

YFP expression was detected in putative transgenic shoots after 4 weeks of selection. YFP positive shoots emitted yellow green light under blue lights (upper photos, green arrows). Non-transgenic shoots emitted red fluorescence (red arrow). YFP positive putative transgenic shoots were small and did not grow on selection medium (SEM1), resulting in failure to elongate shoots.

Table 3. 4. Comparisons of regeneration and transformation efficiency using half-seed methods (Bunya and Bragg).

Cultivars	Batches	Number of infected explants[A]	Number of regenerated shoot clumps	Number of YFP positive shoot clumps[B]	Number of elongated shoots	Regeneration efficiency (%)	Transformation efficiency (%)
Bunya	1	98	24	0	0	24.5	0.0
	2	95	33	0	0	34.7	0.0
	3	98	28	0	0	28.6	0.0
	Total	291	85	0	0	29.2±5.16	0.0
Bragg	1	85	69	2	0	81.2	2.4
	2	85	49	1	0	57.6	1.2
	3	87	53	1	0	60.9	1.2
	Total	257	171	4	0	66.5±12.75	1.6±0.69

Half-seed explants of Bunya showed a significant reduction of regeneration efficiency after infection with *Agrobacterium*. Half-seed explants of Bragg had a high regeneration frequency (66.5%) after infection and produced four YFP positive shoots during selection (transformation efficiency 1.6%). Transformation efficiency=B/A*100.

3.2.2.3 Effect of Adenine hemisulfate and PVP 40,000 on shoot elongation

Although half-seed explants of Bragg showed high regeneration efficiency (83.3%) and ability to elongate shoots in regeneration experiments, putative transgenic shoots showed stunted growth during selection. Total four YFP positive shoots were detected on putative transgenic explants on SEM2. However, all YFP positive explants failed to elongate and died after 6 weeks of selection. To overcome this elongation problem, the effect of adenine hemisulfate (AD) and polyvinylpyrrolidone mol. wt. 40,000 (PVP) were evaluated on shoot elongation. 6-week-old shoot clumps were placed on SEMAP (SEM containing 40 mg/L of AD and 500 mg/L of PVP) and SEM (SEM medium without AD and PVP). Explants were subcultured every 2 weeks. Total numbers of elongated shoots were examined after 8 weeks of culture. Shoot clumps on SEMAP produced 6 times higher elongated shoots than explants on SEM (Table 3.5). Moreover, the shoots from SEMAP had longer lengths and bigger leaves than those of shoots on SEM (Figure 3.9). This result indicated that the addition of AD and PVP on SEM have a positive effect on stimulation of shoot elongation.

Table 3. 5. Analysis of effects of Adenine hemisulfate and PVP on shoot elongation.

Treatment	Total number of shoot clumps	Total number of elongated shoots
SEM	13	2
SEMAP	13	12

Total numbers of elongated shoots (>4cm long) were recorded after 8 weeks of culture on SEM.
SEM: SEM medium without AD and PVP, SEMAP: SEM medium containing 40 mg/L of AD 500 mg/L of PVP.

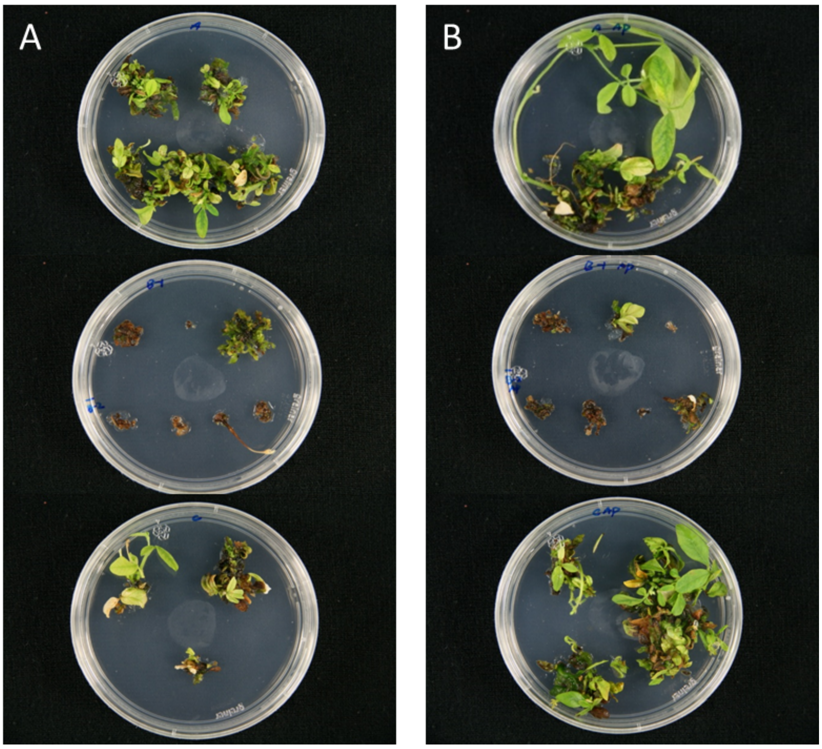


Figure 3. 9. Addition of Adenine hemisulfate and PVP improves shoot elongation of half-seed explants.

Shoot clumps (6 weeks old) were cultured on (A) SEM (SEM medium without AD and PVP) and (B) SEMAP (SEM containing 40 mg/L of AD and 500 mg/L of PVP). Higher numbers of shoots were produced from SEMAP after 8 weeks of culture.

3.2.2.4 Optimization of soybean transformation using half-seed explants of Bragg

Since the addition of AD and PVP on SEM have a positive effect on shoot elongation, AD and PVP were added on SEM in soybean transformation. Six independent transformation experiments were conducted with the addition of AD and PVP on SEM. After 6 weeks of co-cultivation, YFP expression was detected on 14 putative transgenic shoots (Figure 3.10). In addition, YFP positive shoots successfully elongated on SEM containing AD and PVP (Figure 3.11). A total of 23 putative transgenic shoots were produced from 6 batches of experiments and 13 elongated shoots were rooted and transferred into pots. Acclimatized putative transgenic plants showed no morphological differences with non-transformed plants and set seeds in the glasshouse (Figure 3.12).

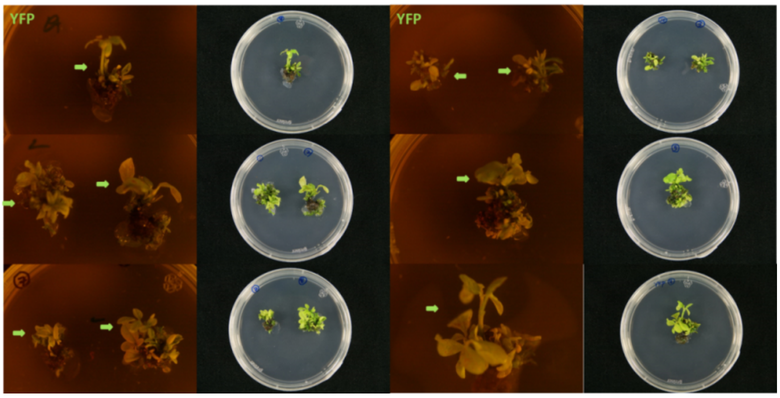


Figure 3. 10. YFP positive putative transgenic shoots.

YFP expression was detected on putative transgenic shoots (after 6 weeks of co-cultivation). YFP positive shoot emitted yellow green light under blue lights (green arrows). Non-transgenic shoots emitted red fluorescence.

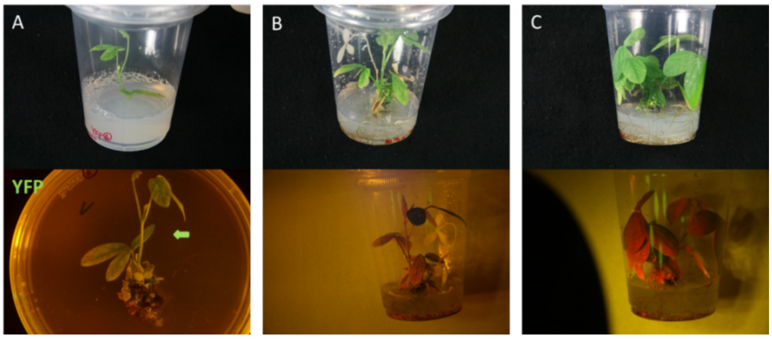


Figure 3. 11. Elongated putative transgenic shoots from selection medium.

Putative transgenic shoots successfully elongated on SEM2. (A) A well rooted plant on rooting medium (upper) showed a strong YFP expression. (B) and (C) putative shoots were well rooted on RM and showed a very weak YFP expression.

Figure 3. 12. Putative transgenic plants in the glasshouse (T0 generation).

Putative transgenic plants were grown in the glasshouse. Putative transgenic plants (T0 generation) showed no morphological differences as compared to non-transformed plants and set seeds in the glasshouse.

3.3.3 Confirmation of transgenic plants

3.3.3.1 Basta paint assay

To confirm the expression of *bar* gene in putative transgenic plants, tolerance to damage by application of basta solution (100 mg/L of ammonium glufosinate) was examined. 100 mg/L of basta solution was brushed 5 times on adaxial surface of leaves and tolerance was examined after 5 days. A total of 13 putative transgenic lines were tested. Basta-susceptible lines and wild-type had damages on leaf surface (chlorosis and necrosis) (Figure 3.13). However, three independent T0 lines (T0-3, T0-8 and T0-11) showed strong resistance to basta (Figure 3.13A).

Figure 3. 13. Basta paint assay (T0 generation).

100 mg/L of basta solution was brushed 5 times on a leaf of each line. After 5 days, basta resistance was examined. (A) 3 putative transgenic lines showed basta resistance (lines T0-3, T0-8 and T0-11). (B) Wild-type (WT) and basta-susceptible lines showed necrosis on leaves (red arrows). +: basta-applied side. -: non-treatment side.

3.3.3.2 Detection of yellow fluorescence in putative transgenic plants (T0 generation)

Total three lines (line T0-3, line T0-8 and line T0-11) showed basta resistance. To examine YFP expression, leaves of basta-resistant lines and non-transformed plants (wild-type) were placed under blue light. The leaves of transgenic plants are expected to emit green yellow light while wild-type and non-transgenic plants are expected to emit red light. Although lines T0-3, T0-8 and T0-11 showed strong basta resistance, line T0-3 only showed strong YFP expression. YFP expression in line 11 was only detected on petiole of leaves (Figure 3.14).

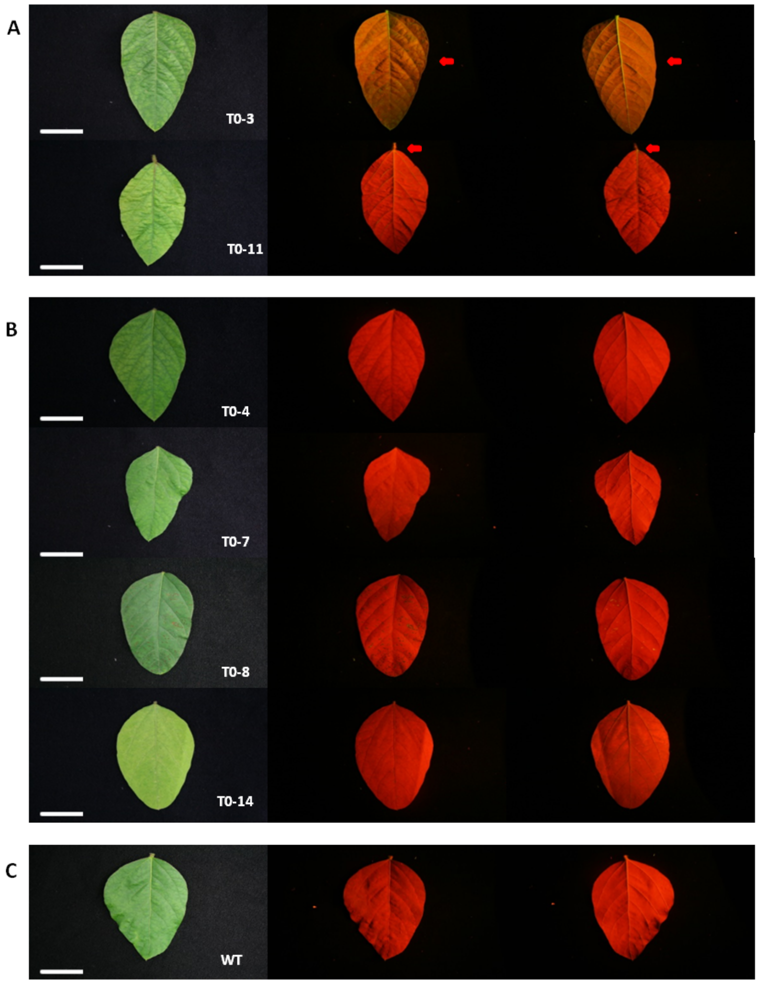


Figure 3. 14. YFP fluorescence emission of T0 generation.

(A) YFP expression was detected on leaves from two basta resistance lines (T0-3, T0-11). Line T0-3 showed strong YFP expression at main vein and moderate level of expression on entire of leaf (red arrows). Line T0-11 showed weak YFP expression at petiole (red arrows). (B) Yellow fluorescence was not detected on leaves of putative transgenic plants (lines T0-4, 7, 8 and 11) (C) Leaves of wild-type emitted red light. All photos on left at (A), (B) and (C) were taken under white fluorescent light. Middle and right photos at (A), (B) and (C) were taken under blue light with a long-pass filter to detect YFP expression. Middle: adaxial side of leaves, Left: abaxial side of leaves. WT: wild-type. White bar: 2.5cm.

3.3.3.3 Molecular analysis of transgenic plants

To confirm the presence of *bar* gene in putative transgenic plants, PCR analysis was carried out using *bar* gene specific primers. 439 bp fragment of *bar* gene was detected on 6 putative transgenic lines (lane 3, 4, 7, 8, 11 and 14) (Figure 3.15).

Four PCR positive lines (lane 7, 8, 11 and 14) and non-transformed wild-type plants were used for RT-PCR analysis to confirm the expression of *bar* gene. Total RNA was extracted from leaves of putative transgenic and wild-type plants and 1 μg of total RNA was used for cDNA synthesis. The expression of *bar* gene was detected on lines T0-8 and T0-11 (Figure 3.16). Both lines showed strong basta resistance in basta brush test, suggesting that *bar* gene was successfully expressed in putative transgenic lines (T0-8 and T0-11).

Line T0-3 showed strong basta resistance and YFP expression in leaves. To confirm the numbers of transgene copies, southern blot hybridisation was carried out. Genomic DNA of putative transgenic plants and wild-type was digested with restriction enzyme (*BamHI*) and probed with DIG labelled *bar* gene. Successful single insertion of T-DNA was detected in line T0-3 which showed strong expression of *bar* and YFP gene (Figure 3.17).

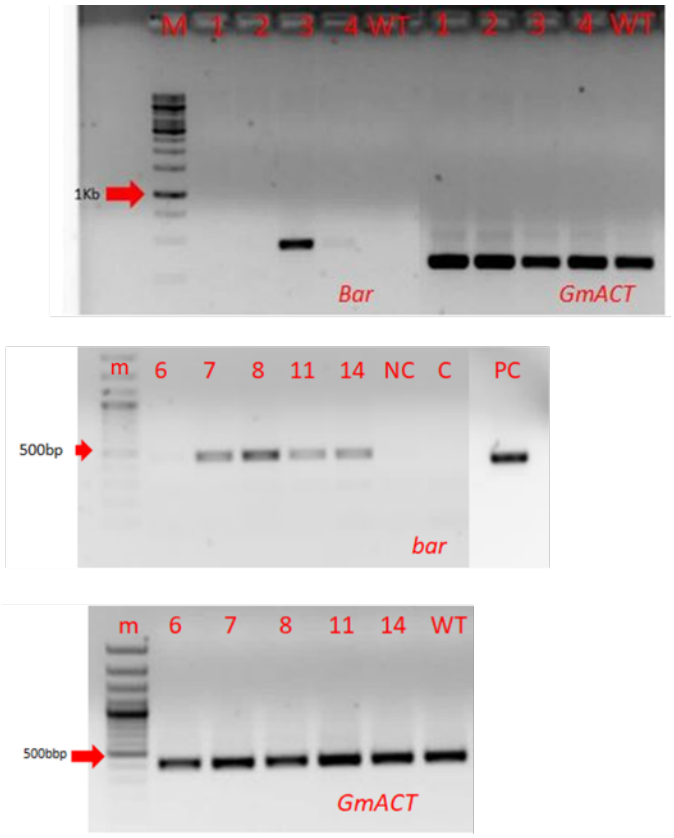


Figure 3. 15. PCR analysis of putative transgenic plants of soybean (Bragg).

Genomic DNA of acclimatized putative transgenic plants and wild-type were extracted. PCR analysis was performed to detect presence of *bar* gene in individual lines. One strong band was detected in lane 3 (line T0-3) and weak bands appeared in lane 4, 7, 8, 11 and 14 (line T0-4, T0-7, T0-8, T0-11 and T0-14). No amplicon of *bar* gene was detected in non-transformed plants (WT). Lane 1, 2, 3, 4, 6, 7, 8, 11 and 14: putative transgenic plants. WT: wild-type, PC: positive control. M: 1Kb DNA ladder, m: 100bp DNA ladder. *GmACT* was used as internal control.

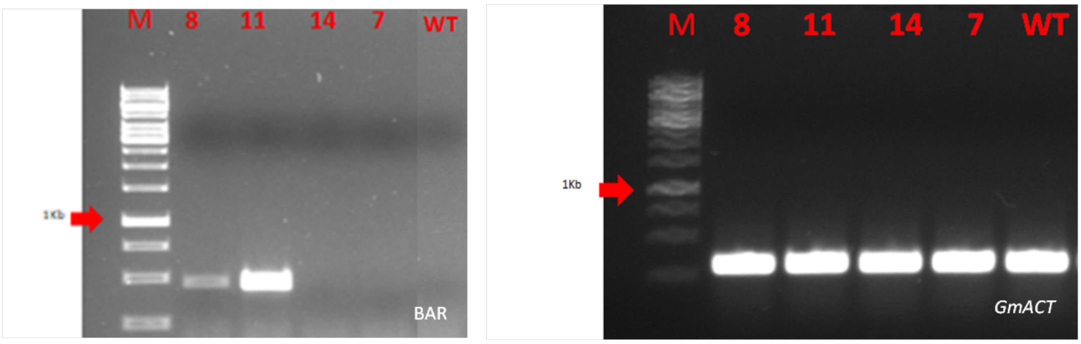


Figure 3. 16. RT-PCR analysis of *bar* gene expression in putative transgenic plants (T0 generation).

Total RNA of five independent putative transgenic lines (lines T0-7, T0-8, T0-11 and T0-14) was extracted from mature leaves and used for cDNA synthesis. Strong expression of *bar* gene was detected in line 11 (left). No expression of *bar* gene was detected in lines 7 and 14. M: 1Kb DNA ladder. WT: wild-type, *GmACT* was used as internal control.

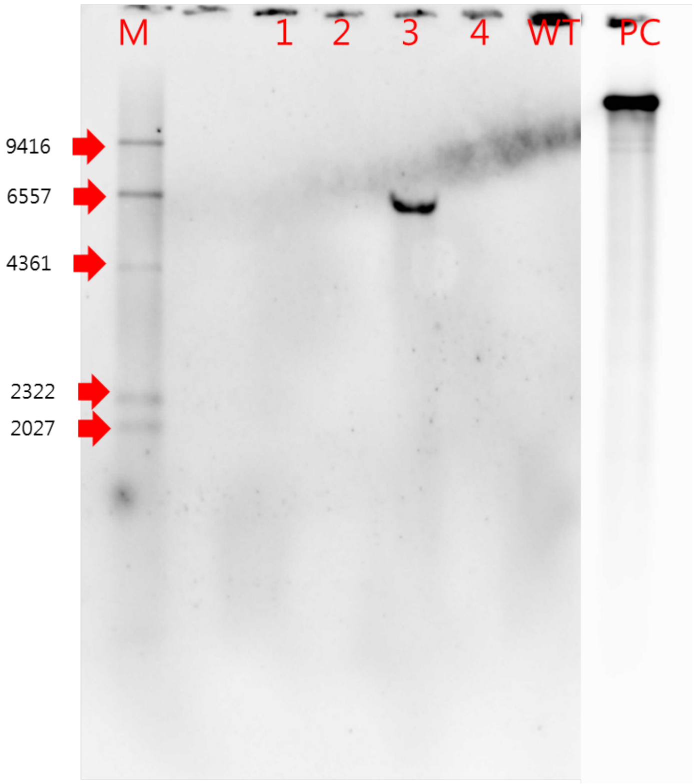


Figure 3. 17. Southern blot analysis of putative transgenic plants of soybean (T0 generation).

Primary transgenic lines (lane 1-4) were hybridized with DIG labeled *bar* probe. Insertion of single copy of T-DNA was detected in lane 3 (line T0-3). No band was detected in non-transformed soybean plants (WT). M: DIG-labeled DNA ladder (DNA molecular weight marker II), lane 1-4: independent putative transgenic soybean plants. WT: wild-type (non-transformed) soybean plant, PC: pUQC10255 plasmid.

In this study, the *Agrobacterium*-mediated soybean transformation system for a commercial cultivar of Bragg was established (Figure 3.18). Total 23 putative transgenic plants were produced using half-seed explants method. Total 13 plants were rooted on rooting medium and transferred to pots. After acclimatization, putative transgenic plants were grown in the glasshouse. No morphological variations and fertility were observed in all putative transgenic plants grown in the glasshouse. Three independent lines (T0-3, 8 and 11) had herbicide resistance and yellow fluorescence was detected on two basta resistance lines (T0-3 and 11). The presence and expression of *bar* gene was confirmed by PCR and RT-PCR analyses. Single copy of gene insertion in T0-3 line was confirmed by southern blot hybridisation. Based on herbicide resistance, total transformation efficiency was 0.6% (Table 3.6). Stable transmission and expression of T-DNA were confirmed in T1 and T2 generation of T0-3 line (Table 3.7, Figure 3.19 and 20).

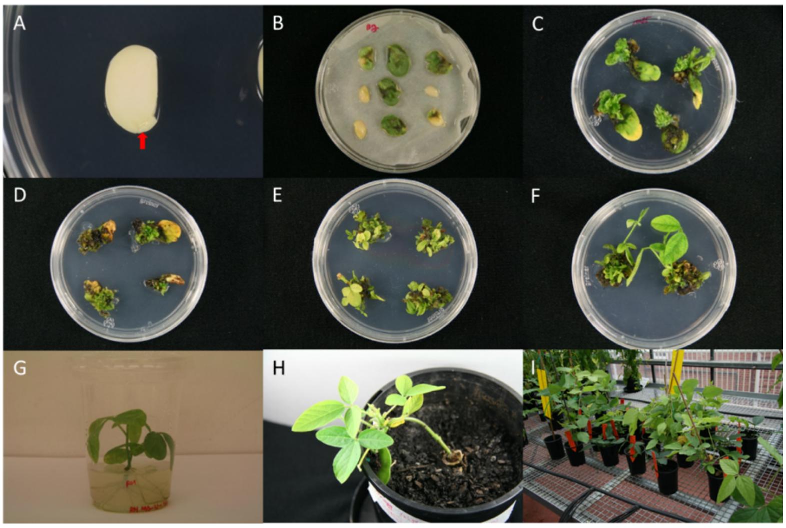


Figure 3. 18. Procedures of plant transformation using half-seed explants of a commercial cultivar of soybean (Bragg).

(A) Half-seed explants were obtained from imbibed seeds. Meristematic region was target tissue for transformation (red arrow). (B) Explants turned green after 5 days of co-cultivation. (C) Multiple shoots regenerated from explants on SIM1 after 2 weeks of culture. (D) Putative transgenic shoots regenerated from explants on selection medium, SIM2 after 2 weeks of selection. (E) Shoots elongated on SEM1. (F) Putative transgenic shoots elongated. (G) A surviving shoot (over 4 cm long) was rooted on RM. (H) A rooted plant was transferred to sterilized soil for acclimatization. (I) Acclimatized plants in the glasshouse.

Table 3. 6. Efficiency of *Agrobacterium*-mediated transformation of Bragg using half-seed explants.

Batch number	Number of infected explants^A	Number of elongated shoots^B	Number of basta resistance plants^C	Number of YFP positive plants	Number of PCR positive plants	Number of RT-PCR positive plants	Transformation efficiency^D
1	104	10	1	1	2	nt	1.0
2	86	0	0	0	0	0	0.0
3	85	4	0	0	2	1	0.0
4	84	5	2	1	2	1	2.4
5	90	1	0	0	0	0	0.0
6	85	3	0	0	0	0	0.0
Total	534	23	3	2	6	2	0.6

The efficiency of soybean transformation using half-seed explants of Bragg is 0.6%.

A: total numbers of infected half-seed explants of Bragg. B: numbers of 4 cm long elongated shoots were counted. C: basta resistant plants in basta brush test. D: transformation efficiency: C/A*100. nt: not tested

Table 3. 7. Segregation analysis herbicide resistant gene (*bar* gene) in T2 transgenic plants.

T2 transgenic lines	Copy numbers in T0	Total number of resistant plants (H^R)	Total number of susceptible plants (H^S)	Segregation ratio	χ^2 value (1:0)
1	1	17	0	1:0	-
2	1	11	0	1:0	-
3	1	10	1	10:1	0.09
4	1	11	2	5.5:1	0.31

T2 generations were derived from four independent T1 generations (T0-3 line). Data were obtained after 5 days of application of basta solution (100 mg/L of glufosinate). H^R: herbicide resistant plants showed tolerant to basta solution. H^S: herbicide susceptible plants exhibited chlorosis and necrosis on leaves. Stable expression of *bar* gene was observed in T2 generation. Two homozygous lines (T2-1 and T2-2) were obtained. χ^2 values for 1*df*= 3.84 at 5% *P*-level.

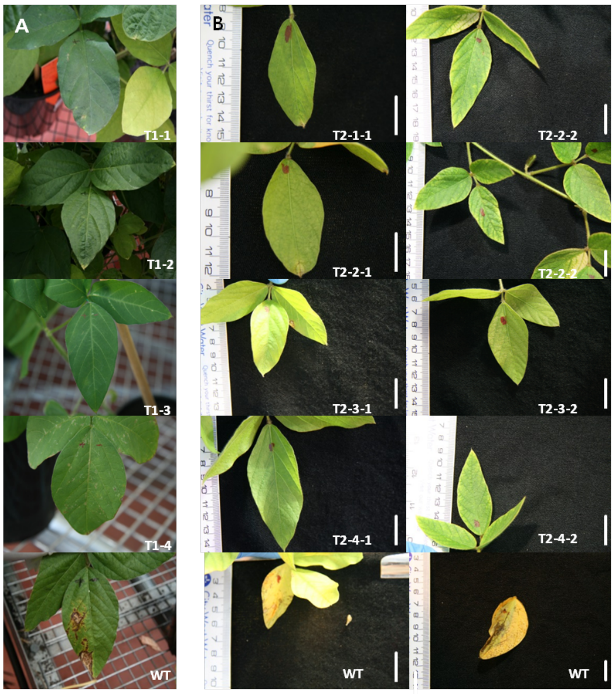


Figure 3. 19. Analysis of basta resistance in T1 and T2 generations of a transgenic plant.

Stable *bar* gene expression was confirmed in T1 (A) and T2 (B) generations of T0-3 line by basta paint assay. White bar: 2cm

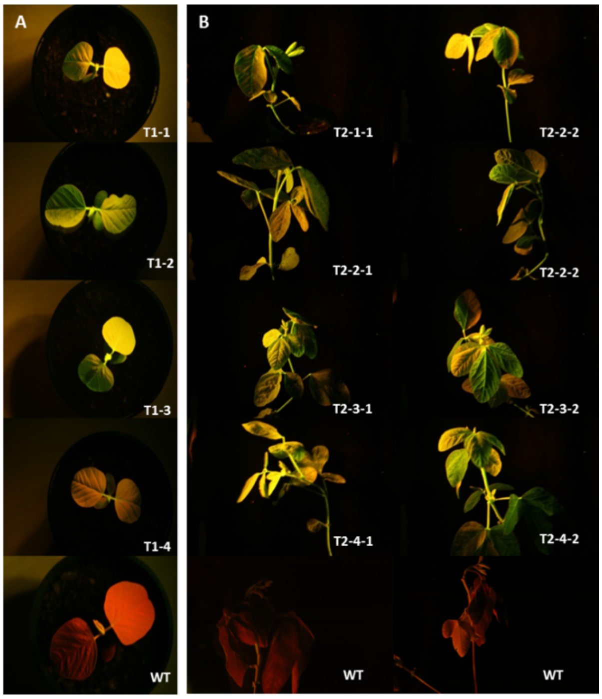


Figure 3. 20. Detection of YFP fluorescence emission from transgenic plants (T1 and T2 generation of T0-3 line).

(A) Strong YFP expression was detected from three transgenic plants (lines 1, 2 and 3 of T1 generation). One line (line 4) showed moderate YFP expression. (B) Strong YFP expression was detected in T2 generation. WT: wild-type, T1: T1 generation, T2: T2 generation.

3.4. Discussion

In soybean transformation, the efficiency of transformation is influenced by various factors such as strains of *Agrobacterium,* types of plasmid, infection and co-cultivation scheme, selection regime and regeneration ability (Raza et al., 2017). In this study, firstly the effects of different types of selection reagents (hygromycin and herbicide: glufosinate) on transformation were examined to optimize transformation systems for two commercial cultivars of soybean (Bunya and Bragg) as hygromycin and herbicide-based selection systems have been widely used for soybean transformation. In a preliminary study on selection systems for two commercial cultivars of soybean (Bunya and Bragg), glufosinate-based selection system was more suitable for soybean transformation of these two cultivars compared to hygromycin-based selection regime. The explants of Bragg had a lower efficiency of multiple-shoot formation (7.9%) under hygromycin selection than that of glufosinate-based selection (49.5%) (Lee et al., 2017). Although successful soybean transformation systems for cv. Bert with hygromycin-based selection system was reported by Olhoft et al. (2003), this selection system was proved suitable only for a specific soybean cultivar (Bert) indicating that hygromycin selection is genotype-dependent (Zeng et al., 2004). Recently, there have been reports of improved soybean transformation efficiency using herbicide-based selection systems (Jack Purple, Williams, Williams82 and Throne), in a Chinese cultivar (Tianlong 1) and in a Korean cultivar (Kwangan) (Zeng et al., 2004; Paz et al., 2006; Kim et al., 2012b; Song et al., 2013; Li et al., 2017). Paz and colleagues (2006) reported high transformation efficiency (3.8%) was obtained via *Agrobacterium*-mediated transformation method using half-seeds as explants and glufosinate (herbicide) as a selective reagent. In addition, half-seeds transformation system showed higher transformation efficiency (1.5 fold) than cotyledonary node method in American cultivars, Williams, Williams82 and Throne (Paz et al., 2006). Therefore, the modified half-seed transformation method was used to develop soybean transformation systems in this study.

The ability to regenerate is one of the most important factors for soybean transformation (Paz et al., 2004; Raza et al., 2017). In previous study on the analysis of regeneration ability of nine commercial cultivars of soybean, Bunya (86-100%) and Bragg (60-100%) both had a high regeneration efficiency in three different types of explants, hypocotyl, split-hypocotyl and cotyledonary node (Raza et al., 2017). In addition, half-seed explants have been used for soybean transformation using herbicide-based selection systems (Paz et al., 2006; Kim et al., 2012b; Song et al., 2013). In this study, regeneration efficiency from half-seed explants of Bunya and Bragg were evaluated. Both cultivars showed a high regeneration frequency (Bunya 80%, Bragg 83.3%). The high regeneration ability of Bunya and Bragg indicate that these cultivars have a strong potential for genetic transformation of soybean.

Regeneration and transformation efficiency were also evaluated using half-seed explants from Bunya and Bragg. Interestingly, a significant reduction of regeneration efficiency was detected from half-seed explants of Bunya after co-cultivation (Table 3.4). Non-infected explants of Bunya showed similar regeneration efficiency (76.7±15.28%) in previous experiments (80%) after co-cultivation without bacterium infection. In contrast, infected half-seed explants of Bunya showed 29.2±5.16% regeneration frequency after co-cultivation (Table 3.4). Furthermore, about 70% of the seeds remained without the change of colour to green and further growth, indicating that embryogenic development process was hindered by infection of *Agrobacterium*. These results indicated that Bunya has a poor susceptibility to *Agrobacterium* and more sensitive response to pathogen-attack during early embryogenic stage, resulting in reduction of regeneration efficiency. The highly sensitive reaction to *Agrobacterium* may hamper the delivery of T-DNA to soybean and recovery of transgenic plants from *Agrobacterium*-infected explants (Zhang et al., 2016). Gene expression analysis on response of soybean to *Agrobacterium* during infection was conducted by Zhang et al. (2016). Cotyledonary node explants from Chinese soybean cultivar (Jidou17) were infected with *Agrobacterium* strain EHA105 and samples after 5 hours of co-cultivation were used for sequence analysis. They reported that *Agrobacterium* infection triggered expressions of host defence related genes. Total 2,158 genes were differentially expressed compared to non-infected explants. Among them, plant-pathogen interaction, plant hormone signal transduction and phenylpropanoid biosynthesis showed significant expression changes. In plant, phytohormes such as salicylic acid, jasmonic acid, and ethylene are involved in defence system (Anand et al., 2008). Up-regulation of WRKY transcription factor family (involved in biotic and abiotic stress response) and ethylene responsive factors was detected in *Agrobacterium*-infected explants. In addition, expression of brassinosteroid-metabolism-related genes were significantly changed. As one of the endogenous plant growth regulators, brassinosteroid regulate the elongation and division of cells (Clouse et al., 1996). Brassinosteroid insensitive 1-associated receptor kinase 1 (BAK1) is a key regulator to modulate brassinosteroid signalling and pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI). The expression of BAK1 significantly increased in *Agrobacterium*-infected soybean explants. Furthermore, phenylpropanoid biosynthesis (94 genes), flavonoid biosynthesis (77 genes) and isoflavonoids biosynthesis (19 genes) were enriched in infected explants. The metabolites derived from these biosynthesis pathways play important roles in plant stress and immune response by acting as anti-microbial molecules (Dakora and Phillips, 1996). Isoflavonoids are a group of polycyclic compounds present mostly in leguminous plants and particularly abundant in soybean (Gutierez-Gonzalez et al., 2010). The genes related in biosynthesis of isoflavonoids were significantly up-regulated after 5 hours of infection. This result indicated that soybean stimulates its defence response to *Agrobacterium* infection, resulting in lower transformation efficiency in soybean. Zhang et al. (2015) validate adverse roles of isoflavone in *Agrobacterium*-soybean infection by use of daidzein (a class of legume specific isoflavone). Addition of 2 mM daidzein inhibited *Agrobacteriu*m growth and respiration by 95% and 35% respectively. To reduce the negative effect of isoflavone in *Agrobacterium*–mediated transformation, they tested the effect of α-aminooxyacetic acid (AOA) and sonication treatment to alleviate isoflavone synthesis from infected explants. α-aminooxyacetic acid (AOA) acts as an inhibitor of isoflavone synthesis by inhibition of phenylalanine ammonialyase (PAL: first enzyme in the pathway of plant secondary metabolism) (Li et al., 2009a). Sonication treatment makes micro-wounds on the surface of explants, thereby *Agrobacterium* infecting more efficiently (Trick and Finer, 1997). Addition of 20 µM AOA in co-cultivation media caused a reduction of expression level of genes involved in isoflavone synthesis (14.3-47.0%). Moreover, sonication treatment during infection maintained the expression level of those isoflavone synthesis genes at a lower level (32.6-56.5%). With these treatments to reduce isoflavone synthesis during *Agrobacterium* infection, they reported about 3.6 times higher transient GUS expression than that of non-treatment batches (Zhang et al., 2015).

In this study, putative transgenic explants showed a low elongation rate during selection. Although half-seed explants of Bragg had a high frequency of shoot elongation (1-10 shoot /explant), four YFP positive shoots did not elongate and eventually died on selection medium. In addition, browning and necrosis on the surface of infected explants were observed. Reactive oxygen species (ROS) will be generated from explant after infection with *Agrobacterium* and it causes cell death and browning of tissue, which lead to the reduction of transformation efficiency (Li et al., 2017). To alleviate tissue browning, antioxidants such as DTT (dithiotheritol), L-cysteine and PVP (polyvinylpyrrolidone) were used in *Agrobacterium*-mediated transformation (Dan, 2008). In this study, infected explants successfully formed multiple shoots on selection medium during shoot induction, whilst most of explants had tissue browning symptoms on surfaces of cotyledons and explants finally failed to elongate shoots during shoot elongation stages. Tissue browning was considered as a main factor for poor shoot elongation as many shoot primordia were easily detached from browning parts before shoot elongation and ultimately causing shoot primordia to die. In plant tissue culture, adenine hemisulfate has positive effects on cell growth and formation of shoots by enhancing plant growth regulators such as cytokinin (Bantawa and VanStaden, 2009). Adenine hemisulfate acts as a precursor in cytokinin synthesis, resulting in reinforcement of shoot differentiation and growth (Gatica et al., 2010). Furthermore, addition of 100 mg/L of adenine hemisulfate to 10 µm of BA significantly improved multiple shoot induction (about 40%) and reduced 90% of shoot tip necrosis and chlorosis (Naaz et al., 2014). PVP prevents accumulation of phenolic compounds, which are produced from explants during tissue culture resulting in improvements of shoot differentiation and formation of embryogenic callus (Reustle and Natter, 1994; Passos et al., 1999). In this study, the effects of Adenine hemisulfate (AD) and PVP 40,000 (PVP 40,000) on shoot elongation efficiency were evaluated. Addition of 40 mg/L of adenine hemisulfate and 500 mg/L of PVP 40,000 on SEM increased the numbers of elongated shoots (Figure 3.8). Explants cultured on SEMAP (shoot elongation medium contains 40 mg/L of adenine hemisulfate and 500mg/L of PVP 40,000) showed 6 times higher number of elongation shoots than that of SEM (without adenine hemisulfate and PVP 40,000) (Table 3.5). With the additions of AD and PVP, total 23 elongated shoots were produced from 6 batches of transformation experiments.

In this study, 13 putative transgenic plants (in pots) were obtained from *Agrobacterium*-mediated transformation (Table 3.6). In PCR analysis, the presence of *bar* gene was confirmed in 6 putative transgenic plants. However, three putative transgenic plants (T0-3, T0-8 and T0-11) showed herbicide resistance in basta brush test (Figure 3.12). These herbicide resistant transgenic plants (T0-8 and T0-11) showed *bar* gene expression in RT-PCR analysis (Figure 3.15). In contrast, three lines (T0-4, T0-7 and T0-14) did not show basta resistance and expression of *bar* gene. These results indicated that the silencing of transgene may occur in these transgenic lines. The silencing of transgene in transgenic plants is a common phenomenon and an obstacle to development transformants (Meins and Kunz, 1995). This inactivation of transgene has been reported in plants such as rice (Kohli et al., 1999), citrus (Dominguez et al., 2002) and soybean (Srinivasa Reddy et al., 2003). When transgenes are inserted into plant genome, the silencing of transgenes can take place by rearrangements of transforming plasmid prior to or during insertion into the host plant genome (Kohli et al., 1999). In rice, silencing of *gusA* gene expression was caused by aberrant mRNA expression of GUS gene. During transgene integration into rice genome, promoter region was truncated by rearrangement of genomic DNA leading to methylation of *gusA* gene (Kohli et al., 1999). In citrus, over 30% of transgenic plants derived from non-selective conditions had silenced transgenes and even single insertion of transgene was able to trigger transgene silence (Dominguez et al., 2002). Srinivasa Reddy et al. (2003) reported that transgenic soybean plants (with over 100 transgene copies) obtained from particle bombardment transformation method showed different protein expression level of transgene resulting in variable GUS expression.

This study demonstrated genetic transformation of a commercial cultivar of soybean (cv. Bragg). Insertion of single copy of *bar* gene was confirmed in a putative transgenic line and stable expression of *bar* gene and YFP gene was confirmed in T1 and T2 generations. Recent studies on soybean defence reaction during *Agrobacterium* infection provide alternative ways to improve soybean transformation (Zhang et al., 2016). Further investigation on pathogen attack mechanisms and alleviation of the response to *Agrobacterium* in soybean will provide information useful for development of transformation systems for commercial cultivars of soybean.

3.5 Conclusions

In this study, *Agrobacterium*-mediated transformation system for commercial cultivars of soybean (cv. Bragg) was demonstrated. Bragg showed a high regeneration ability with half-seed explants (83.3%). Although Bunya showed high regeneration frequency with half-seed explants, a significant reduction in regeneration efficiency (80.0%→29.2%) was observed after infection with *Agrobacterium*. This result indicated that susceptibility of *Agrobacterium* is important factors to establish *Agrobacterium*-mediated soybean transformation system.

The ability of shoot elongation during selection may be one of the key factors for successful soybean transformation. In this study, addition of PVP 40,000 and adenine hemisulfate increased the shoot elongation rate suggesting that antioxidant and adenine hemisulfate has effect on recover of putative transgenic plants.

In this study, stable expression of transgene and transmission to progeny (T1 and T2) was observed in the transgenic plant. Final efficiency of *Agrobacterium*-mediated transformation system using half-seed explants of a commercial cultivar of soybean (cv. Bragg) was 0.6%. This transformation system paves the way to improve important agronomical traits and enables a functional analysis of soybean genes.

Chapter 4. Functional analysis of *GmLFY1* (Glyma.06G163600.1), a *LFY* homolog of soybean

Abstract

LEAFY (*LFY*) gene is a plant-specific transcription factor and is found in all land plants. *LFY* gene plays an important role in the onset of flowering and floral development. In soybean, two *LFY* homologs (Glyma.04g202000.1 and Glyma.06G163600.1) are annotated in its genome database (phytozome v12.1). A high sequence conservation of soybean *LFY* homologs with Arabidopsis *LFY* was observed in sequence alignment analysis, suggesting that these two *LFY* homologs may play similar roles as Arabidopsis *LFY* during flowering. In addition, high conservations of key amino acids involved in DNA-binding were observed in two conserved domains (N- and C-domain) of soybean *LFY* homologs. In Glyma.04G202000.1, substitutions of amino acids in the N-domain were detected and it suggests putative functional variations in DNA-binding of this *LFY* homolog. In temporal expression analysis, two *LFY* homologs were expressed in shoot apical meristem (SAM) and the expression gradually increased under flowering inductive conditions (short day). Glyma.06G163600.1 showed a higher expression (2 fold at short day 4) than Glyma.04G202000.1. In situ hybridization of Glyma.06G163600.1 in shoot apical meristem (SAM) revealed that it was expressed in peripheral regions of SAM and leaf primordia during vegetative development and strong expression was detected in floral meristem. These temporal and spatial expressions of Glyma.06G163600.1 under flowering inductive conditions indicated its involvement in flowering initiation. Ectopic expression of Glyma.06G163600.1 in transgenic Arabidopsis and tobacco plants promoted the initiation of flowering process. Glyma.06G163600.1 overexpressing transgenic Arabidopsis plants started flowering about 1 week earlier (31.3±2.42 DAG) than wild-type plants (39.8±3.60 DAG). Transgenic tobacco plants also showed early flowering phenotypes (58.5±7.59 DAG) than wild-type plants (73.3±9.03 DAG). In addition, overexpression of Glyma.06G163600.1 in transgenic tobacco plants caused adult traits (narrow leaf shape) in leaves during vegetative development. Leaves of transgenic tobacco plants were narrower in shape (L/W ratio 2.9±0.41) than those of wild-type (2.6±0.24). Up-regulations of genes involved in the initiation of flowering (*AP1, SOC1, and LFY*) in transgenic Arabidopsis and tobacco plants were detected by qRT-PCR analysis. In transgenic Arabidopsis, T2 homozygous lines showed an up-regulation of floral pathway integrators. *AtAP1* showed a higher expression (1.5 fold at line 14 and 1.8 fold at line 15) than wild-type. Significantly increased expression of *AtSOC1* was also detected in both transgenic lines 14 (14.5 fold) and 15 (15.8 fold). On the other hand, no significant difference in the expression of endogenous Arabidopsis *LFY* gene was detected among transgenic plants and wild-type, indicating that overexpression of *GmLFY1* promoted initiation of flowering by up-regulations of floral pathway integrators. *GmLFY1* overexpressing transgenic tobacco plants also showed an up-regulation of flowering pathway integrator genes, *NtAP1a* (3.4 fold), *NtAP1b* (1.8 fold), *NFL1* (1.8 fold), *NtSOC1a* (3.7 fold) and *NtAG* (2.8 fold). During floral development, strong *GUS* expressions were detected mainly in the sepal of flowers of transgenic tobacco plants harbouring *LFY::GUS::OCS* (5' regulatory fragment of Glyma.06G163600.1: 2.5Kb upstream from ATG). *GUS* expression was detected throughout all floral primordial stages and strong *GUS* expression was only observed on the sepal and the top region of pistil after the emergence of stamen and pistil. These expression patterns became strong during mature flower developmental stages. This result suggests that *GmLFY1* may have a role in flower development.

In this study, the roles of *GmLFY1* have been identified in the initiation of flowering. Ectopic expression of *GmLFY1* in Arabidopsis and tobacco plants showed early flowering phenotypes. However, abnormal growth of flowers was not observed in Arabidopsis and tobacco plants, suggesting the confined functions of *GmLFY1* in flowering. This observation may be explained by the fact that functional divergence of *GmLFY1* occurred during genome duplication.

Key words: soybean, *LEAFY*, flowering, Arabidopsis, tobacco

4.1 Introduction

Flowering plants undergo different developmental stages during its life cycle, namely, germination, vegetative growth, flowering and setting of seeds. Among this, a shift from a vegetative state to a reproductive state is a major developmental transition in the plant's life cycle. Since flowering is the first step of sexual reproduction which leads to formation of seeds, it bears a huge significance in agriculture (Bernler et al., 1993). Control of flowering is important in order to maximize a plant's reproductive success, as it plays a crucial role in gaining yield and producing dry matter in plants (Corkram et al., 2007; Kim et al., 2012a). Early flowering phenotypes are valuable to avoid unfavourable growth conditions, conversely, late flowering allows plants to accumulate and produce more resources for seed production under optimal growth environment (Roux et al., 2006). In this regard, a detailed understanding of flowering regulatory mechanisms is crucial to improve and enhance important agricultural characteristic of crops. To understand flowering mechanisms, comprehensive molecular analyses of flowering have been conducted on a model plant, *Arabidopsis thaliana*. In Arabidopsis, flowering is initiated via various types of genetic pathways, namely, photoperiod, vernalisation, autonomous flowering pathways, gibberellins (GA) and miRNAs (Roux et al., 2006; Wu et al., 2009; Yamaguchi and Abe, 2012). Environmental and endogenous cues from these pathways are converged in order to control the expression of flowering pathway integrator genes, *SOC1*, *FT* and *LFY*. These genes activate the expression of floral meristem identity genes, thereby initiating the flowering process (Roux et al., 2006; Kim et al., 2009).

Among these genes, *LFY* gene is a plant-specific transcription factor which plays an important role in the onset of flowering and reproductive development (Moyroud et al., 2010, Tang et al., 2016). In Arabidopsis, *LFY* expression is observed in increasing levels during the vegetative phase and when expression reaches a critical level, plants flower through direct activation of *AP1* (Parcy, 2005). *LFY* plays a pivotal role in specification of floral identity of meristems and is expressed at a very early stage of flowering (Moyroud et al., 2010). Furthermore, *LFY* gene is involved in the differentiation of cells from shoot apical meristem into floral primordia by regulation of downstream floral homeotic genes, *AG* and *AP3* (floral organ identity C genes) and *AP1* (a floral organ identity A gene) (Weigel et al., 1992; Ahearn et al., 2001). The loss of function of *LFY* shows partial or complete flower-to-shoot conversions, indicating that *LFY* plays an important role in regulating the floral organ identity genes (Coen et al., 1990; Souer et al., 1998; Molinero-Rosales et al., 1999; Bomblies et al., 2003). In monocots, two *LFY* homologs *in Zea mays* L., *ZFL1* and *ZFL2* have conserved roles in flowering and branching of inflorescence via regulation of ABC floral organ identity gene (Bomblies et al., 2003). However, in rice (*Oryza sativa*) *LFY* homolog, *RFL* is responsible for the control of inflorescence structure and its expression has not been detected in floral meristem (FM), indicating that *RFL* lost partial functions in floral identity (Kyozuka et al., 1998; Rao et al., 2008; Ohmori et al., 2009). In woody plants, a *LFY* homolog of *Jatropha curcas* (*JcLFY*) has an effect on flower identity and development of fruit shape (Tang et al., 2016). Ectopic expression of *JcLFY* in Arabidopsis promoted flowering with formation of precocious flowers in inflorescence. In addition, co-suppression of *JcLFY* in jatropha produced abnormal flowers and fruits, suggesting its roles in reproductive developments (Tang et al., 2016).

Soybean (*Glycine max* L.) is one of the most important crops in the world due to its seeds containing 40% protein and 20% oil. Soybean is used for animal feed as a good protein source. Besides, its usages for humans vary from processed foods to secondary metabolite sources (e.g. tocopherols, isoflavones, lecithin and saponin) (Sugano, 2005). In addition, the unique ability of soybean, nitrogen fixation, makes it an important crop in sustainable agricultural practice. Rhizobia, a group of soil bacteria, have an ability to fix nitrogen from the atmosphere. This symbiotic bacterium of soybean infects the roots of soybean and forms nodules, where they fix nitrogen and the fixed nitrogen is used for soybean growth (Liew et al., 2014). Because of the agronomical and economic importance of soybean as sources of oil, protein and other uses, soybean production has been rapidly growing (Hartman et al., 2011). Therefore, a good understanding of flowering mechanisms of soybean can help achieve better yields. In spite of the significance of flowering in reproductive success and crop yield, limited studies are available on the molecular pathways and mechanisms of flowering in soybean (Liew et al., 2014). In addition, although the knowledge gained from model plants such as Arabidopsis allow a basic understanding of plant biology, such findings are not suitable for direct application to important crop plants, such as soybean (Haerizadeh et al., 2009). Moreover, there are major developmental differences between Arabidopsis and soybean. Soybean is a short day plant whereas Arabidopsis is a long day plant. Soybean has a genome size of 1.1-gigabase as opposed to Arabidopsis, whose genome size is much larger. The soybean genome was duplicated approximately 59 and 13 million years ago, which means nearly 75% of its genes are present in multiple copies (Schmutz et al., 2010; Jung et al., 2012). This is one of the features of soybean which poses a difficulty in direct application of genetic information obtained from model plants and a challenge on the analysis of flowering pathways in soybean.

This study aims to identify roles of the soybean *LFY* gene. The objectives of this chapter are as follows:

- Comparison of sequence structures in highly conserved DNA-binding domains of *LFY*
- Investigation of temporal spatial expression of two soybean *LFY* homologs
- Examination of roles of Glyma.06G163600.1 in flowering and floral development via transgenic Arabidopsis and tobacco plants which harbour overexpressed Glyma.06G163600.1
- Examination of Glyma.06G163600.1 expression during flower development in transgenic plants which harbour 5' regulatory region of Glyma.06G163600.1

4.2 Materials and methods

4.2.1 Phylogenetic analysis

The sequence information of two homologs of the *LFY* gene in soybean (*GmLFY1*; Glyma.06G163600.1 and *GmLFY2*; Glyma.04G202000.1) was obtained from the phytozome database (phytozome v12, www.phytozome.jgi.doe.gov). Protein sequences of *LEAFY* homologs from different plant species were obtained from phytozome (phytozome v.12) and NCBI database by BLAST search with peptide sequence of the Arabidopsis *LFY* gene (At5g61850). Sequence alignment analysis was carried out using multiple-sequence alignment tool (MUSCLE) with default parameters. Examinations of *cis*-regulatory elements (CREs) were conducted using PLACE (Plant *cis*-regulatory DNA elements) database (Higo et al., 1999). The phylogenetic tree was constructed with neighbour-joint-analysis, 1,000 replicates bootstrap using MEGA X.

4.2.2 Plant transformation and selection of transgenic plants

4.2.2.1 Preparation of plant tissue culture medium

Compositions of medium, antibiotics and hormones used in this study are presented in appendices (Table A.2).

4.2.2.2 Preparation of Glyma.06G163600.1 overexpressing constructs and promoter construct for plant transformation

Glyma.06G163600.1 overexpressing constructs

The cDNA was synthesized with 1 μg of total RNA which was extracted from soybean SAM (SD 4). The coding sequence of Glyma.06G163600.1 (1230 bp) was amplified with specific primer pairs (Table A3) using Phusion High-Fidelity DNA polymerase. Phusion DNA polymerase reaction mixtures contained 4 μl of 5X Phusion HF buffer, 200 μM each of dATP, dCTP, dGTP, dTTP, 0.5 μM of each primer and 0.2 μl of Phusion DNA polymerase (Thermo Scientific, USA) in a volume of 20 μl with 100 ng of cDNA. PCR reactions were carried out using a Mastercycler® nexus (Eppendorf, Germany) with the condition: an initial denaturation step at 98℃ for 30 sec followed by 30 cycles at 98℃ for 10 sec, 72℃ for 90 sec and one cycle of final extension at 72℃ for 10 min. The amplified PCR products were separated by electrophoresis on 1.0% agarose gel. Gels were visualized with a ChemiDoc MP imaging system (Biorad, USA). 1,230 bp of amplified PCR products was excised from agarose gel and purified using Wizard® SV Gel and PCR clean-up system (Promega, USA) in accordance with the manufacturer's instructions. Purified PCR products were cloned into a T-A cloning vector (pGEM-T easy, Promega, USA). The intermediate plasmid (pART7) and Glyma.06G163600.1 gene in pGEM-T easy vector were digested with *EcoRI* restriction enzyme. Glyma.06G163600.1 was inserted between CaMV35S promoter and OCS terminator in pART7. pART7 plasmid harbouring Glyma.06G163600.1 and binary vector pUQC10255 (which was kindly provided from Professor Bernard Carroll at The University of Queensland) were digested with *NotI* restriction enzyme (Figure 4.2). The digested Glyma.06G163600.1 overexpression (*GmLFY1*) construct *(35S::GmLFY1::OCS*) from pART7 was ligated with binary vector pUQC10255. The binary vector harbouring *35S::GmLFY1::OCS* was introduced into *Agrobacterium tumefaciens* strain EHA105 by heat shock method and was used for plant transformation.

Promoter construct

Putative 5' regulatory region of Glyma.06G163600.1 (2.5 kb upstream from start codon) was amplified from the genomic DNA of soybean (cv. Bragg). The isolated 5' regulatory region was introduced into pGEM-T easy plasmid (Promega, USA). The 5' regulatory fragment in pGEMT-easy plasmid and the intermediate plasmid (pRITA) were digested with *EcoRI* and *NcoI* restriction enzyme. The 5' regulatory fragment was inserted in front of *GUS::*OCS construct in pRITA. The pART7 plasmid harbouring putative 5' regulatory region of Glyma.06G163600.1 were digested with *NotI* restriction enzyme and the digested 5' regulatory region of Glyma.06G163600.1 construct (*LFY::GUS::OCS*) from pART7 were introduced into and binary vector pUQC10255 (Figure 4.2B). The binary vector harbouring *LFY*::*GUS*::OCS was inserted into *Agrobacterium tumefaciens* (strain EHA105) and was used for used for plant transformation.

Plant transformation: Arabidopsis

Arabidopsis transformation was carried out using the floral dip method (Zhang et al., 2006). Wild-type Arabidopsis plants (Col-0) which contained 20 to 30 inflorescences were used for transformation. *Agrobacterium* which harbours Glyma.06G163600.1 overexpression construct was grown on YEP-agar medium. The developed single colony was inoculated into 5 ml YEP medium in a 50 ml tube and was cultured for 16 h at 220 rpm (28℃, dark). 500µl of *Agrobacterium* culture was added into 500 ml of liquid YEP medium in a sterilized 1 L conical flask and incubated at 220 rpm (28℃, dark) until O.D.$_{600}$ value reaches between 1.5 and 2.0. *Agrobacterium* culture was aliquoted into four 50 ml tubes and centrifuged for 10 min at 4,000 g at room temperature to collect

Agrobacterium cells. The pellets were resuspended with 500ml of 5% (w/v) sucrose solution in a sterilized container. One hundred microliter of the non-ionic surfactant (Silwet L-77, Phytotechnology Laboratories, USA) was added into *Agrobacterium*-resuspended culture and mixed well with a stirring bar. Inflorescences of Arabidopsis plants were dipped into the *Agrobacterium* suspension for 10 sec with gentle agitation and were drained infected plants for 5 sec. The infected plants were placed on a new container and were covered with plastic wrap to maintain high humidity. After 16 h of incubation, plastic covers were removed and plants were grown in a plant growth room (light intensity 150 μmol m^{-2} s^{-2}, 16 h/8 h, 22±1℃) until seeds matured.

Transgenic plants were screened using basta spray method. Putative transgenic seeds which were obtained from T0 generation were sown on sterilized soil and were grown in the plant growth room (light intensity 150 μmol m^{-2} s^{-2}, 16 h/8 h, 22±1℃). After 7 days of germination, 200 mg/L of Basta® (Bayer Crop Science, Germany) solution were sprayed onto the putative transgenic seedlings (3 times per a week) to select transgenic plants. The survived transgenic plants were grown in the plant growth room (light intensity 150 μmol m^{-2} s^{-2}, 16 h/8 h, 22±1℃) until seeds matured. T1 seeds of survived transgenic plants were harvested individually and randomly selected 20 lines were screened by application of basta [spray basta solution (200mg/L of Basta®) three times per a week] to obtain T2 homozygous transgenic lines. Two homozygous transgenic lines (line 14 and line 15) were used in this study to analyse the functions of *GmLFY1* gene.

Plant transformation: Tobacco

Tobacco transformation was conducted using leaf disc method (Horsch et al., 1985). *Agrobacterium* harbouring *35S::GmLFY1::OCS* was cultured on YEP medium (solid) for 20 h (28℃, dark). A single colony was cultured in 5 ml of liquid YEP medium (liquid) for 20 h, shaking at 200 rpm (28℃, dark). *Agrobacterium* culture was resuspended with 10 times volume of MS liquid medium for infection. Leaf segments (1cm x 1cm) were prepared and were inoculated with *Agrobacterium* suspension for 2 min at room temperature. Infected explants were placed on sterilized filter papers to remove excessive infection medium and were co-cultivated for 2 days (25℃, dark). After co-cultivation, explants were transferred to selection medium containing 4 mg/L of glufosinate (Sigma, USA), 400 mg/L of carbenicillin (GoldBio, USA) and cultured in a tissue culture room (150 µmol m^{-2} s^{-2}, 16/8 h, 25±1℃). Shoots were regenerated from infected explants within 2 weeks. Explants were subcultured every 2 weeks into fresh selection medium. Survived putative transgenic shoots were excised from explants and were rooted on rooting medium containing 4 mg/L of glufosinate, 400 mg/L of carbenicillin.

Putative transgenic plants with developed roots were transferred on sterilized commercial soil mix (Debco, Australia) and were placed in plastic containers then covered with plastic wrap to retain high humidity for acclimatization. The acclimatized plants were incubated in a plant growth incubator (150 µmol m^{-2} s^{-2}, 16/8 h, 25±1v) for 3 days and were transferred to glasshouse (light intensity 250 µmol m^{-2} s^{-2}, 16/8 h, 25±1℃) and grown till maturity and seeds were collected.

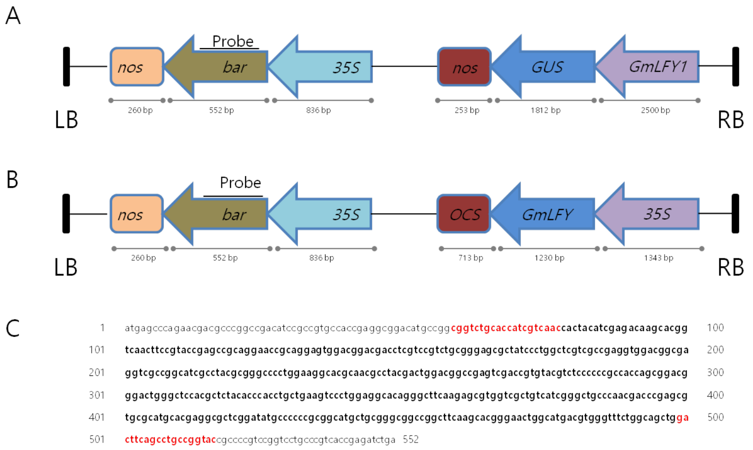


Figure 4. 1. Schematic diagram of Glyma.06G163600.1 overexpression and promoter constructs.

(A) Full length of Glyma.06G163600.1 promoter construct. T-DNA region of *35S::GmLFY1::OCS* and *LFY::GUS::nos* were inserted into pUQC10255 vector. (B) Glyma.06G163600.1 overexpression construct. (C) The nucleotide sequence of *bar* gene: primer annealing regions for amplified DIG labelled *bar* probe for southern blot analysis are indicated in red. Bold sequences indicate whole sequences of *bar* probe. LB: left border, *nos*: nopaline synthase terminator, *bar:* herbicide bialaphos *resistant gene,* 35S: CaMV 35S promoter, *GmLFY1*: Glyma.06G163600.1, OCS: octopine synthase terminator, *NPT* Ⅱ : neomycin phosphotransferase gene, RB: right border, Probe: probe region for southern blot analysis.

4.2.3 Morphological analysis of transgenic plants harbouring overexpressed Glyma.06G163600.1

4.2.3.1 Flowering time measurements and morphological analysis

The days until first open flower was recorded for flowering time analysis. Days until first flowering was calculated from germination to first flower opening. Plant morphological traits such as plant height, total number of leaves, leaf shape and area were examined. Plant height was measured from ground level to the tip of shoot apex. The length from leaf apex to the junction between leaf lamina and petiole along the mid vein was measured for leaf length. Leaf width was measured end-to-end between widest lamina. Leaf area was measured using a LI-3100 leaf area meter (LI-COR, USA). Total 40 biggest leaves from 40 lines were measured. Average value of three measurements from one largest leaf of one line were used for leaf area analysis.

4.2.3.2 Measurement of chlorophyll concentration and photosynthesis rate

Chlorophyll and photosynthesis rates were measured on two different points of intact leaf lamina at different locations (top, middle and bottom) on 8-week-old plants. SPAD 502 Plus Chlorophyll Meter was used for quantification of total chlorophyll photosynthesis yield data (Y (Ⅱ) = (Fm'-F)/Fm) and measurements were taken using MINI-PAM (Walz, Germany).

4.2.3.3 Pollen viability

Pollens were extracted from three preanthesis flowers of transgenic and wild-type plants. Pollens were placed on the slides with a drop of solution containing 40 ul of 10% sucrose with 2 ul of Propidium iodide (1 mg/ml) and 2 ul of Fluorescein diacetate (2 mg/ml). Pollen viability was observed under optical microscope (BX60 microscope, Olympus, Japan).

4.2.4 Molecular analysis of gene expression

4.2.4.1 Genomic DNA isolation

Modified CTAB methods were used for genomic DNA extraction (Doyle and Doyle, 1990). For genomic DNA extraction from Arabidopsis, 0.1 g of fully expanded leaves from each plant were homogenized using a TissueLyser LT (Qiagen, Germany). Pre-warmed (55℃) 0.5 ml of CTAB solution (2 g of CTAB, 100 mM Tris-HCl pH8.0, 20 mM EDTA, 1.4 M NaCl, 1 g of PVP 40,000 in 100 ml, pH5.0) were added into homogenized samples and were mixed by vortexing. The mixtures were incubated at 55℃ in water bath for 15 min with occasional mix. Cell debris were precipitated by centrifugation at 12,000 g at room temperature for 5 min. Supernatants were carefully transferred to new 1.5 ml tubes and 0.25 ml of chloroform:isoamyl alcohol (24:1) was added into each tube. The tubes were mixed by inversion followed by centrifugation at 13,000 rpm at room temperature for 1 min. Upper aqueous phase was transferred to new 1.5 ml tubes. 50 μl of 7.5 M ammonium acetate was added and then ice-cold absolute EtOH (500 μl) was added to precipitate genomic DNA. The tubes were carefully inverted 5 to 7 times and were incubated at -20℃ for 16 h. Genomic DNA was precipitated by centrifugation at 13,000 rpm for 5 min at 4℃. Genomic DNA was washed with 70% (v/v) ethanol by inverting and was centrifuged at 13,000 rpm for 1 min at 4℃. Supernatant was discarded thoroughly using a pipette and pellets were dried for 5 min at room temperature. Genomic DNA was dissolved in TE buffer and concentration of DNA was measured using NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, USA).

For genomic DNA extraction from putative transgenic tobacco plants, 1 g of fully expanded leaves were ground in liquid nitrogen using mortar and pestle. Homogenized leaves were mixed with 10ml of pre-warmed (55℃) 2% CTAB solution (2 g of CTAB, 100 mM Tris-HCl pH8.0, 50 mM EDTA, 0.1% v/v β-mercapoethanol in 100 ml) and incubated at 55℃ in water bath for 15 min with gentle shake. The samples were centrifuged at 4,500 rpm at room temperature for 10 min. Supernatants were transferred to new 50 ml tubes and were mixed with 5ml of chloroform:isoamyl alcohol (24:1) by inversion. The mixtures were centrifuged at 4,500 rpm at room temperature for 10 min and then the upper aqueous phase was transferred to new 50 ml tubes. 1 ml of 10% CTAB solution (10 g of CTAB, 100 mM Tris-HCl pH 8 in 100 ml) was added into each tube and mixed by inverting. After 5 min of incubation at room temperature, 2 ml of chloroform:isoamyl alcohol (24:1) were added and were mixed by inversion. The mixtures were centrifuged at 4,500 rpm for 10 min and the upper phase was transferred to new 50 ml tubes. The samples were treated again with 1 ml of 10% CTAB solution and chloroform:isoamyl alcohol (24:1). After centrifugation (at 4,500 rpm for 10 min), the upper phase was transferred to new 50 ml tube. To precipitate of genomic DNA, 2/3 volume of isopropanol (ice-cold) was added into each tube and the mixtures were stored on ice for 1 hour to precipitate genomic DNA. The tubes were centrifuged at 4,500 rpm for 10 min and the pellet was washed with 70% (v/v) ethanol. After the washing step, the DNA pellets were dried for 5 min at room temperature. Genomic DNA was dissolved in TE buffer and DNA purity (260/280 nm) was measured using nano-drop.

4.2.4.2 PCR analysis

The presence of *bar* gene in transgenic plants was confirmed by Polymerase chain reaction (PCR). Specific primer pairs were used for amplification of coding region of *bar* gene (439 bp) (Table A.3). *GmACT* was used as an internal control. PCR analysis was carried out using *Taq* DNA polymerase (Invitrogen, USA) following manufacturer's instructions with 50 ng of genomic DNA. PCR reactions were conducted using a Mastercycler® nexus (Eppendorf, Germany) under the conditions: initial denaturation step at 94℃, for 3 min, 1 cycle, followed by 30 cycles of 94℃ for 45 sec, 55℃ for 30 sec, 72℃ for 90 sec and 1 cycle of final extension at 72℃ for 10 min. The amplicons were loaded on 1.0% agarose gel and examined using a ChemiDoc MP imaging system (Biorad, USA).

Primers used in this study are presented in the appendices (Table A.3).

4.2.4.3 Southern blot analysis

The presence and copy numbers of the *bar* gene in putative transgenic lines were confirmed using Southern blot hybridisation (Southern, 2006). DIG-labelled probe of *bar* gene (439 bp) was amplified from a pUQC10255 plasmid using DNA DIG-labelling kit

(Roche, USA). DIG-labelling reaction mixture contained 5 µl of 10X PCR Buffer with $MgCl_2$, 5 µl of PCR DIG probe synthesis mix, 5 µl of 0.5 µM forward and reverse primer and 0.75 µl of enzyme mix in a volume of 50 µl with 50 ng pUQC10255 plasmid harbouring the *bar* gene. PCR reactions were carried out to amplified DIG-labelled probe using a Mastercycler® nexus (Eppendorf, Germany) under the condition: an initial denaturation step at 95℃, for 2 min followed by 30 cycles of 95℃ for 30 sec, 60℃ for 30 sec, 72℃ for 40 sec and one cycle of final extension at 72℃ for 7min.

Genomic DNA from putative transgenic plants was digested with restriction enzyme, *BamHI* (single-cutter of cassette). The mixture containing 10 µg of genomic DNA, 10 µl of 10X buffer *BamHI*, 100 units of *BamHI* in a total volume of 100 µl was incubated for 16 h at 37℃ and was concentrated to 20 µl using DNA concentrator after overnight incubation. The DNA samples were loaded on 0.8% agarose gel in 1X TAE buffer for 16 h at 20V and were transferred to a positively charged nitrocellulose membrane (Roche, USA) by capillary transfer. The DNA was immobilized by exposure to short-wavelength ultraviolet light in the UV-crosslinker (120,000 µJ, 2 min). The membranes were pre-hybridised with 40 ml of hybridization solution [2.85 ml of 1 M $Na_2H_2PO_4$, 12.15 ml of 1 M Na_2HPO_4, 120 µl of 0.5 M EDTA pH 8.0, 30 ml of 20% SDS (Sodium dodecyl sulfate, Sigma, USA), 0.3 g of Blocking reagent (Roche, USA), 14.8 ml of sterilized distilled water in 60 ml] for 4 h before hybridisation. After pre-hybridisation, the membrane was hybridised with a DIG-labelled *bar* probe for 16 h at 60℃ followed by wash at low stringency washing buffer (2 x SSC, 0.1% SDS) at room temperature, 5 min for 2 times, and then wash with high stringency washing buffer (0.1 x SSC, 0.1% SDS) at 60℃, 15 min for 2 times. After the washing steps, the membrane was incubated with anti-DIG solution (2 µl of Anti-Digoxygenin-AP in 20 ml of 1% blocking solution) at room temperature for 1 h and 1 ml of detection buffer [10 µl of CDP-star (Roche, USA), 100 µl of 1 M Tri-HCl pH 9.5, 0.1 M NaCl, 890 µl of sterilized distilled water] was applied to the membrane and incubated for 5 min at room temperature. Chemilluminescent was examined in a ChemiDoc MP imaging system (Biorad, USA).

4.2.4.4 Total RNA isolation

Total RNA from transgenic and wild-type plants was extracted using Trizol reagent following manufacturer's instructions (Invitrogen, USA). Plant tissues (0.1 g) were ground using a TissueLyser LT. TRizol reagent (1 ml of each sample) was added to homogenized samples and mixed by inversion. The samples were incubated for 5 min at room temperature and centrifuged at 12,000g for 10 min at 4℃. Supernatant was transferred carefully to a new 1.5 ml tube. 200 µl of chloroform was added to the tube and immediately mixed by inversion followed by incubation for 5 min at room temperature. The mixtures were separated into three phases (lower, inter and upper phases) by centrifugation at 12,000g for 15 min at 4℃. The upper aqueous phase was carefully transferred into a new tube and 500 µl of ice-cold isopropanol was added. The mixtures were incubated for 10 min at room temperature and were centrifuged at 12,000 g for 10 min at 4℃ for precipitation of total RNA. The RNA pellet was washed with 750 µl of 70% (v/v) EtOH and was centrifuged for 5 min at 12,000 g at 4℃. Supernatant was discarded with a pipette and RNA pellets were dried for 2 min at room temperature. The pellets were resuspended with 40 µl of RNase-free water. Total RNA was treated with TURBO^{TM} DNase (Thermo Fisher Scientific, USA) following the manufacturer's instructions to remove possible DNA contamination.

4.2.4.5 Complementary DNA (cDNA) synthesis

Complementary DNA (cDNA) was synthesized. One µg of total RNA was used as a template and cDNA was synthesized using Superscript^{TM}III Reverse Transcriptase (Invitrogen, USA) with oligo (dT) primer. One µl of 50 µM oligo(dT) primer and 1 µl of 10 mM dNTP mix (10 mM each dATP, dGTP, dCTP, dTTP) were added into 1 µg of total RNA. The mixture was incubated at 65℃ for 5min and then placed on ice for 1 min. 7 µl of cDNA Synthesis mix [4 µl of 5X First strand buffer, 1 µl of 0.1M DTT, 1 µl of RNase OUT^{TM} (Invitrogen, USA), 1 µl of Superscript^{TM}III Reverse Transcriptase (Invitrogen, USA) was added to the RNA mixture and the mixture was incubated at 50℃ for 30 min to synthesis of cDNA. After reverse transcript, 80 µl of RNase-free water was added to a total 20 µl of each cDNA template.

4.2.4.6 Gene expression analysis

For temporal expression analysis of two *LFY* homologs of soybean (Glyma.04G202000.1 and Glyma.06G163600.1), soybean plants (*Glycine max* L. cv. Bragg) were grown under long day (LD) conditions (16/8h, 400 μmol/m^{-2}s^{-1}, 25±1℃) for 10 days and then transferred to short day (SD) conditions (10/14h, 400 μmol/m^{-2}s^{-1}, 25±1℃). Different plant tissues (SAM, mature leaves, stem and roots) were collected at different time points (SD 0, 1, 2, 4 and 6). cDNA of each sample was synthesized with 1 μg of total RNA from different samples using SUPERSCRIPT^{TM}III Reverse Transcriptase (Invitrogen, USA). qRT-PCR was carried out with specific primer pairs for Glyma.04G202000.1 and Glyma.06G163600.1 to examine temporal expression patterns respectively (Table A3).

Expression levels of flowering pathway integrators gene in transgenic plants were examined by qRT-PCR. Total RNA was extracted from shoot apex from transgenic plants. cDNA was synthesized with Superscript^{TM}III Reverse Transcriptase (Invitrogen, USA) according to manufacturer's protocol. One μl of cDNA template was used in 10 μl of reaction mixture for qRT-PCR analysis. The qRT-PCR analysis was carried out under the following conditions: pre-incubation at 95℃ for 3 min, followed by 40 cycles of denaturation at 95℃ for 20 sec, annealing at 60℃ for 20 sec, and extension at 72℃ for 20 sec, in the Agilent Mx3000p (Applied Biosystems) using Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent Technologies, USA). PCR specificity was confirmed by melting curve and the 2-ΔΔCt method was used for data analysis (Livak and Schmittgen, 2001). Three biological replicates were performed. Primers used for qRT-PCR analysis were given in Table A3.

4.2.4.7 In situ hybridization

Ten-day-old soybean plants (*Glycine max* L. cv. Bragg), grown under long day (LD) conditions (16/8h, 400 μmol/m^{-2}s^{-1}, 25±1℃) were transferred to short day (SD) conditions (10/14h, 400 μmol/m^{-2}s^{-1}, 25±1℃). The shoot apex was collected between 10am and 11am at different time points (SD 0, 1, 2, 4, 6, 8 and 10 days). The tissues were immersed immediately into a fixative solution (4% paraformaldehyde in 1X PBS buffer, pH 7.0) after dissection. The samples were vacuumed for 10 min and were incubated overnight at 4℃. The samples were dehydrated with graded ethanol series (15%, 30%, 50%, 70% and 80% for 1 h each step) followed by overnight staining with 0.1% Eosin Y (Sigma, USA). The samples were embedded in paraplast (Sigma, USA) after Histo-clear (Sigma, USA) treatments.

In situ hybridisation was carried out, followed by modified protocols (Jackson, 1991; Harizadeh et al., 2009). The embedded samples were sectioned at 8μm thickness using a microtome machine (Zeiss, Germany). The sections were placed on slides [coated with (3-Aminopropyl) triethoxysilane (Sigma, USA)] and stored at 4℃.

DIG-labelled antisense RNA probe were transcribed from SP6 promoter of pGEMT-easy vector (Promega, USA) harbouring 255bp coding sequence of Glyma.06G163600.1 using DIG RNA Labelling Kit (Roche, USA) following the manufacturer's instructions. Before hybridisation, DIG labelled probe was denatured using heat treatment. For one slide hybridisation, 4 μl of DIG-labelled probe was added into 36ul of 50% formamide (Sigma, USA) and was incubated at 80℃ for 5 min to denature DIG-labelled antisense RNA probe. After incubation, the probe was kept on ice before use.

For hybridisation, 160 μl of hybridise solution [25 μl of 10X In-situ salts (3M NaCl, 100 mM Tris-HCl pH8.0, 100 mM Sodium phosphate buffer pH6.8, 50mM EDTA pH8.0), 100 μl of deionized formamide (Sigma, USA), 50 μl of 50 % Dextran sulphate (50% concentration), 5 μl of Denhardt's solution {2% (w/v) Bovine serum albumin, 2% (w/v) Ficoll 400, 2% (w/v) Polyvinylpyrrolidone}, 2.5 μl of t-RNA (Roche, USA)] was mixed with 40 μl denatured DIG-labelled RNA probe. Total 200 μl of hybridise solution containing 40 μl of DIG-probe was added on to samples on a slide. After addition of hybridise solution, slides were covered with parafilm and placed on a container filled with 1/3 volume of sterilized distilled water to maintain high humidity. The slides were hybridised for 16 h at 50℃. After hybridisation, the slides were washed with sterilized distilled water and were observed for development of signals using a BX60 microscope (Olympus, Japan). Photos were taken using a DP70 digital camera (Olympus, Japan) mounted on a BX microscope.

4.2.4.8 Histochemical GUS assay

To examine the expression of Glyma.06G163600.1 during flower development, flowers from T2 homozygous transgenic tobacco plants harbouring *LFY::GUS::OCS* construct were used. Floral buds and flowers at different developmental stages were collected from transgenic tobacco plants (line 5-2). Explants were fixed with 90% Acetone in ice for 30min and subsequently washed with GUS staining washing solution (0.5mM $K_4Fe(CN)_6$, 100mM $NaHPO_4$ pH 7.0, 10mM EDTA pH 8.0, 0.1% Triton X-100). After washing 2 times, explants were incubated in GUS solution (0.5mM $K_4Fe(CN)_6$, 100mM $NaHPO_4$ pH 7.0, 10mM EDTA pH 8.0, 0.1% Triton X-100, 1mM 5-bromo-4chloro-3-indolyl-β-D-glucuronic acid) at 37℃, dark, overnight. After incubation, explants were transferred into 30% EtOH solution for 6 hours and followed by 50% EtOH solution for overnight to remove chlorophyll. After decolourization, *GUS* activity was examined under optical microscope (BX60 microscope, Olympus, Japan).

4.3 Results

4.3.1 Sequence and *cis*-regulatory elements information of two homologs of *LFY* gene in soybean

Detailed sequence information of two *LFY* homologs are presented in Figure 4.2. Both *LFY* homologs of soybean, Glyma.04G202000.1 (full length 3079bp) and Glyma.06G163600.1 (full length 2931bp), consist of 3 exons, 2 introns and 3'untranslated region (UTR). Two introns contain the canonical GT-AG dinucleotide splice site junctions. Glyma.06G163600.1 has longer coding sequences (1230 bp) than Glyma.04G202000.1 (972 bp) (Figure 4.2A). Two *LFY* homologs of soybean are 73.4% (Glyma.04G202000.1) and 69.5% (Glyma.06G163600.1) identical to Arabidopsis *LFY* (At5G61850.1) and Glyma.04G20200.1 is 96.27% identical to Glyma.06G163600.1 in peptide sequences alignment analysis (Figure 4.2B).

cis-regulatory elements (CREs) are located in the upstream part of the genes in the promoter regions, which are transcription factor binding sites. Examination of 2500 bp promoter sequences upstream of the transcription start codon of soybean *LFY* homologs resulted in the identification of 99 and 109 CREs in *GmLFYs*

(Glyma.06G163600.1 and Glyma.04G202000.1), respectively. Among them, the duplication frequencies and locations of ten highly repeated CREs are shown in Figure 4.2C and D. The ten highly duplicated CREs in soybean *LFY* homologs are composed of various elements which are responsible for carbon metabolism (DOFXOREZM: 5'-AAAG-3'), light-responsive elements (GATABOX: 5'GATA-3', GT1CONSENSUS: 5'-GRWAAW-3', EBOXBNNAPA: 5'CANNTG-3'), cytokinin signalling (ARR1AT: 5'-NGATT-3'), plant tissue specific {pollen-specific (POLLEN1LELAT52: 5'-AGAAA-3', GTGANTG10: 5'-GTGA-3'), mesophyll (CACTFTPPCA1: 5'YACT-3'), seed development (CAATBOX1: 5'-CAAT-3'), root apex and vascular organ specific (ROOTMOTIFTAPOX1: 5'-ATATT-3')} (Figure 4.2C). DOFCOREZM is the most abundant *cis*-regulatory motif in *GmLFYs*. It presented 37 to 44 duplication ranges in 2.5 Kb promoter regions of both Glyma.04G202000.1 and Glyma.06G163600.1, respectively. In addition, the light responsive CREs including GT1CONSENSUS, CACTFTPPCA1 and GATABOX are highly present in both soybean *LFY* homologs (Figure 4.2D). Gibberellin acid (GA) is an endogenous hormone and plays regulatory roles in expression of *LFY* gene in Arabidopsis. GA-responsive CRE (P-box) was also identified in the promoter region of two soybean *LFY* homologs (Figure 4.2). Interestingly, high duplications of seed development-specific *cis*-regulatory element (CAATBOX1) in both Glyma.04G202000.1 (26 duplications) and Glyma.06G163600.1 (25 duplications) were observed, suggesting that soybean *LFY* homologs may have undergone functional diversification.

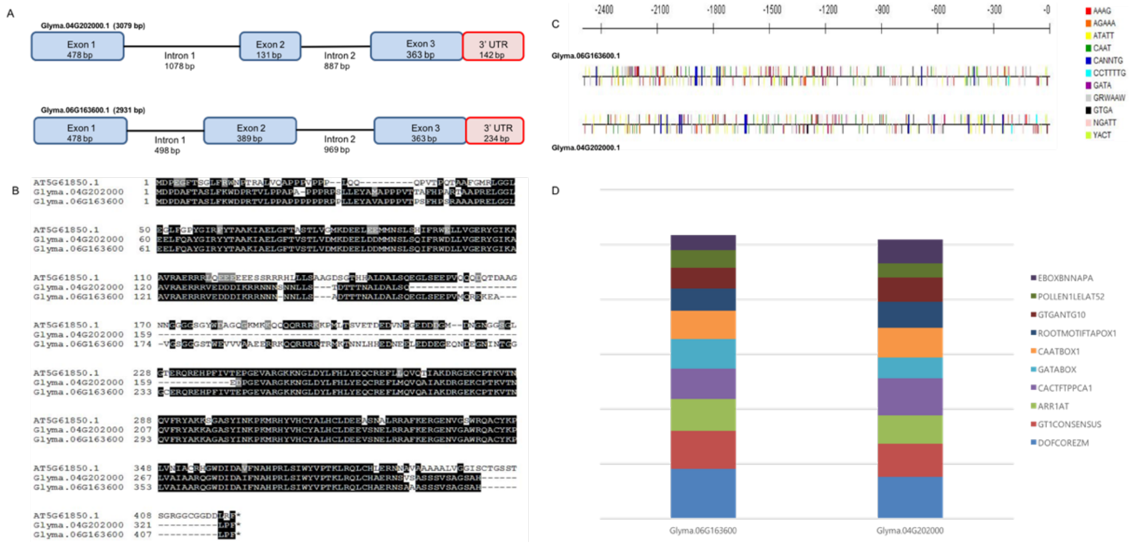


Figure 4. 2. Gene structures and *cis*-regulatory elements of two *LFY* homologs in soybean.

(A) Genomic sequence of soybean *LFY* homologs consist of 3 exons (blue boxes), 2 intros (black lines) and 3’ UTR (red boxes). (B) Peptide sequence alignment analysis showed that two *LFY* homologs have 96.27% similarity. Black backgrounds indicate identical peptides. Grey backgrounds indicate weaker conservation of peptides. (C) The locations of ten most highly duplicated *cis*-regulatory elements [carbon metabolism (DOFXOREZM: 5’-AAAG-3’), light-responsive (GATABOX: 5’GATA-3’, GT1CONSENSUS: 5’-GRWAAW-3’, EBOXBNNAPA: 5’CANNTG-3’), cytokinin signalling (ARR1AT: 5’-NGATT-3’), plant tissue specific {pollen-specific (POLLEN1LELAT52: 5’-AGAAA-3’, GTGANTG10: 5’-GTGA-3’), mesophyll (CACTFTPPCA1: 5’YACT-3’), seed development (CAATBOX1: 5’-CAAT-3’), root apex and vascular organ specific (ROOTMOTIFTAPOX1: 5’-ATATT-3’)}] in the promoter regions of two soybean *LFY* homologs. (D) The frequency of ten most highly duplicated *cis*-regulatory elements were shown. DOFCOREZM is the most abundant and presented 37 to 44 duplication ranges in 2.5 Kb promoter regions of both Glyma.04G202000.1 and Glyma.06G163600.1, respectively. The light-responsive CREs including GT1CONSENSUS, CACTFTPPCA1, GATABOX are also highly duplicated in both soybean *LFY* homologs.

4.3.2 Phylogenetic analysis of two homologs of *LFY* gene in *Glycine max* L. (cv. Bragg)

LFY gene encodes a transcription factor and has been mostly presented in single copy in terrestrial plants from moss to angiosperm (Bomblies et al., 2003; Maizel et al., 2005). In soybean genome database (phytozome v12.1), two homologs of *LFY* gene (Glyma.04G202000.1 and Glyma.06G163600.1) were annotated. To examine the relationship of soybean *LFY* homologs to different species, a total of 38 *LFY* peptide sequences from 29 plant species were used to construct a phylogenetic tree. *LFY* homologs were classified into four clades (fern, gymnosperm, eudicot and monocot) showing that *LFY* gene was differentiated from lower (fern) to higher (monocot) plants.

Two homologs of soybean *LFY* and *LFY* homologs in Fabaceae family (*Medicago truncatula, Phaseolus vulgaris, Trifolium paratense*) are clustered within the same clade (Figure 4.3).

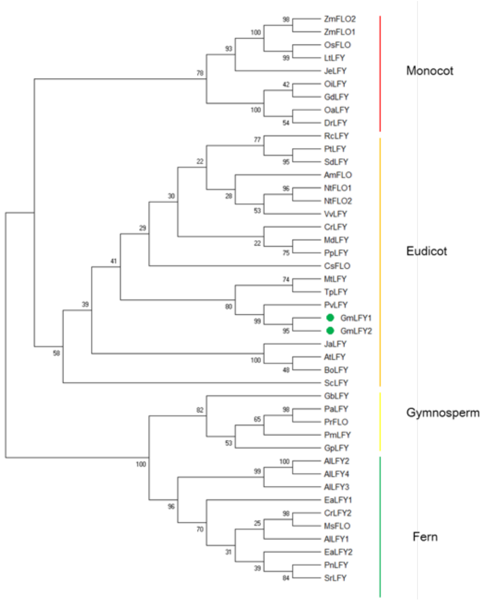


Figure 4. 3. Phylogenetic analysis of soybean *LFY* homologs.

Phylogenetic tree was constructed using MEGA X with neighbour-joint-analysis, 1000 replicates bootstrap. Amino acid sequences were obtained from NCBI databases, Arabidopsis database (TAIR 10) and phytozome databases (v12.1). The bootstrap values are shown at each node. The abbreviated species names were used in this figure. The abbreviations and accession number: *Silene coeli-rosa LFY* (ScLFY, CAC86163.1), *Sceptridium robustum LFY* (SrLFY, BAB88864.1), *Psilotum nudum LFY* (PnLFY, BAB88863.1), *Ceratopteris richardii LFY* (CrLFY2, BAB41070.2), *Matteuccia struthiopteris FLO* (MsFLO, AAF77608.1), *Equisetum arvense LFY* (EaLFY2, BAB90844.1; EaLFY1, BAB88866), *Angiopteris lygodiifolia LFY* (AlLFY4, BAB93543; AlLFY3, BAB88869; AlLFY2, BAB88868; AlLFY1, BAB88867), *Podocarpus matudae LFY* (PmLFY, AAY35059), *Picea abies LFY* (PaLFY, AAV49504), *Gnetum parvifolium LFY* (GpLFY, BAA93436), *Dactylorhiza romana LFY* (DrLFY, BAC55083), *Orchis anthropophora LFY* (OaLFY, BAC55072), *Orchis italica LFY* (OiLFY, BAC54955), *Juncus effusus LFY* (JeLFY, AAF77077), *Lolium temulentum LFY* (LtLFY, AAG41992), *Zea mays FLO* (Zm FLO1, NP_001105201; ZmFLO2, AAV68220), *Oryza sativa FLO* (OsFLO, BAA21547), *Brassica oleracea LFY* (BoLFY, Q05536), *Jonopsidium acaule LFY* (JaLFY, AAF00503), *Cucumis sativus FLO* (CsFLO, AAC64705), *Malus* x *domestica LFY* (MdLFY, BAD10949), *Vitis vinifera LFY* (VvLFY, XP_002284664), *Antirrhinum majus FLO* (AmFLO, P23915), *Nicotiana tabacum FLO* (NtFLO1, Q40504; NtFLO2, Q40505), *Citrus reticulata LFY* (CrLFY, ABJ97283), *Salix discolor LFY* (SdLFY, AAO73539), *Populus trichocarpa LFY* (PtLFY, AAO53547), Trifolium paratense *LFY* (TpLFY, Tp57577), Phaseolus vulgaris *LFY* (PvLFY, Phvul.009g160900.1), Medicago truncatula *LFY* (MtLFY, Medtr3g098560.1), *Ricinus communis LFY* (RcLFY, XP_002511083), *Arabidopsis thaliana LFY* (AtLFY, AT5G61850), *Glycine max* L. *LFY* (GmLFY1, Glyma.06G163600.1; GmLFY2, Glyma.04G202000.1). Two homologs of soybean *LFY* are clustered within the same clade.

4.3.3 Analysis on highly conserved two domains in *LFY* gene

LFY gene has two highly conserved domains (N- and C-domain) which are involved in binding to downstream genes of *LFY* (Sayou et al., 2016). N-domain is a Sterile Alpha motif (SAM) consisting of five α-helices connected with four loops (Figure 4.4) and plays important roles in oligomerisation and cooperative binding to target genes in *Ginkgo biloba LFY* homolog (*Gb LFY*) (Sayou et al., 2016). To examine whether this domain is conserved in soybean *LFY* homologs, sequence alignment analysis was performed. High conservations of N-domain were observed in soybean *LFY* homologs (Figure 4.4A). Two soybean *LFY* homologs (Glyma.04G202000.1 and Glyma.06G163600.1) have identical N-domain (Figure 4.4). This highly conserved N-domain in soybean suggests that this domain in *LFY* homologs of soybean may have similar functions in oligomerisation and cooperative binding to target genes. The Sterile Alpha motif domain is made up of mid-loop (ML) and end-helix (EH) surfaces which are related to the formation of oligomers (Kim et al., 2001). Three negative and polar residues (T72, T75 and E83) on the ML surface and two amino acids (R112 and R116) on the EH surfaces have effects on oligomerisation efficiency and cooperative DNA-binding (Sayou et al., 2016). In sequence alignment analysis, no peptide substitutions at key amino acids (T72, T75, E83, R112 and R116) in soybean *LFY* homologs were observed (Figure 4.4B). Moreover, the peptides which are involved in interactions with Sterile Alpha motif monomers via lateral and the main chain in protein were also highly conserved in soybean *LFY* homologs (Figure 4.4B). However, amino acid substitutions on two amino acids which are involved in the interaction between Sterile Alpha motif monomer (P55A: main chain interaction, G78D: lateral chain interaction) were observed in soybean *LFY* homologs (Figure 4.4B). These amino acid replacements may suggest the alteration on oligomerisation of *LFY*.

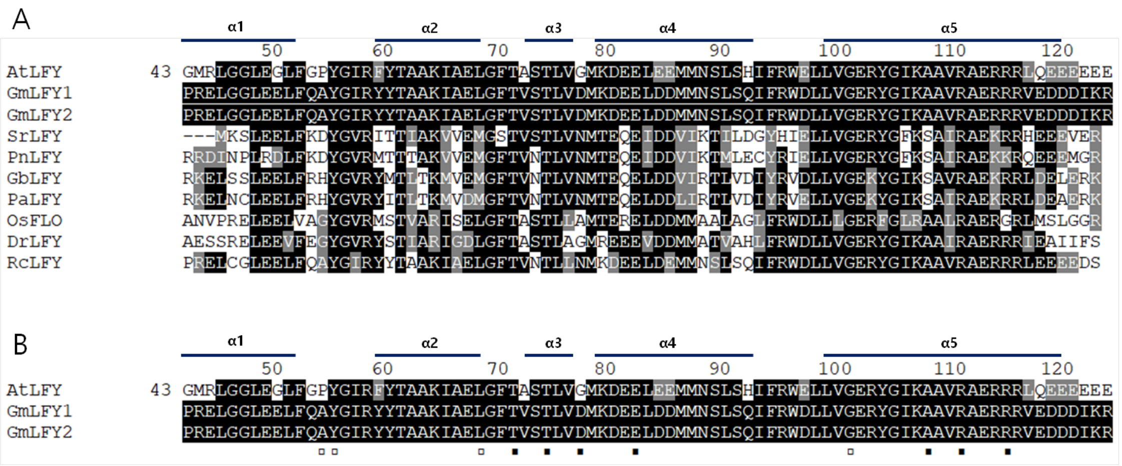


Figure 4. 4. Sequence alignment analysis on *LFY* N-domain.

(a) sequence alignment analysis of *LFY* homologs from moss to angiosperm showed high conservation of N-domains. (b) N-domains of soybean *LFY* homologs are very similar to Arabidopsis' *LFY* N-domains. Amino acid substitutions in key components in the formation of lateral and main chain were examined (P55A and G78D). The amino acid numbering refers to AtLFY (Arabidopsis thaliana *LFY* sequence). Secondary structures (α1-α5) were observed on the sequence alignment. The key amino acids involved in the formation of secondary structures appeared in rectangular shapes under the sequence; filled rectangles: lateral chain formation (T72, T75, G78, E83), empty rectangles: main chain formation (P55, Y56, L69) (Sayou et al., 2016). Abbreviations and accession numbers: *Sceptridium robustum LFY* (SrLFY, BAB88864.1), *Psilotum nudum LFY* (PnLFY, BAB88863.1), *Picea abies LFY* (PaLFY, AAV49504), *Ginko biloba LFY* (GbLFY AAF77075.1), *Arabidopsis thaliana LFY* (AtLFY, AT5G61850), *Ricinus communis LFY* (RcLFY, XP_002511083), *Oryza sativa FLO* (OsFLO, BAA21547), *Dactylorhiza romana LFY* (DrLFY, BAC55083), *Glycine max L. LFY* (GmLFY1, Glyma.06G163600.1; GmLFY2, Glyma.04G202000.1)

The C-domain of *LFY* (LFY-C) was identified as a DNA-binding domain (DBD) (Hamès et al., 2008). In Arabidopsis, LFY-C is made up of two beta-strands, β-1 and β-2, followed by seven alpha helices, α1 to α7 (Figure 4.5) (Hamès et al., 2008). To examine the differences in LFY-C structures between Arabidopsis *LFY* and soybean *LFY* homologs, LFY-C sequences were compared. The sequence alignment results showed that soybean *LFY* homologs had highly conserved LFY-C domains (Figure 4.5). Most of key amino acids (E235, T240, R277, K280, N287, R291, N302, P304, K305, Y309, R327, H384 and R387) which are critical elements in interactions with DNA-base, backbone of DNA and dimerization, respectively, were well conserved in soybean *LFY* homologs (Figure 4.5). These results indicate that two soybean *LFY* homologs have similar structures to recognise and bind target site of downstream genes of *LFY* in soybean. In addition to mutations in DNA-bindings, other mutations that have an effect on the formation of secondary protein structure were also examined to investigate the differences in structure-function of soybean *LFY* genes. Glyma.04G202000.1 has a shortened subset of amino acid in its exon 2 (131 bp) compared with that of Glyma.06G163600.1 (389 bp), resulting in shortened peptides in this region. In sequence alignment analysis, deletions of amino acids in the region which has an effect on the formation of secondary structure (Arg233, Glu234 and Pro236) was detected in Glyma.04G202000.1 suggesting that these peptide deletions may have an effect on the structure of protein, resulting in the affinity of DNA-binding (Figure 4.5). In contrast, Glyma.06G163600.1 has mostly similar C-domains with Arabidopsis and no differences were observed in key residues which are involved in DNA-binding affinity (Figure 4.5). This structural similarity of C-domain in Glyma.06G163600.1 suggested it could be a candidate for the *LFY* gene in soybean.

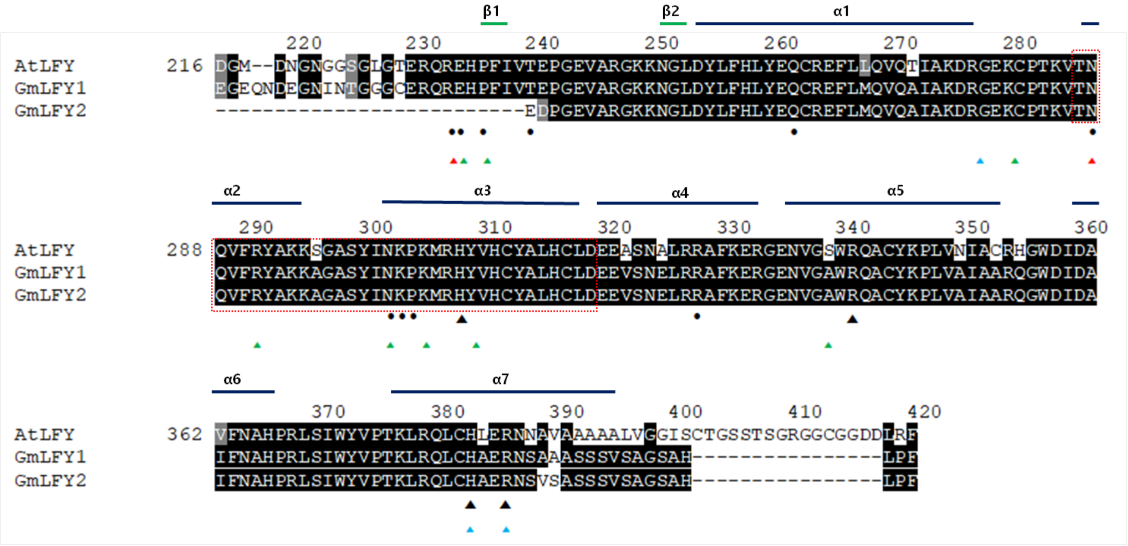


Figure 4. 5. Comparisons of conserved key amino acids of *LFY* C-domain in soybean homologs.

Sequence alignment analysis of the *LFY* C-domain showed high conservation of peptides in both soybean *LFY* homologs and Arabidopsis *LFY*. The amino acid numbers refer to Arabidopsis *LFY* sequence. *LFY* C-domain is comprised of two beta-strands, β-1 and β-2, and seven alpha helices, α1 toα7. The key amino acids involved in Arabidopsis *lfy* mutants and DNA-binding affinity are indicated under the sequences. Filled circles show identified mutations in Arabidopsis *LFY* (R234: *lfy-4*, P236L: *lfy-5*, T240M: *lfy-3*, N302D: *lfy-20*, P304L: *lfy-28*) and the amino acids involved in DNA-binding affinity. Filled triangles indicate that key amino acids are responsible for DNA-binding. Red triangles show residues involved in DNA-based interaction. Green triangles show the residues having an effect on backbone interaction. Blue triangles show the residue is involved in dimerization. No differences in identified key amino acids (Hamès et al., 2008) were detected between Glyma.06G163600.1and Arabidopsis *LFY*. The absences of residues in beta-strand 1 region (Arg233, Glu234 and Pro236) were detected in Glyma.04G202000.1).

4.3.3 Analysis of expression patterns of two *LFY* homologs (Glyma.04G202000.1 and Glyma.06G163600.1) in soybean

In previous studies using transcriptome analysis of soybean shoot apical meristem (SAM), expressions of two soybean *LFY* homologs were detected in flowering inductive conditions (short day, SD) (Figure 4.6A) (Wong et al., 2013). To confirm the temporal expression patterns of soybean *LFY* genes, expression levels of two *LFY* homologs of soybean were investigated across different flowering inductive periods (short day, SD 0, 1, 2, 4 and 6). Expression of soybean *LFY* homologs increased under flowering inductive conditions and the expression level of Glyma.06G163600.1 was higher than Glyma.04G20200.1 under flowering inductive conditions (Figure 4.6B). In SD 0 and SD 1, expression levels of both *LFY* homologs were similar and the expression of Glyma.06G163600.1 was slightly higher (SD 0: 1.2 fold, SD 1: 1.3 fold) than that of Glyma.04G202000.1 (Figure 4.6B). The differences in levels of expression became striking from SD 2. Glyma.06G163600.1 showed 1.6 fold higher expression than Glyma.04G202000.1 on SD 2. The difference in expression levels peaked at SD 4 (Glyma.06G163600.1 showed 2.0 fold higher expression than Glyma.04G202000.1), the difference reduced slightly on SD 6 (Glyma.06G163600.1: 1.5 fold higher expression) (Figure 4.6B). These expression patterns of two soybean *LFY* homologs are similar to the previously reported transcriptome data (Wong et al., 2013). Although two soybean *LFY* homologs are expressed under flowering inductive conditions, the expression levels of Glyma.06G163600.1 was higher than that of Glyma.04G20200.1. In addition, Glyma.06G163600.1 has two conserved DNA-binding domains of *LFY*, whilst Glyma.04G202000.1 has a smaller size of exon 2 than Glyma.06G163600.1, resulting in loss of amino acids in beta sheet of C-terminal region. Taken together, the focus of this study is the functional analysis of Glyma.06G163600.1. This gene is designated as *GmLFY1* hereafter.

For precise examination of spatial and temporal expression of *GmLFY1* (Glyma.06G163600.1), in-situ hybridisation was carried out. Wild-type soybean plants (cv. Bragg) were grown in long day conditions for 10 days, and then transferred to short days. Shoot apex was dissected from plants before the shift (SD 0) and at SD 1, 2, 4, 6, 8, and 10 days after transfer. Samples were embedded and were hybridised with DIG-labelled RNA *GmLFY1* probe. In coincidence with temporal expression, expression of *GmLFY1* was observed across flowering inductive conditions (Figure 4.7). *GmLFY1* was expressed in the peripheral regions of SAM and leaf primordia and the expression levels of *GmLFY1* increased under flowering inductive conditions. On SD 0, expression of *GmLFY1* was observed in the flank of SAM and leaf primordia (Figure 4.7A). The expression of *GmLFY1* was confined to the top of leaf primordia on SD 1 and 2 (Figure 4.7 B, C). Expression of *GmLFY1* became strong on SD 4. The expression expanded over the entire area of leaf primordia and *GmLFY1* expression in the peripheral regions of SAM had increased (Figure 4.7D). It was reported that the formation of undifferentiated axillary meristem near trifoliate primordia is the evidence that soybean plants has transitioned to reproductive phase (Washburn and Thomas, 2000). This axillary meristem was observed on SD 6 and suggested that shoot apical meristem had transformed into inflorescence meristem. Moreover, the spatial expression of *GmLFY1* had changed on SD 6. Strong expression was observed at the top of leaf primordia and moderate levels of *GmLFY1* expression was detected in both the flank region of SAM and undifferentiated axillary meristems near young leaf primordia (Figure 4.7E). These expression patterns became more evident on SD 8. The expressions of *GmLFY1* were detected in the upper layers of SAM and axillary meristems (Figure 4.7F). The strongest signal was detected on floral meristems (FM) in SD 10. The expression of *GmLFY1* was observed across the whole area of floral meristem (Figure 4.7G, H). These temporal and spatial expression patterns of Glyma.06G163600.1 suggested that it is involved in development of floral meristem under flowering inductive conditions.

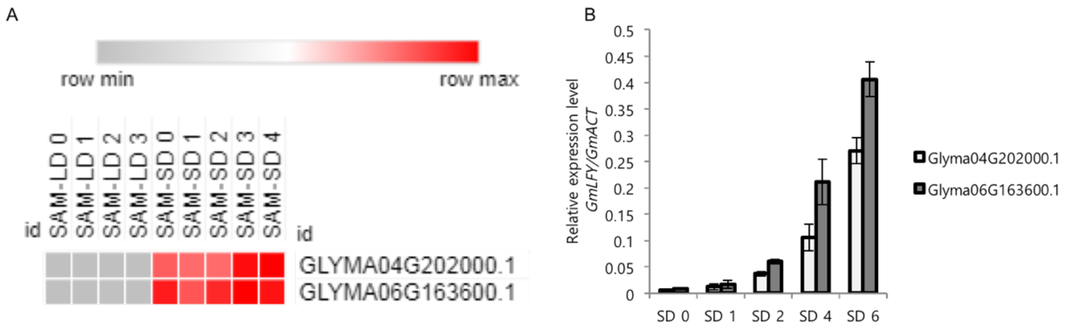


Figure 4. 6. Analysis of expression profiles and patterns of soybean *LFY* genes.

(A) Profile of expression of two soybean *LFY* homologs (Glyma.04G202000.1 and Glyma.06G163600.1) in shoot apical meristem (SAM) during flowering inductive conditions (short day, SD 0 to SD 4). Transcriptome data was obtained from a published paper (Wong et al., 2013). A heat map was constructed using Morpheus (https://software.broadinstitute.org/morpheus/). (B) qRT-PCR analysis was performed on SAM to confirm the expression patterns of soybean *LFY* homologs. Samples were derived from shoot apical meristem of soybean were used at different flowering inductive time periods (short day: SD 0, 1, 2, 4, 6). n=15. Expression of Glyma.04G202000.1 and Glyma.06G163600 were increased during flowering inductive condition. Glyma.06G163600 exhibited strong expression levels (1.2 to 2.0 fold) than Glyma.04G202000.1. The qRT-PCR data was obtained from three biological replicates.

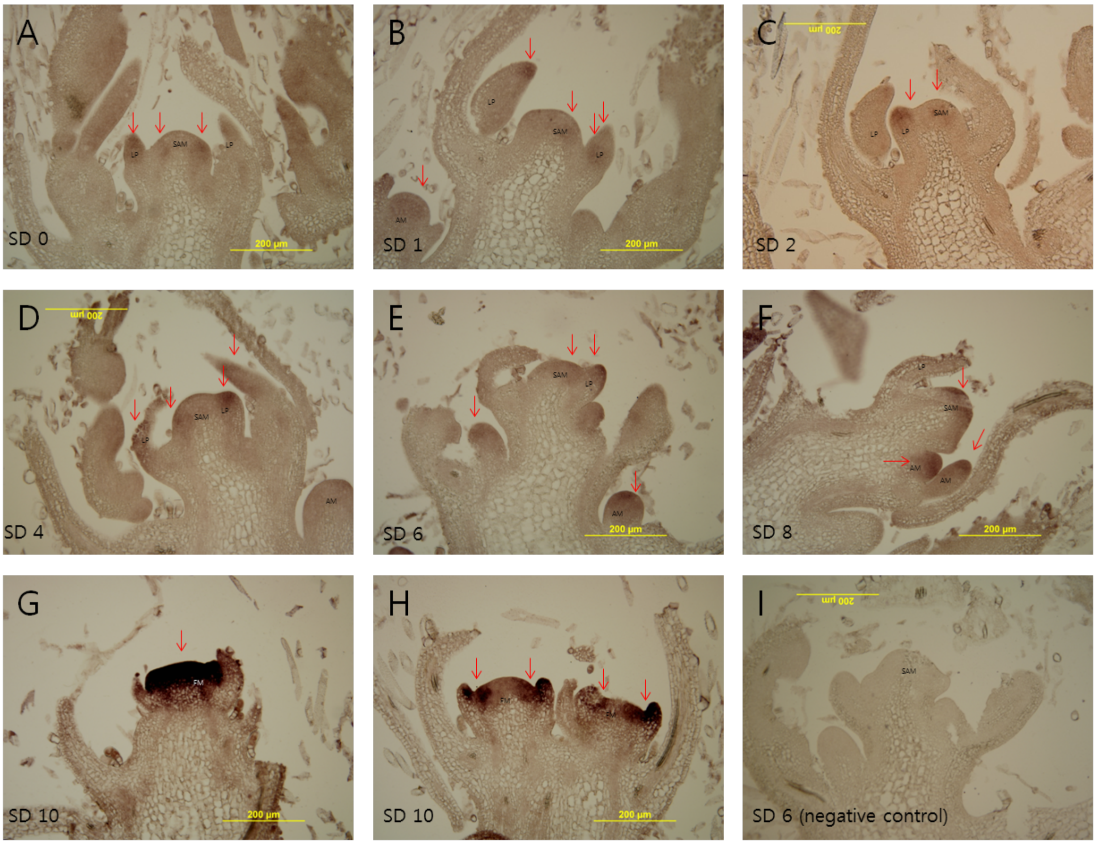


Figure 4. 7. Examination of expression of *GmLFY1* (Glyma.06G163600.1) using in situ hybridization at soybean shoot apical meristem (SAM).

In situ hybridization was conducted to examine temporal and spatial expression of *GmLFY1* on SAM. The signal of expression of *GmLFY1* is indicated in red arrows. (A) Expression of *GmLFY1* was detected on peripheral regions of SAM and leaf primordia on SD 0. (B) and (C) the concentrated expression of *GmLFY1* was observed at the upper layers of leaf primordia on SD 1 and 2. Weak expression of *GmLFY1* was detected on the centre region of SAM on SD 1 and 2. (D) Increased expression of *GmLFY1* was detected on leaf primordia and SAM on SD 4. (E) Expression of *GmLFY1* was increased at axillary meristem and peripheral regions of SAM on SD 6. (F) Strong expression of *GmLFY1* was detected in upper layers of SAM and leaf primordia on SD 8. (G) Strong expression of *GmLFY1* was detected on floral meristem on SD 10. (H) Strong expression of *GmLFY1* was detected from peripheral regions of SAM and upper layers of leaf primordia on SD 10. Moderate expression of *GmLFY1* was also detected in the middle part of SAM on SD 10. (I) No signal was detected on negative control (sense probe of *GmLFY1*, SAM on SD 6). GmLFY1 expression was detected in the peripheral region of SAM and leaf primordia from SD 0. The expression increased during SD. Strongest expression of *GmLFY1* was detected on floral meristem.

4.3.3 Ectopic expression of *GmLFY1* in Arabidopsis inducing early flowering

To determine whether *GmLFY1* is involved in the regulation of flowering initiation, two model plants for genetic analysis, Arabidopsis and tobacco, were used in this study. Firstly, transgenic Arabidopsis plants harbouring overexpressed *GmLFY1* were produced. T0 and T1 generations of transgenic plants were selected using basta spray selection. After the application of basta solution (200 mg/L), transgenic plants survived and continued to grow, whilst non-transgenic and wild-type plants died after 1 week of basta application (Figure 4.8A, B). In T1 generation, 20 transgenic lines were randomly selected and were examined for flowering time. All T1 lines started flowering earlier (35.9±1.18 days) than wild-type (42.6±0.60 days) (Figure 4.8C and 4.9).

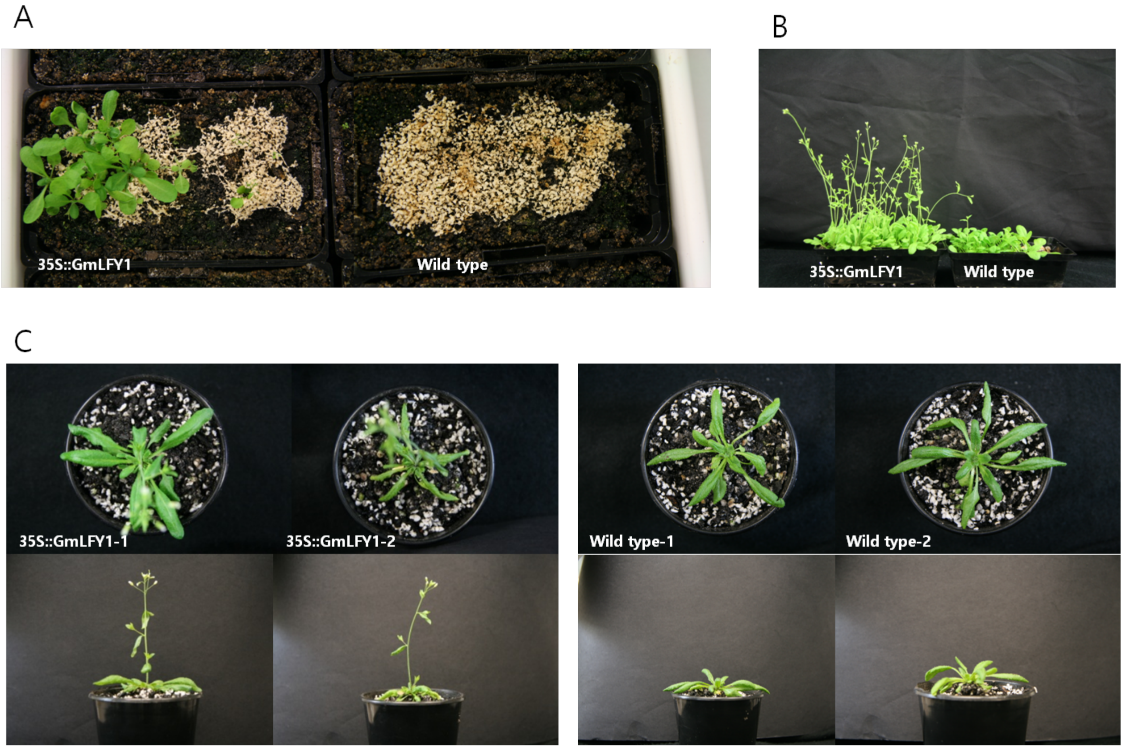


Figure 4. 8. Screening of *GmLFY1* overexpression Arabidopsis transgenic plants.

(A) Transgenic plants survived after basta application (200 mg/L). Transgenic plants (5 weeks old) showed normal growth (green plants), whilst non-transgenic and wild-type plants died (yellow plants). (B) Primary *GmLFY1* overexpression transgenic plants (T0 generation) exhibited early flowering phenotypes. (C) T1 transgenic plants started flowering earlier than wild-type. Plants were grown in a plant growth chamber (16/8h, 150 μmol/m^{-2}s^{-1}, 25±1℃).

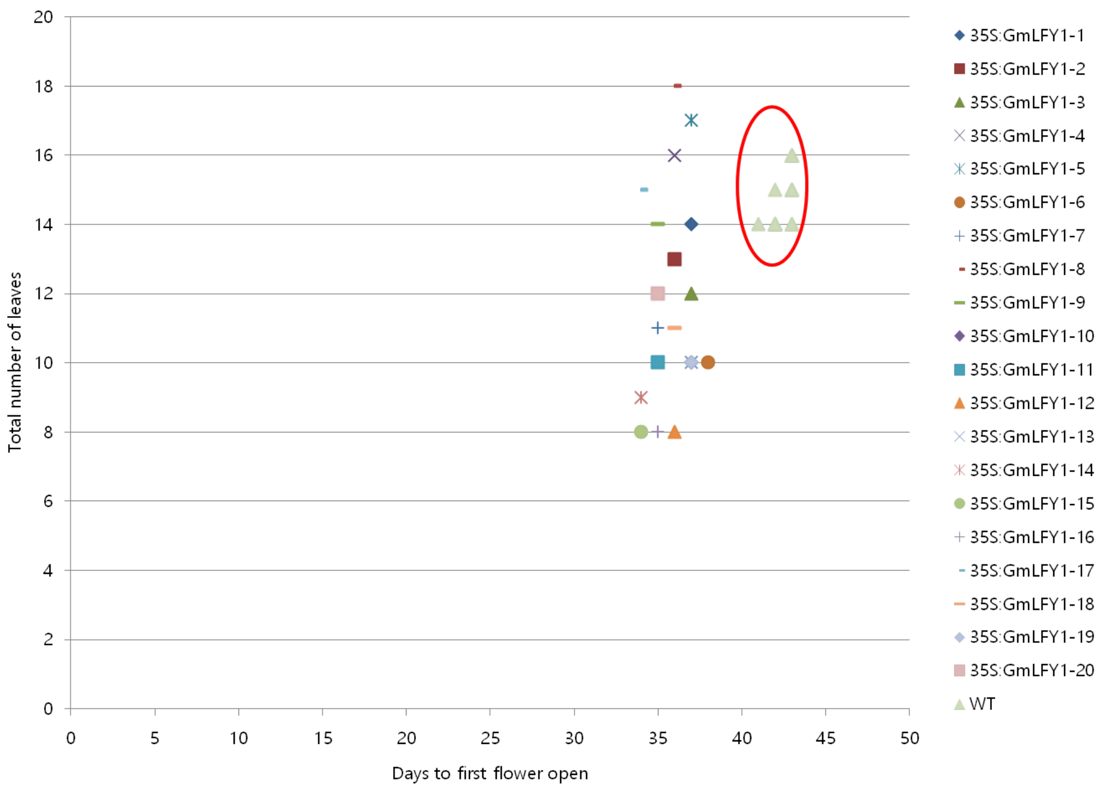


Figure 4. 9. Ectopic expression of *GmLFY1* in Arabidopsis promotes initiation of flowering in T1 generation.

Flowering time measurements of wild-type (WT) and 20 lines of T1 transgenic Arabidopsis plants are presented in a scatter plot. Transgenic plants showed early flowering phenotypes. Red circle: wild-type, blue circle: transgenic Arabidopsis plants overexpressing *GmLFY1* (T1 generation). Plants were grown under LD conditions (16/8h).

After selection of homozygous lines from T1 generation, two homozygous transgenic Arabidopsis lines (lines 14 and 15) were used for flowering time analysis. These homozygous T2 lines started flowering at 33.8±2.23 days (line 14) and 31.3±2.42 days (line 15), whilst wild-type flowered at 39.8±3.60 days under LD condition (16/8h, 150 μmol/m^{-2}s^{-1}, 25±1℃). In phenotype analysis, *GmLFY1* overexpressing transgenic plants had 6 to 7 fewer rosette leaves (line 14: 10.2±0.58 and line 15: 10.1±0.90) than wild-type (14.3±2.22) at 35 days after germination (DAG). In addition, transgenic lines produced fewer branches (line 14: 3.2±1.12 and line 15: 3.2±0.71) than wild-type (4.3±0.75) at 49 DAG (Figure 4.10 and Table 4.1).

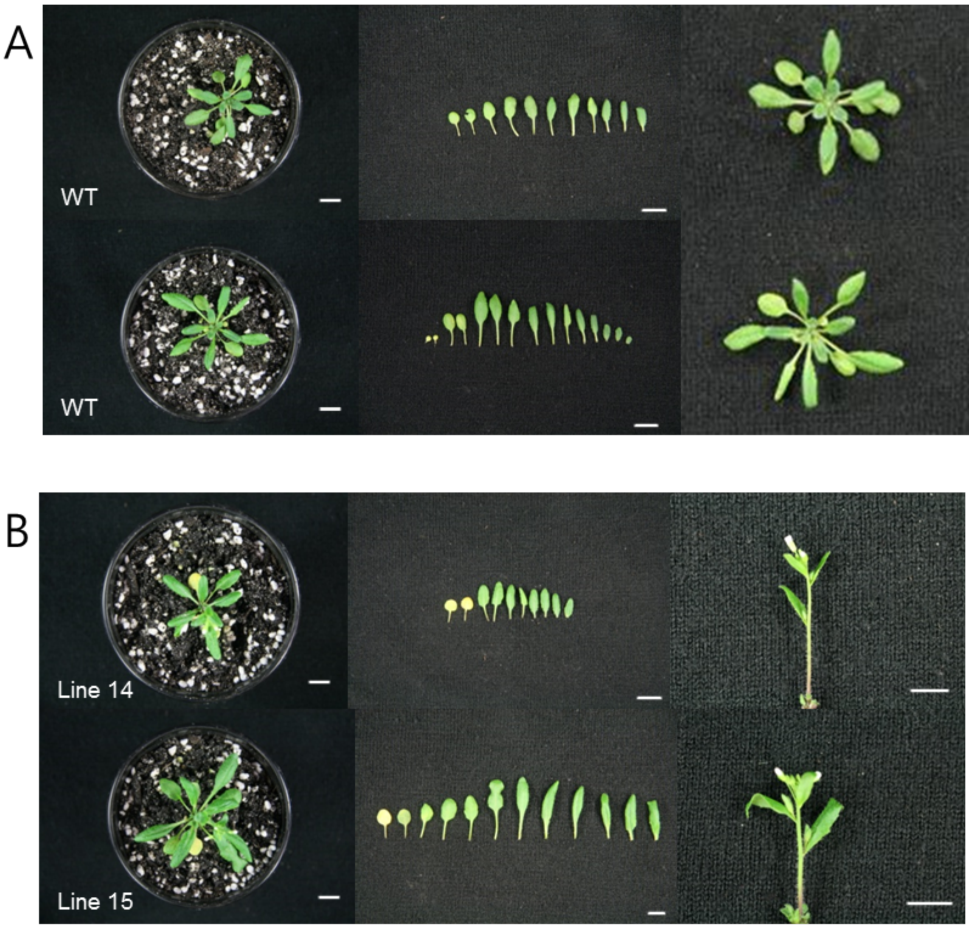


Figure 4. 10. Phenotypes of *GmLFY1* overexpressing transgenic Arabidopsis plants.

(A) 4-week-old wild-type (Col-0) plants in the vegetative phase. (B) Transgenic lines 14 and 15 in the flowering stage. Inflorescence of transgenic plants. Transgenic plants had fewer leaves than wild-type at 5 weeks after germination. White bar: 1cm

Table 4. 1. Ectopic expression of *GmLFY1* in transgenic Arabidopsis plants promotes flowering.

Lines	n	Rosette leaves	Days to first flower open	Height/cm	Branches
WT	15	14.3±2.22	39.8±3.60	30.1±7.00	4.3±0.75
Line 14	15	10.2±0.58	33.8±2.23	28.4±4.32	3.2±1.12
Line 15	15	10.1±0.90	32.6±2.42	31.3±5.11	3.2±0.71

Wild-type (Col-0) and two independent homozygous T2 transgenic Arabidopsis plants grown under LD conditions were examined for phenotype analysis (rosette leaves, day of first flower opening, height and number of branches). The values are presented as the mean ±standard deviation.

4.3.4 Functional analysis of *GmLFY1* in transgenic tobacco plants

4.3.4.1 Production of transgenic plants

To further examine the functions of *GmLFY1*, transgenic tobacco plants harbouring *GmLFY1* overexpression driven by CaMV 35S promoter were produced using leaf disc method. Total 38 putative transgenic plants were regenerated from infected explants on selection mediums (4 mg/L of glufosinate) and 23 plants successfully produced roots on rooting medium. Randomly selected 15 putative transgenic plants were transferred in pots containing sterilized soil for acclimatization. Total 11 acclimatized putative transgenic plants survived in the glasshouse and showed normal growth and no difference in morphology was observed as against wild-type. All putative transgenic plants produced fertile flowers and set seeds.

Copy numbers of transgene were determined by southern blot analysis. Single or double copies of T-DNA insertion were observed in 8 putative transgenic lines. Single T-DNA insertion was observed in 6 independent lines (lines 3, 4, 6, 8, 9 and 10). Two lines (lines 2 and 7) had two copies of transgene in the genome (Figure 4.11). Lines 3 and 8 which displayed single gene insertion were used for further analysis.

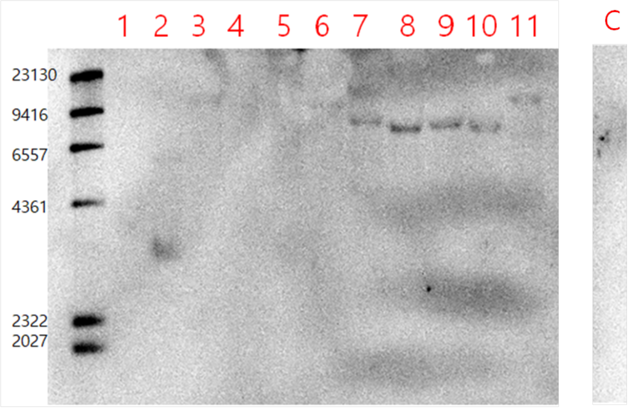


Figure 4. 11. Southern blot analysis of transgenic tobacco plants.

Genomic DNA of 10 independent putative *GmLFY1* transgenic tobacco plants was digested with *BamHI* and was hybridized with DIG-labelled *bar*-specific probe. Single or double copies of gene insertion were detected in transgenic plants. No positive band of gene was detected in non-transformed tobacco plants (wild-type). C: non-transformed control. Ladder: DNA molecular weight marker II (Roche, USA). Two single gene insertion lines (lines 3 and 8) were used for further analysis.

4.3.4.2 Segregation analysis

Inheritance of the T-DNA in the progeny of T0 transgenic plants was examined using herbicide selection test (4 mg/L of glufosinate). Sterilized T1 generation of mature seeds and non-transformed plants (wild-type) were germinated on selection medium (MS basal medium + Sucrose 30 g/L + Agar 7 g + 4 mg/L glufosinate, pH 5.8) in tissue culture room (16/8 h, 150 µmol/m^{-2}s^{-1}, 25±1℃). Herbicide resistant plants were expected to show normal growth on selection medium. After 4 weeks of culture, herbicide resistance was examined. Herbicide susceptible plants turned yellow after germination and failed to grow on selection medium (Figure 4.12). In contrast, basta resistant plants showed normal growth similar to wild-type plants (in non-selection medium: MS basal medium + Sucrose 30 g/L + Agar 7 g, pH 5.8) (Figure 4.12).

In T1 generation, Mendelian inheritance pattern (3:1) was observed in single T-DNA copy inserted lines (lines 3, 6 and 8) (Table 4.2). However, lines 2 and 7 (double T-DNA copies in genome) showed deviations from monogenic segregation. Mature seeds of two T1 lines (lines 3 and 8) were screened on selection medium to discriminate homozygous lines. After 4 weeks of selection, two homozygous from T1 line 8 (designated lines 8-1 and 8-5) were obtained and were used for analysis (Table 4.2).

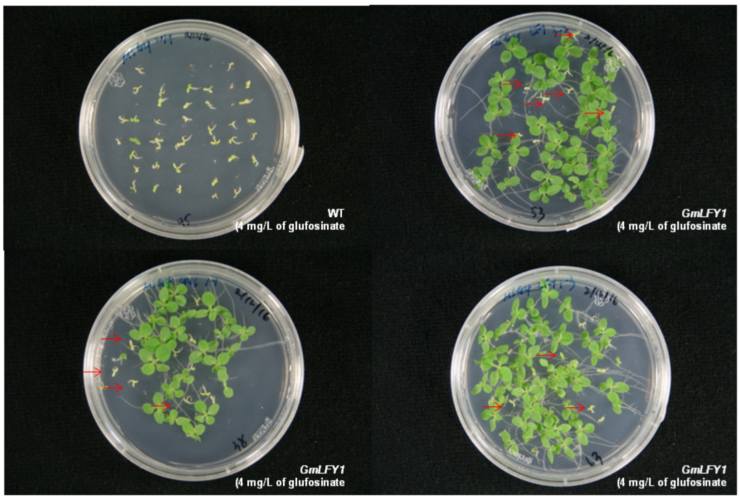


Figure 4. 12. Selection of T1 generation of transgenic tobacco plants harbouring *GmLFY1*.

Herbicide resistant transgenic seedlings showed normal growth on selection medium (glufosinate 4 mg/L). Wild-type and herbicide susceptible seedlings showed stunted growth on selection medium after 4 weeks of culture (Red arrows).

Table 4. 2. Analysis of segregation of transgenic tobacco plants harbouring *GmLFY1*.

Generation	Lines	Copy numbers in T0	Total numbers of resistant plants (G^R)	Total numbers of susceptible plants (G^S)	Segregation ratio	Single gene insertion	χ^2 value
T1	2	2	89	15	5.93:1	3:1	6.01
	3	1	97	31	3.13:1	3:1	0.04 $^{(h.s.)}$
	6	1	102	24	4.25:1	3:1	2.34 $^{(h.s.)}$
	7	2	93	15	6.2:1	3:1	6.88
	8	1	43	10	4.3:1	3:1	1.04 $^{(h.s.)}$
T2	3-2	1	51	36	1.42:1	1:0	25.41
	3-7	1	93	17	5.47:1	1:0	3.11 $^{(h.s.)}$
	8-1	1	79	0	1:0	1:0	0.0 $^{(h.s.)}$
	8-5	1	111	0	1:0	1:0	0.0 $^{(h.s.)}$

Data was obtained after 4 weeks of selection (4 mg/L of glufosinate). G^R: glufosinate resistant plants showed normal growth. G^S: Glufosinate susceptible plants exhibited stunted growth. T1 lines 3, 6 and 8 showed Mendelian inheritance patterns (3:1), (H.S.): significant deviations from monogenic segregation. χ^2 values for 1*df*= 3.84 at 5% *P*-level. (H.S.): significant deviations from monogenic segregation.

4.3.4.3 Phenotype analysis of *GmLFY1* overexpressing transgenic tobacco plants

4.3.4.3.1 Leaf morphology

Leaf shape is one of the most reliable traits for the purposes of distinguishing between vegetative and reproductive phases (Poethig, 1990; Feng et al., 2016). In vegetative development phase, tobacco (*Nicotiana tabacum* L. cv. NC98) leaf is rounder in shape and smaller in size than that of wild-type (Feng et al., 2016). Therefore, leaf shape was examined in both *GmLFY1* transgenic plants and wild-type plants using length/width ratio (L/W ratio). Expected value of a round shape of leaf (i.e. circular shape) is 1 and the value will increase as the shape becomes long and narrow. Leaves of *GmLFY1* overexpressing transgenic plants have narrow shapes compared to wild-type. In T0 and T1 generations, *GmLFY1* transgenic plants had a bigger value of L/W ratio [T0: 3.11±0.56, T1: 1.94±0.15 (line 3), 1.92±0.17 (line 8)] than that of wild-type (T0: 2.9±0.36, T1: 1.86±0.24) (Table 4.3, 4.4). Narrow shape of leaves were also observed in T2 generation (Figure 4.15). Fully expanded leaves of wild-type plants had smaller values (2.6±0.24) than that of *GmLFY1* transgenic plants (line 8-1: 2.9±0.41 and line 8-5: 2.8±0.20) (Figure 4.16A).

To examine the detailed differences in leaf morphology between *GmLFY1* overexpressing transgenic plants and wild-type plants, factors of leaf morphology such as leaf length, leaf width, leaf area, petiole length and petiole width were examined. T0 generation of *GmLFY1* transgenic plants had smaller leaf length (16.6±2.41 cm), width (5.4±0.99 cm) and leaf area (48.1±12.65 cm^2) than that of wild-type (leaf length: 18.9±2.61 cm, leaf width: 6.6±1.34 cm and leaf area: 72.9±21.99 cm^2) (Table 4.3) (Figure 4.13). In contrast, T1 generation of *GmLFY1* transgenic plants possessed bigger leaves than wild-type in leaf length, width and area (Table 4.4). Two transgenic lines with single transgene insertion exhibited longer and wider shape of leaves [length: 12.7±2.53 cm (line 3), 13.5±2.32 cm (line 8), width: 6.6±1.37 cm (line 3), 7.1±1.28 cm (line 8)] than that of wild-type (length: 11.5±2.08 cm, width: 6.3±1.43cm) (Table 4.4). These longer and wider shapes of leaves in *GmLFY1* transgenic *GmLFY1* plants resulted in bigger leaf area of transgenic plants (line 3: 54.0±19.05 cm^2, line 8: 58.0±18.21 cm^2 and wild-type: 45.2±17.01 cm^2) (Table 4.4) (Figure 4.14). In contrast to leaf morphology of T1 generation, leaves of homozygous T2 transgenic tobacco plants (*35S::GmLFY1*) were slightly shorter in length (line 8-1: 12.7±1.11 cm and line 8-5: 13.0±1.22 cm) than wild-type (13.4±1.22 cm). In leaf width, however, *GmLFY1* shows a significantly narrower width (line 8-1: 4.5±0.56 cm and line 8-5: 4.6±0.45 cm) than wild-type (5.2±0.60 cm), contributing to forming a narrower shape of leaves (Figure 4.15, 4.16B, C). In addition, *GmLFY1* had a smaller leaf area (line 8-1: 34.6±4.88 cm^2 and line 8-5: 35.8±8.13 cm^2) than wild-type (42.1±8.29 cm^2) (Figure 4.16D). In petiole morphology, no difference was observed in petiole lengths between *GmLFY1* (line 8-1: 2.35±0.37 cm and line 8-5: 2.41±0.40 cm) and wild-type (2.32±0.37 cm). However, has a narrow petiole width (line 8-1: 0.33±0.07 cm and line 8-5: 0.36±0.10 cm) than wild-type (0.42±0.07 cm) (Figure 4.16E, F). These differences in leaf morphology suggested that *GmLFY1* have an effect on the regulation of flowering initiation resulting in shortened vegetative developmental phase.

Table 4. 3. Morphological analysis of T0 transgenic tobacco plants harbouring *35S::GmLFY1*.

Construct	n	Leaf length (cm)	Leaf width (cm)	L/W ratio	Leaf area (cm^2)	Petiole length (cm)
Wild type	8	18.9±2.61	6.6±1.34	2.9±0.36	72.9±21.99	3.1±0.44
GmLFY1	11	16.6±2.41	5.4±0.99	3.11±0.56	48.1±12.65	2.0±0.56

Data was recorded 7 weeks following transfer to a glasshouse. Fully expanded leaf from each line was used for measurement of leaf length, width, area and petiole length. The values are presented as the mean ±standard deviation.

Figure 4. 13. Leaves of primary transgenic (*35S::GmLFY1*) and wild-type plants.

(A) Represents fully expanded leaves of wild-type and (B) *GmFLY1* overexpressing (T0 generation) transgenic tobacco plants. Leaves of *GmLFY1* overexpressing transgenic tobacco plants have smaller and narrower leaf shapes than that of wild-type. White bar: 10 cm.

Table 4. 4. Morphological analysis of T1 transgenic tobacco plants (*35S::GmLFY1*).

Line	n	Leaf length (cm)	Leaf width (cm)	L/W ratio	Leaf area (cm^2)	Petiole length (cm)	Petiole width (cm)
Wild type	13	11.5±2.08	6.3±1.43	1.86±0.24	45.2±17.01	1.89±0.35	0.40±0.07
Line 3	9	12.7±2.53	6.6±1.37	1.94±0.15	54.0±19.05	1.80±0.26	0.40±0.08
Line 8	9	13.5±2.32	7.1±1.28	1.92±0.17	58.0±18.21	1.70±0.31	0.40±0.09

Data was recorded 4 weeks after transfer to a glasshouse. Fully expanded leaf from each line was used for measurement of leaf length, width, area and petiole length and width. The values are presented as the mean ±standard deviation.

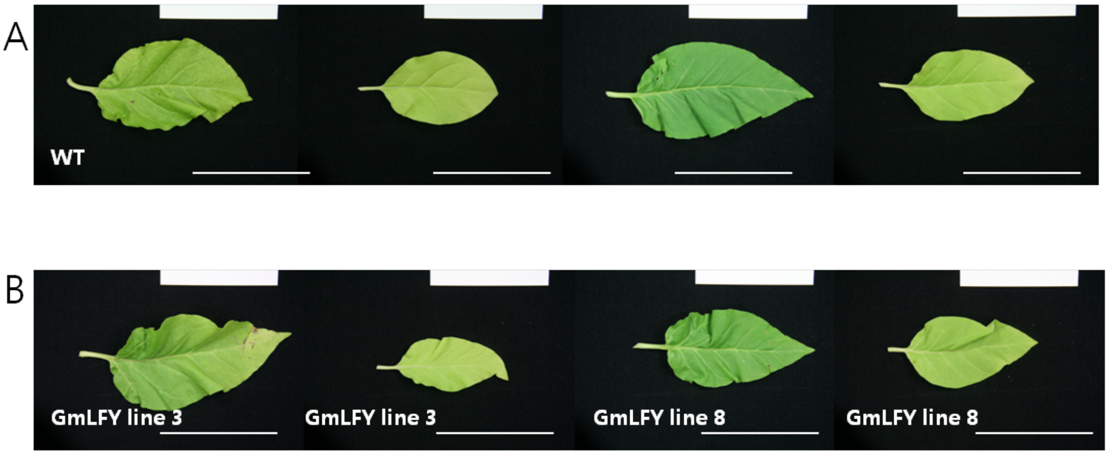


Figure 4. 14. Leaf shapes of *GmLFY1* overexpressing transgenic plants (T1 generation).

(A) Represents fully expanded leaves of wild-type (WT) and (B) GmLFY1 overexpressing (T1 generation, lines 3 and 8) transgenic tobacco plants. Leaves of wild-type plants are bigger and rounder in shape than transgenic tobacco plants (*35S::GmLFY1*). White bar: 10 cm.

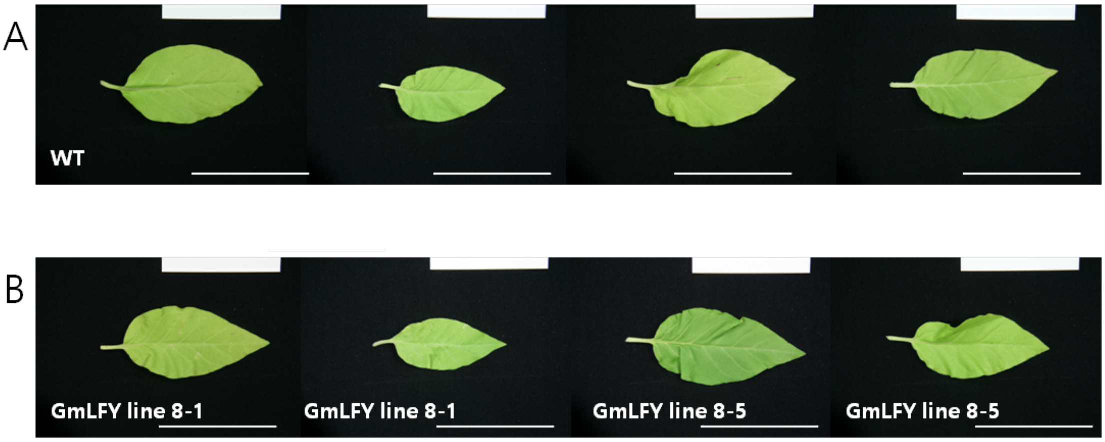


Figure 4. 15. Morphological differences in leaf shape of transgenic tobacco plants (T2 generation).

(A) Fully expanded leaves of wild-type (WT) and (B) *GmFLY1* overexpressing (T2 generation: lines 8-1 and 8-5) transgenic tobacco plants of T2 generation. Leaves of *GmLFY1* overexpressing transgenic tobacco plants have a narrower shape than that of wild-type. White bar: 5cm.

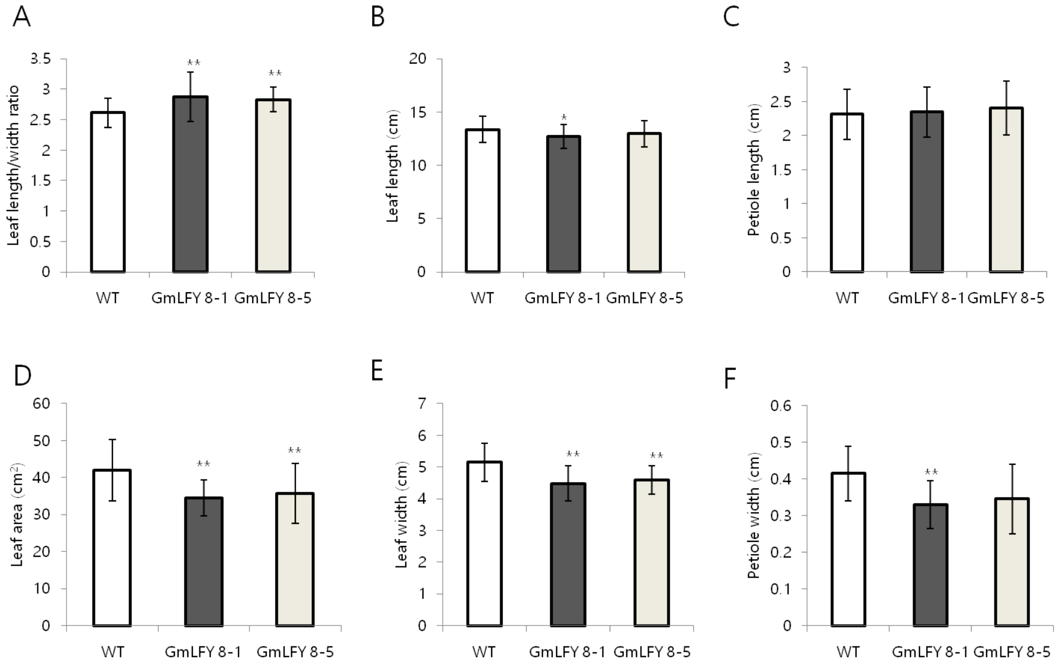


Figure 4. 16. Comparison of length and width ratio of tobacco leaves (T2 generation).

Leaf morphological factors on fully expanded leaves of wild-type plants (WT) and transgenic tobacco plants (lines 8-1 and 8-5) were examined. (A) L/W ratio wild-type and transgenic plants. (B) and (C) length and width of leaves. Transgenic plants had smaller leaves than wild-type. (D) Transgenic plants showed smaller leaf area than wild-type. (E) No significant differences between wild-type and transgenic plants were observed in petiole length. (F) Narrower petiole width was observed in transgenic lines. n=20. Bar: standard deviation (±SD). Significant differences were calculated using student *t*-test. Asterisk: *P>0.05, **P>0.01.

4.3.4.3.2 Determination of total chlorophyll content and photosynthesis rate.

In tobacco plants, leaves in juvenile phase are round in shape with lower concentration of chlorophyll and photosynthesis rate (Feng et al., 2016). To examine whether ectopic expression of *GmLFY1* has an effect on chlorophyll content and photosynthesis rate, both physiological factors were measured in leaves of *GmLFY1* transgenic tobacco plants. Total chlorophyll content and photosynthesis rate were measured on leaves from three locations of plants, apice (top), medial (middle) and basal (bottom). A slight reduction of total chlorophyll content was observed in *GmLFY1* transgenic tobacco plants. Transgenic plants had lower total chlorophyll content [line 8-1 (top: 19.4±3.16, middle: 11.4±2.15, bottom: 6.0±1.73) and line 8-5 (top: 19.8±3.28, middle: 13.3±3.58, bottom: 8.8±3.0)] than that of wild-type (top: 20.36±3.17, middle: 16.0±3.72, bottom: 9.2±2.43) (Figure 4.17A). In contrast, the photosynthesis rate of *GmLFY1* transgenic tobacco plants was similar to wild-type (Figure 4.17B).

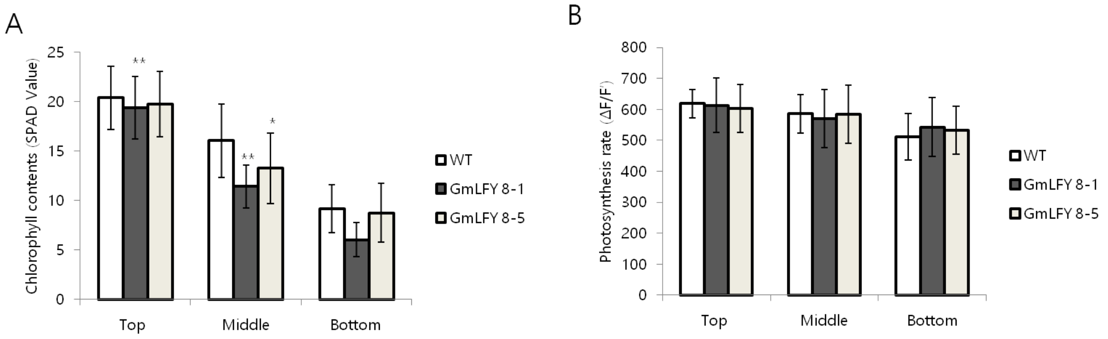


Figure 4. 17. Comparison of total chlorophyll content and photosynthesis rate of transgenic tobacco plants (T2 generation).

(A) Total chlorophyll content and (B) photosynthesis rate were measured at three different points of plants, apical (top), medial (middle) and basal (bottom) leaves. Reductions of total chlorophyll contents were observed in transgenic plants. No significant differences were detected in photosynthesis rate. WT: wild-type plants, homozygous T2 lines (GmLFY 8-1 and GmLFY 8-5), n=20. Bar: standard deviation (±SD). Asterisk: student *t*-test (*P>0.05, **P>0.01).

4.3.4.4 Ectopic expression of *GmLFY1* promotes flowering

LFY gene is one of floral pathway integrator genes and is involved in flowering initiation. Temporal and spatial expression patterns of *GmLFY1* in soybean and the analysis of ectopic expression of *GmLFY1* in Arabidopsis suggested the involvement of

GmLFY1 in the onset of flowering. To confirm the roles of *GmLFY1* in flowering time regulation, days to first flower opening was examined in transgenic tobacco plants which harbour overexpressed *GmLFY1*. Primary transgenic plants did not show early flowering phenotypes (Figure 4.18). Wild-type plants started flowering at 47.8±3.66 days after transfer to a glasshouse whilst *GmLFY1* transgenic plants flowered at 48.1±3.66 days after transfer to a glasshouse (Figure 4.18A, 4.19). However, the first flowering time of *GmLFY1* transgenic plants were more varied (38 to 61 days) than that of wild-type (42 to 48 days). Earliest flowering phenotypes (line 8) started flowering at 38 days after transfer to a glasshouse. In T1 generation, single gene inserted lines (lines 3 and 8) were used for flowering time analysis. After selection with herbicide (4 mg/L), survived plants were transferred to a glasshouse and used for flowering time analysis. T1 generation of *GmLFY1* transgenic plants showed early flowering phenotypes (Figure 4.18B). Two *GmLFY1* transgenic lines started flowering at 68.1±17.16 days after transfer to a glasshouse (line 3) and 67.6±16.52 days after transfer to a glasshouse (line 8) whilst wild-type flowered at 100.9±28.4 days after transfer to a glasshouse (Figure 4.20). In homozygous, T2 generation also showed early flowering phenotypes (Figure 4.21). *GmLFY1* overexpressing transgenic tobacco plants started flowering (line 8-1: 58.5±7.59 DAG and line 8-5: 59.3±7.91 DAG) 14 to 15 DAG earlier than wild-type, at 73.3±9.03 DAG (Figure 4.21, 4.22A). In addition, *GmLFY1* overexpressing transgenic tobacco plants had slightly smaller plant height (line 8-1: 6.5±2.14 cm and line 8-5: 6.7±1.37 cm) but not significantly different from wild-type (7.2±2.06 cm), whilst there was no significant difference in total numbers of leaves of transgenic plants (line 8-1: 9.9±2.03 and line 8-5: 10.1±1.62) and wild-type (9.9±1.97) plants (Figure 4.22B, C). These results indicate that *GmLFY1* plays a role in initiation of flowering.

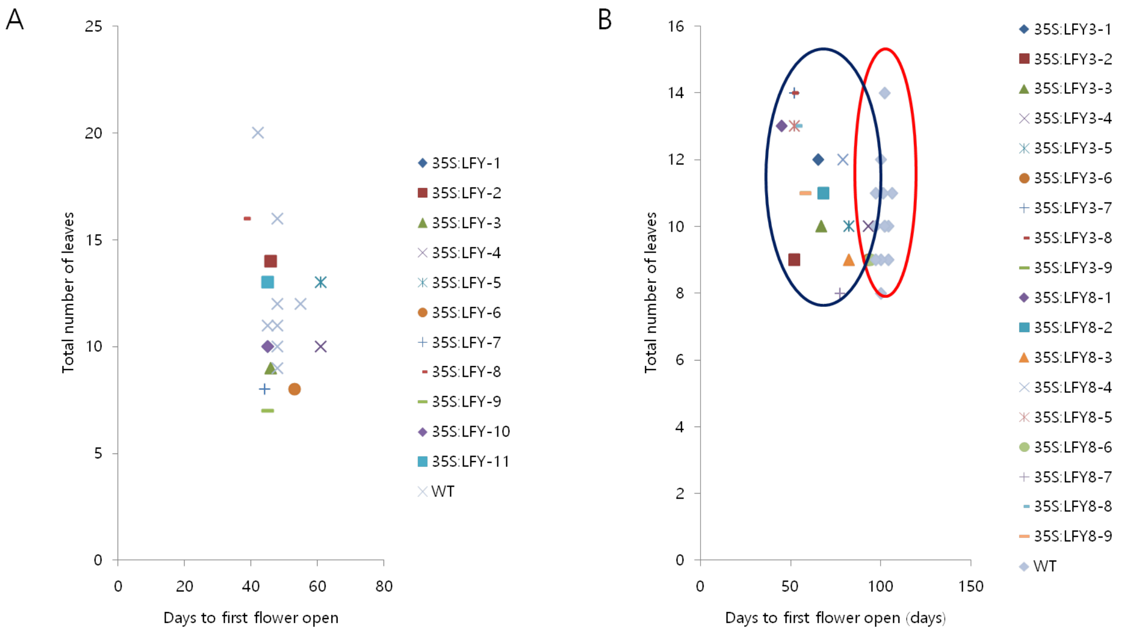


Figure 4. 18. Analysis of the effect of *GmLFY1* overexpression on flowering time in transgenic tobacco plants (T0 and T1 generations).

(A) Flowering time and total leaf numbers were measured in primary transgenic plants (*35S::GmLFY1*) and wild-type (WT). Days of flowering were measured from transfer to glasshouse to first flower opening. Total number of leaves were counted at 7 weeks after transfer to glasshouse. 8 wild-type plants and 11 independent transgenic lines (*35S::GmLFY1*) were used for flowering time analysis. (B) Ectopic expression of *GmLFY1* in transgenic tobacco plants promoted flowering. Two lines of T1 generation (lines 3 and 8) which have single gene insertion in T0 generation were cultivated on selection medium (4 mg/L glufosinate) for 4 weeks. Survived transgenic plants were transferred to soil for further growth. Flowering time was calculated from germination to emergence of first opened flower in T1 generation. n= 13 (wild-type), 9 (transgenic line 3) and 9 (transgenic line 8).

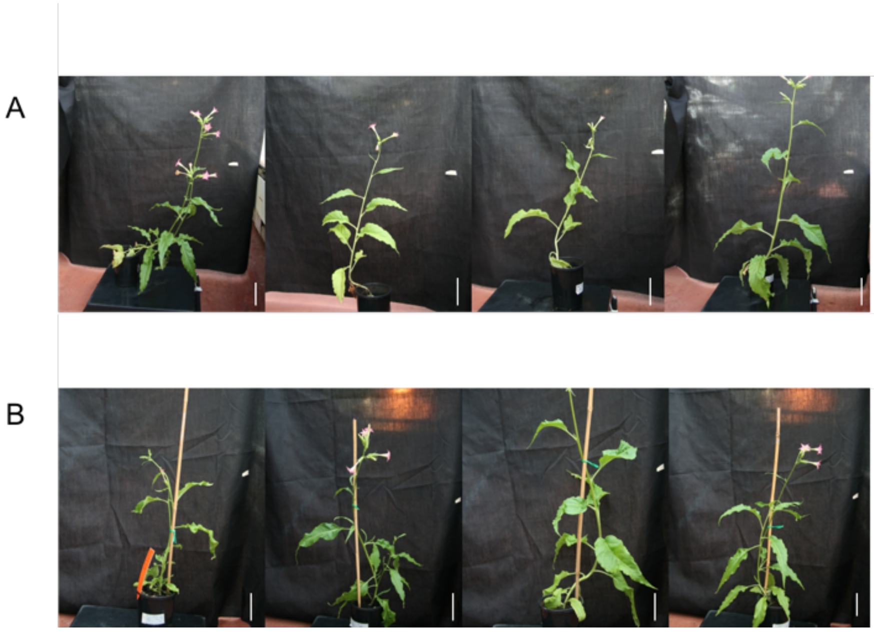


Figure 4. 19. Phenotypes of primary transgenic (*35S::GmLFY1*) and wild-type plants (T0 generation).

(A) Wild-type plants and (B) *GmLFY1* overexpressing transgenic plants started flowering after 6 weeks of acclimatization. No differences in flowering time and flower developments were observed. White bar: 10cm.

Figure 4. 20. T1 generation of *GmLFY1* transgenic tobacco plants showed promoted flowering time.

(A) 10-week-old wild-type plants still in the vegetative developmental phase (left) or have floral bud at shoot apex (right). (B) Transgenic plants overexpressing *GmLFY1* (10-week-old, two transgenic plants of line 3 on left, two transgenic plants of line 8 on right) have inflorescence with fully opened flowers. White bar: 10cm.

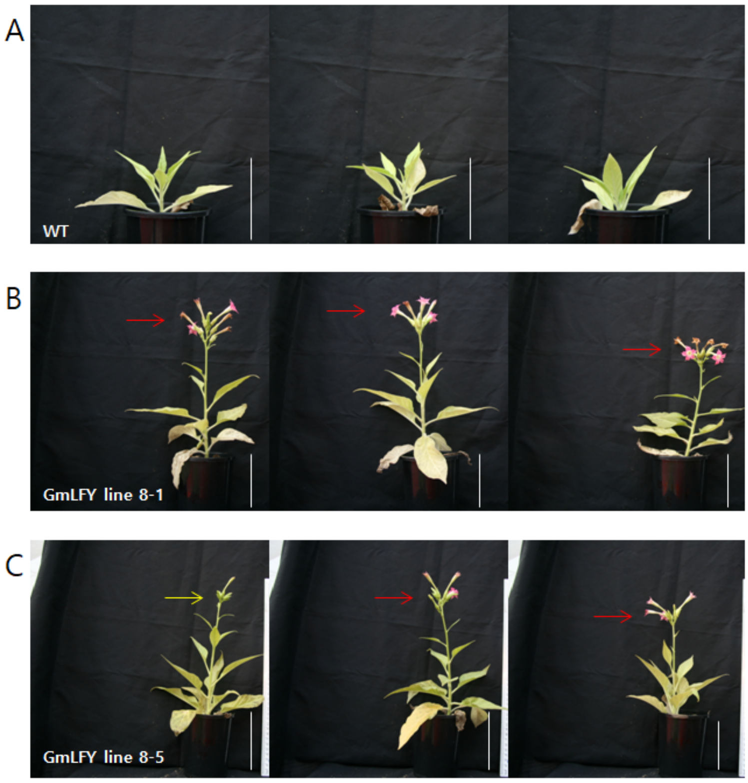


Figure 4. 21. Ectopic expression of *GmLFY1* induced early flowering (T2 generation, homozygous transgenic plants).

(A) Wild-type plants showed normal growth. (B) and (C) ectopic expression of *GmLFY1* in transgenic tobacco plants (line 8-1 and 8-5) caused early flowering. White bar: 10 cm. Red arrows: flowers, yellow arrows: floral buds.

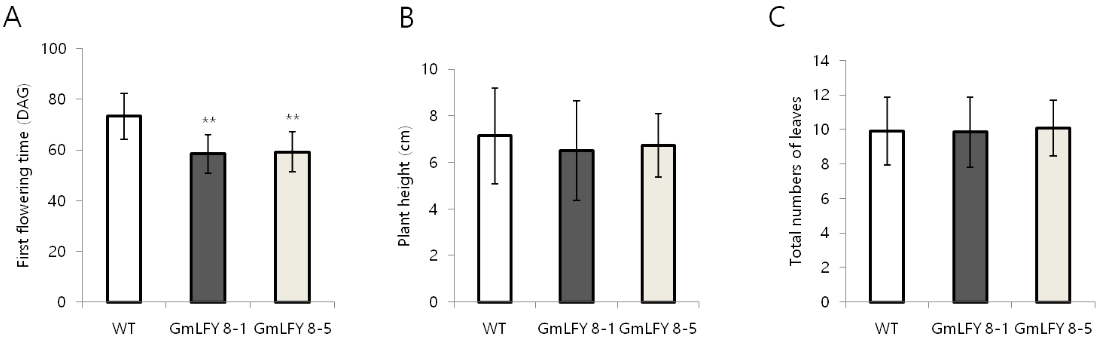


Figure 4. 22. Analysis of flowering time, plant height and leaf numbers of *GmLFY1* overexpressing transgenic tobacco plants.

Ectopic expression of *GmLFY1* in transgenic tobacco plants promoted flowering initiation. (A) Flowering time was recorded from germination to emergence of first open flower. (B) Plant height was measured at base of stem to shoot tip. (C) Total number of leaves was recorded from 5-week-old wild-type and *GmLFY1* transgenic plants. wild-type (WT), homozygous T2 generation transgenic *GmFLY1* plants (line 8-1 and 8-5). n=20, Bar: standard deviation (±SD). Asterisk: student *t*-test (*P>0.05, **P>0.01)

4.3.4.4.1 Flower morphology

To examine whether ectopic expression of *GmLFY1* has any effect on flower development, morphological traits of flowers such as the length and width of flower, sepal and pedicel were measured in flowers from homozygous T2 generation. Flowers of *GmLFY1* overexpressing transgenic plants were slightly smaller than those of wild-type (Figure 4.23). *GmLFY1* transgenic plants were shorter in flower length (line 8-1: 4.7±0.20 cm and line 8-5: 4.8±0.47 cm) and width (line 8-1: 2.8±0.20 cm and line 8-5: 2.7±0.23 cm) than those of wild-type (length: 4.9±0.40 cm, width: 3.0±0.26 cm). In sepal morphology, *GmLFY1* overexpressing lines had shorter sepal lengths (line 8-1: 2.0±0.16 cm and line 8-5: 1.9±0.17 cm) than wild-type (2.1±0.14 cm), whilst *GmLFY1* transgenic plants had wider sepal (line 8-1: 0.78±0.079 cm and line 8-5: 0.75±0.100 cm) than wild-type (0.63±0.06 cm) (Figure 4.23). In addition, morphological differences on pedicel were observed in *GmLFY1* transgenic plants. Transgenic plants had longer but narrower pedicel than wild-type (Figure 4.23). Pedicel lengths of *GmLFY1* transgenic plants were 0.94±0.15 cm (line 8-1) and 0.91±0.17 cm (line 8-5). The width of pedicel of transgenic plants was 0.15±0.05 cm (line 8-1) and 0.16±0.05 cm (line 8-5). On the other hand, pedicel length and width of wild-type plants were 0.85±0.11 cm and 0.2±0.01 cm (Figure 4.23). Although flowers of *GmLFY1* transgenic plants were smaller than that of wild-type, *GmLFY1* overexpressing transgenic plants produced normal flowers suggesting that overexpressing *GmLFY1* has no effect on flower development.

Pollen developments are regulated by combined expression of floral homeotic class B genes. In Arabidopsis, *LFY* gene is involved in the regulation of class B gene by indirect up-regulation of *UFO* (*UNUSUAL FLOWER ORGAN*) which is an upstream gene of *AP3* (*APETALLA 3*) and direct activation of PI (*PISTIALLA*). To investigate whether *GmLFY1* has an effect on pollen development, pollen viability was examined by PI/FDA staining. As FDA stains the membranes of pollen, intact pollens would emit green fluorescence. On the contrary, PI-stained nucleus of cell by infiltration through broken membrane would result in red fluorescence. Therefore, healthy and intact pollens were expected to emit green fluorescence whilst dead pollens were expected to emit red light under the UV light. Fresh pollen grains were obtained from preanthesis flowers of *GmLFY1* overexpressing transgenic plants and wild-type plants. The pollen grains were stained with PI/FDA solution. Strong green fluorescence was detected both wild-type and *GmLFY1* transgenic tobacco plants (Figure 4.24). Pollen viability of *GmLFY1* (line 8-1:

73.4±7.72 % and line 8-5: 76.1±3.24 %) transgenic plants showed high viability similar to wild-type (77.9±6.48 %) (Figure 4.25).

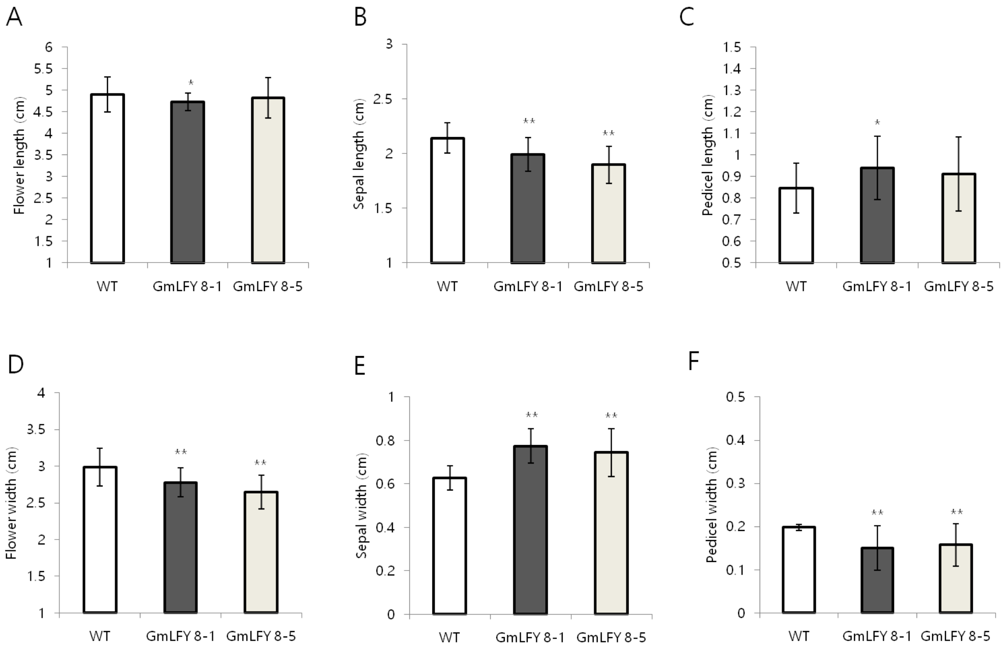


Figure 4. 23. Morphological analysis of flowers (T2 generation).

Differences in flower morphology such as length and width of flowers (A, D), sepals (B, E) and pedicel (C, E) were observed. Flowers from *GmLFY1* overexpressing transgenic tobacco plants showed shortened flower length and width, sepal length and pedicel width. Ten fully opened flowers of wild-type (WT) and homozygous T2 generation transgenic *GmLFY1* plants (line 8-1 and 8-5) were randomly selected from 20 plants. Bar: ±SD (standard deviation). Asterisk: student *t*-test (*P>0.05, **P>0.01).

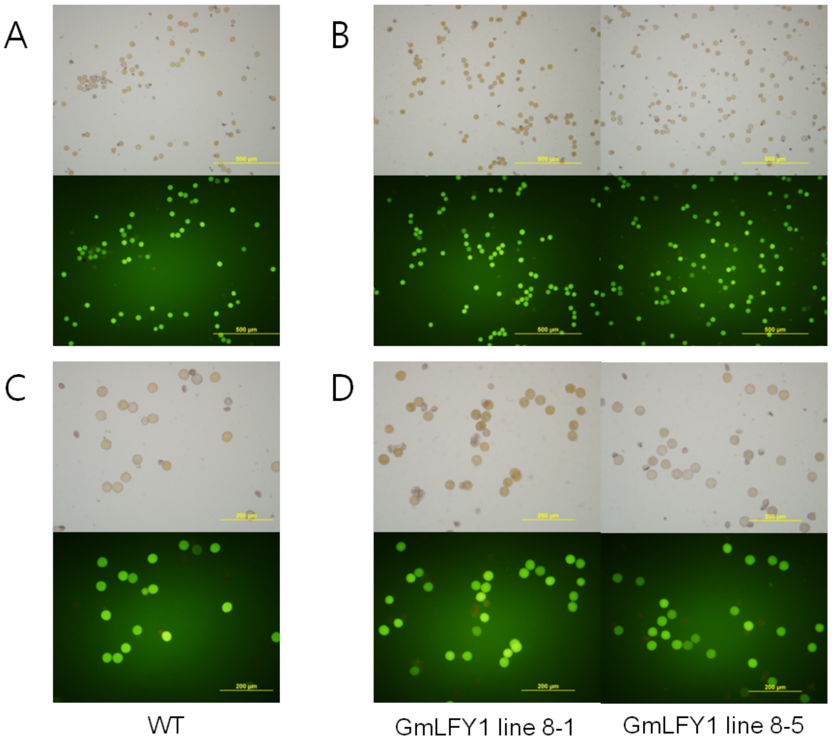


Figure 4. 24. Analysis of Pollen viability of *GmLFY1* transgenic tobacco plants.

Fresh pollen grains of wild-type (A and C) and line 8-5 *GmLFY1* transgenic plants (B and D) were analysed for pollen viability using PI/FDI staining. Stained pollens were examined under a microscope with white light (upper photos with white background) and UV light (lower photos with green background). Green fluorescence indicates viable pollens and red indicates non-viable pollens. Strong green fluorescence was observed both in wild-type and transgenic plants. (A) and (B): 40 x magnification (Yellow bar 500 μm), (C) and (D): 100 x magnification (Yellow bar 200 μm).

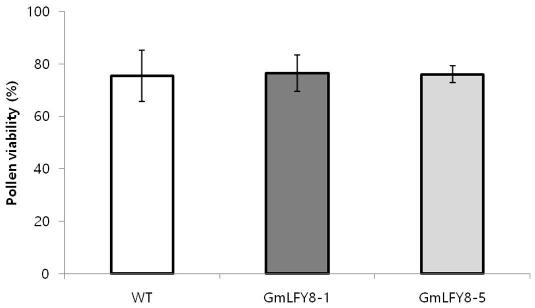


Figure 4. 25. Comparison of pollen viability between wild-type and *GmLFY1* transgenic tobacco plants.

Fresh pollen grains from randomly selected 6 preanthesis flowers of wild-type (WT) and *GmLFY1* transgenic tobacco plants were used. Fertility rate was assessed by (number of sterile pollen/total pollen) x 100. No significant difference in pollen viability (75.6±9.77 % WT), (76.5±6.88 % GmLFY 8-1 and 76.1±3.24 % GmLFY 8-5) were observed. n=471 (WT), 681 (GmLFY 8-1), 377 (GmLFY 8-5).

4.3.4.5 GUS assay

In flowering plants, formation and developmental process of flowers are controlled via a combined interaction between flowering homeotic genes (Bowman et al., 1991). In Arabidopsis, *LFY* acts as an upstream of floral homeotic genes, *AP1* and *AG*, which are involved in sepal and pistil development. To confirm the roles of *GmLFY1* in floral development, putative 5' regulatory region of *GmLFY1* (2.5Kb upstream from ATG) was isolated and fused with *GUS* gene (*LFY::GUS::OCS*). Flowers from transgenic tobacco plants harbouring *LFY::GUS::OCS* constructs were used for *GUS* assay. Strong *GUS* expression was detected mainly in sepals of flowers in different developmental stages. Different flower developmental stages were assigned according to previously described tobacco flower developmental stages (Koltunow et al., 1990). In the early developmental stage (stage -2 to 1), *GUS* expression was detected in all floral primordia (Figure 4.26). During these stages, style and petal were elongated to top of sepals (Koltunow et al., 1990). Strong *GUS* expression was detected not only on sepal but also on young flower organs such as style and petals. After the emergence of stamen and pistil (stage 2), *GUS* expression was only detected in the sepal and the top region of pistil (Figure 4.26). Distinguishable *GUS* expression was detected on sepal and on top of pistil in stages 2 to 3 (anthers and pistil were fully differentiated and corolla emerged from calyx). After elongation of corolla (stage 10), sepals and top of petals showed *GUS* expression while no *GUS* expression was detected on stamen and pistil. In addition, similar *GUS* expression patterns were also detected in fully opened flowers (stage 12). Even though there were no abnormal flowers observed in transgenic Arabidopsis and tobacco plants harbouring overexpressed *GmLFY1, GUS* expression was detected in sepal during flower development. This result suggested that *GmLFY1* plays a role in flower development.

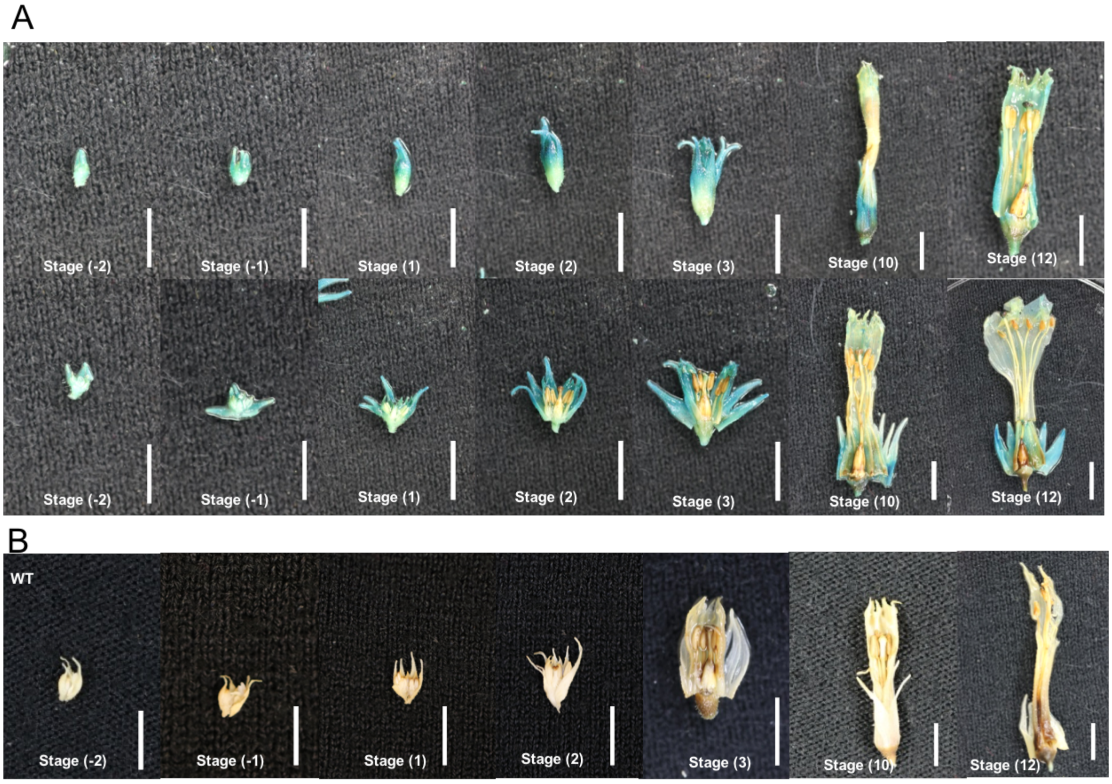


Figure 4. 26. Detection of organ-specific *GUS* expression in transgenic tobacco plants harbouring 5' regulatory fragment of *GmLFY1*.

(A) Strong *GUS* expression in sepal was detected on floral buds and flowers in different developmental stages. In early flower developmental stage (-2 to 1), *GUS* expression was observed in all floral organ primordia (sepals, petals, stamens and pistil). *GUS* expression was limited to sepals and tip of pistil in stages 2 to 3. In later flower developmental stages (10 to 12), *GUS* expressions were only detected in sepals and tip of petals. (B) No *GUS* expression was detected in wild-type flowers. Flowers were collected from T2 homozygous transgenic tobacco line (line 5-2). White bar: 1cm.

4.3.7. Analysis of expression patterns of floral pathway integrator genes in transgenic Arabidopsis and tobacco plants harbouring overexpressed *GmLFY1*

4.3.7.1. Ectopic expression of *GmLFY1* up-regulates the expression of floral pathway integrator genes in transgenic Arabidopsis plants

Overexpression of *GmLFY1* in transgenic Arabidopsis plants exhibited early flowering phenotypes. To determine whether early flowering phenotypes were related with floral pathway integrators, expression levels of *AtLFY, AtAP1, AtSOC1* and *AtAG* genes were examined by qRT-PCR analysis. Total RNA extracted from 4-week-old plants were used for cDNA synthesis. Firstly, overexpression of *GmLFY1* in transgenic

Arabidopsis plants was confirmed (Figure 4.27A). Expression of *GmLFY1* was only detected in transgenic lines (lines 14 and 15). Line 15 showed higher expression level (12 fold) than that of line 14. Expressions of floral pathway integrators were up-regulated in transgenic plants. Expression level of *AtAP1* of transgenic plants showed higher expression (1.5 fold; line 14 and 1.8 fold; line 15) than wild-type. Significantly increased expressions of *AtSOC1*and *AtAG* were detected in both transgenic line 14 (*AtSOC1*:14.5 fold, *AtAG*: 3.1 fold) and line 15 (*AtSOC1*: 15.8 fold, *AtAG*: 2.1 fold). In contrast, no significant differences in *AtLFY* were detected in transgenic plants and wild-type (Figure 4.27). These results indicated that the overexpression of *GmLFY1* promoted initiation of flowering by up-regulation of floral pathway integrators.

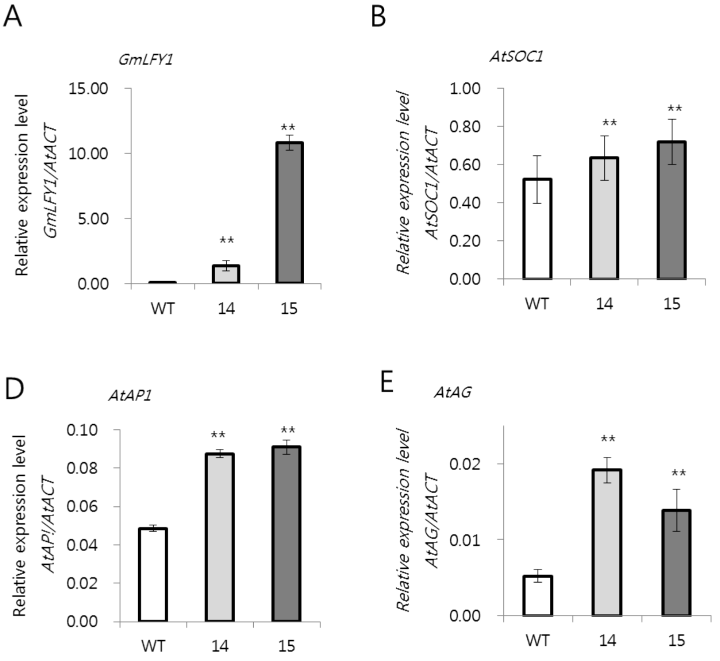


Figure 4. 27. Analysis of expression levels of flowering genes in *GmLFY1* transgenic Arabidopsis plants.

The expression abundances of *GmLFY1* (A)*, AtSOC1* (B)*, AtLFY* (C)*, AtAP1* (D) and *AtAG* (E) were examined using real-time qRT-PCR. The expression levels of *GmLFY1* (A)*, AtSOC1* (B)*, AtLFY* (C)*, AtAP1* (D) and *AtAG* (E) were up-regulated in shoot apex of *GmLFY1* transgenic Arabidopsis plants (line 14 and line 15 respectively). Total RNA was extracted from 10mm shoot apex of 4-week-old plants (n=9). Transcript levels were normalized using *AtACT* as a reference gene. 2-*ΔΔCt* method was used for data analysis. Bar: standard deviation (±SD) of three biological replicates. Asterisk: student t-test (*P>0.05, **P>0.01).

4.3.7.2. Overexpression of *GmLFY1* promotes expression of floral pathway integrator genes in transgenic tobacco plants

Ectopic expression of *GmLFY1* in transgenic Arabidopsis promoted onset of flowering that coordinated with up-regulation of floral pathway integrators. Since overexpressing tobacco plants also showed early flowering phenotypes, expression levels of floral pathway integrators were examined. Up-regulations of floral pathway integrators were detected in transgenic plants. Expression of *NtAP1a* in transgenic plants overexpressing *GmLFY1* showed 13.9 fold (GmLFY 8-1) and 15.5 fold (GmLFY8-5) higher expression than that of wild-type (Figure 4.28). Other floral pathway integrator genes [*NtAP1b* (GmLFY8-1: 1.8 fold, GmLFY8-5: 1.9 fold), *NFL1* (GmLFY8-1: 1.8 fold, GmLFY8-5: 2.5 fold), *NtSOC1a* (GmLFY8-1: 3.7 fold, GmLFY8-5: 4 fold) and *NtAG* (GmLFY8-1: 2.8 fold, GmLFY8-5: 3.7 fold)] were also up-regulated in transgenic plants. These results also indicated that ectopic expression of *GmLFY1* in transgenic tobacco plants accelerates initiation of flowering and via up-regulations of floral pathway integrators.

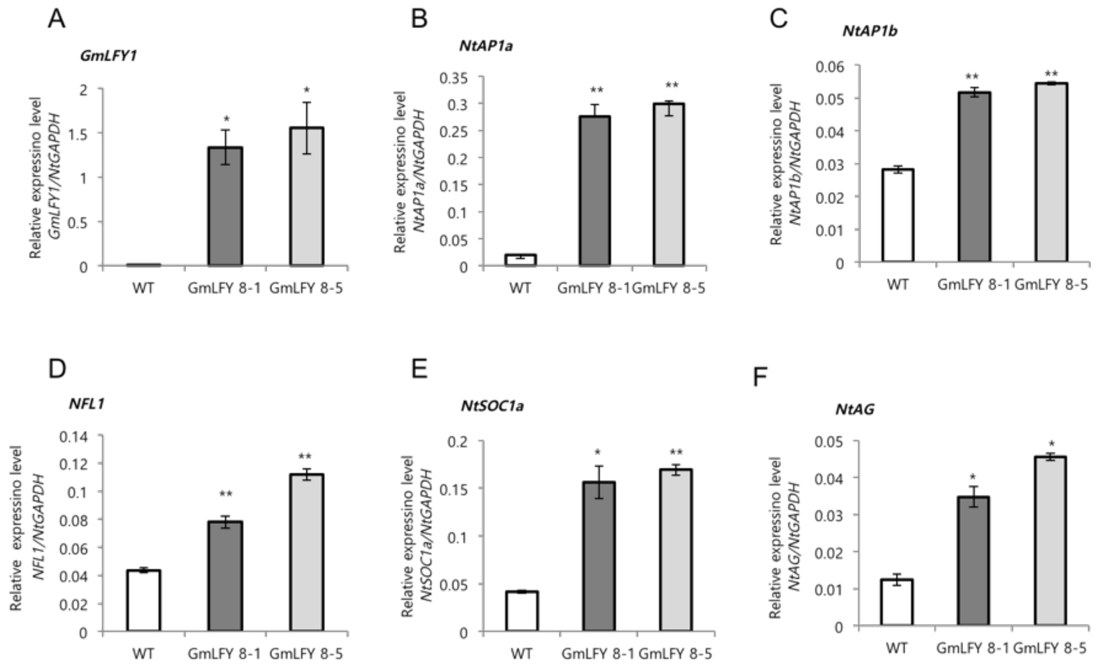


Figure 4. 28. Analysis of expression levels of flowering genes in *GmLFY1* transgenic tobacco plants.

(A) Overexpression of *GmLFY1* in transgenic tobacco plants were observed in GmLFY8-1 and GmLFY8-5. The expression abundances of *NtAP1a* (B)*, NtAP1b* (C)*, NFL1*(D)*, NtSOC1a* (E) and *NtAG* (F) were examined using real-time qRT-PCR. The expression levels of *NtAP1a* (GmLFY8-1: 13.9 fold, GmLFY8-5: 15.5 fold)*, NtAP1b* (GmLFY8-1: 1.8 fold, GmLFY8-5: 1.9 fold)*, NFL1* (GmLFY8-1: 1.8 fold, GmLFY8-5: 2.5 fold)*, NtSOC1a* (GmLFY8-1: 3.7 fold, GmLFY 8-5: 4 fold) and *NtAG* (GmFLY8-1: 2.8 fold, GmLFY8-5: 3.7 fold) were up-regulated in shoot apex of *GmLFY1* transgenic tobacco plants. Total RNA was extracted from 10mm shoot apex of 4-week-old plants grown in the glasshouse (n=9). Transcript levels were normalized using *NtGAPDH* as a reference gene. 2-*ΔΔCt* method was used for data analysis. Bar: standard deviation (±SD) of three biological replicates. Asterisk: student t-test (*P>0.05, **P>0.01).

4.4 Discussion

In this study, the functions of Glyma.06G163600.1 (a soybean *LFY* homolog) in flowering initiation were investigated. In soybean genome database (phytozome v12.1), two *LFY* homologs are annotated (*GmLFY2*: Glyma.04G202000.1 and *GmLFY1*: Glyma.06G163600.1). In the phylogenetic analysis, these two soybean *LFY* homologs are clustered into the same clade, which indicates that these genes were diverged by recent genome duplication (Figure 4.3). Structures of two soybean *LFY* homologs have three exons, two introns and 3′UTR which are similar to the *LFY* homologs in other species (Bomblies et al., 2003; Tang et al., 2016; Yang et al., 2017; Zhao et al., 2017). In sequence alignment analysis, *GmLFY1* and *GmLFY2* exhibited 96.27% identity in peptide sequence.

However, sequential differences on exon2 of Glyma.04G202000.1 were observed in sequence alignment analysis, resulting in the absence of amino acids at conserved region of *LFY* (Figure 4.2). *LFY*, as a transcription factor, regulates the expression of target genes by binding to specific sequences (T/ANNNCCANT/GT/GNNNNT/A) in the regulation region of its target genes (Hamès et al., 2008). It has been revealed that *LFY* gene has highly conserved two domains (N- and C-domain) which play important roles in DNA-binding (Maizel et al., 2005; Hamès et al., 2008; Sayou et al., 2014; Wan et al., 2015; Sayou et al., 2016). The structure of C-domain is made up of two β strands and seven α helices and is responsible for specific DNA-binding via helix-turn-helix motif (Hamès et al., 2008). The other conserved domain is the N-domain which is identified as Sterile Alpha motif (SAM) (Sayou et al., 2016). This is consisting of five alpha helices and is involved in oligomerisation of *LFY* gene (Sayou et al., 2016). The mutations on the two surfaces (mild-loop and end-helix) of a protein structure exhibited reduction on the efficiency of oligomerisation resulting in impaired cooperative DNA binding (Sayou et al., 2016). High conservation of N-domain including these surfaces was confirmed by sequence alignment analysis (Figure 4.4). However, two peptide replacements at two points in the main groove (P55A and G78D) which have an effect on the interactions of Sterile Alpha motif via main and lateral chain were observed in soybean *LFY* homologs (Figure 4.4). In addition, in C-domain, loss of peptide sequences in beta strands was detected in Glyma.04G202000.1 (Figure 4.5). The beta strands have roles in the formation of protein structure by forming core chains. These replacements or deletions of peptides in soybean *LFY* homologs suggest functional divergence of *LFY* genes in soybean by alteration of DNA-binding affinity. Therefore, the structural differences of soybean *LFY* homologs suggest that two soybean *LFY* homologs may have undergone functional divergences during evolution.

Expression of two soybean *LFY* homologs increased during flowering inductive conditions (short day: SD). Under SD conditions, two *LFY* homologs were expressed in the shoot apical meristem and the level of expression gradually increased under SD conditions. Expression levels of Glyma.06G163600.1 were higher (1.2 to 1.9 fold) than Glyma.04G202000.1 in the same time period, suggesting that Glyma.06G163600.1 has overlapping roles with Glyma.04G202000.1. In the analysis of spatial temporal expression of Glyma.06G163600.1, it was expressed at the flank region of shoot apical meristem and leaf primordia between SD 0 to SD 6. In SD 6, expression of

Glyma.06G163600.1 was expanded to the upper region of the shoot apical meristem. In the floral meristem at SD 10, strong Glyma.06G163600.1 expression was detected on floral meristem (Figure 4.7). In Arabidopsis, expression of *LFY* was detected on flank region of shoot apical meristem during vegetative phase and an increased expression was detected on floral primordia (Blázquez et al., 1997). These spatial and temporal expression patterns of Glyma.06G163600.1 suggest it may play conserved roles in initiation of flowering.

Transgenic Arabidopsis and tobacco plants overexpressing Glyma.06G163600.1 exhibited early flowering phenotypes. Ectopic expression of Glyma.06G163600.1 in Arabidopsis promoted the onset of flowering in T2 homozygous transgenic lines (Figure 4.10). In LD conditions, transgenic Arabidopsis plants flowered 7 DAG (days after germination) earlier than wild-type (Col-0) (Table 4.1). Overexpressing endogenous *LFY* and *LFY* homologs from other plants species (aspen, tobacco, rice and jatropha) in Arabidopsis also showed early flowering phenotypes (Weigel and Nilsson, 1995; He et al., 2000; Tang et al., 2016). In transgenic tobacco plants, ectopic expression of Glyma.06G163600.1 also induced early flowering phenotypes (Figure 4.18). Transgenic plants started flowering about 15 DAG earlier (line 8-1: 58.5±7.59 DAG and line 8-5 59.3±7.91 DAG) than wild-type (73.3±9.03 DAG) (Figure 4.22). Moreover, transgenic tobacco plants overexpressing Glyma.06G163600.1 exhibited morphological differences compared to wild-type. Glyma.06G163600.1 overexpressing tobacco plants showed adult phenotypes in leaf morphology (Figure 4.16). Leaf morphology is one of the distinguishable characteristics between vegetative and reproductive developmental phase. In tobacco, the leaves in juvenile vegetative developmental stages are rounder in shape and smaller than the leaves in adult developmental phase (Feng et al., 2016). Glyma.06G163600.1 overexpressing transgenic tobacco plants had narrower shape of leaves and smaller leaf area than that of wild-type. In leaf width, Glyma.06G163600.1 had narrower width (line 8-1: 4.5±0.56 cm and line 8-5: 4.6±0.45 cm) than wild-type (5.2±0.60 cm), contributing to forming a narrower shape of leaves (Figure 4.16B, C). In addition, Glyma.06G163600.1 had a smaller leaf area (line 8-1: 34.6±4.88 cm^2 and line 8-5: 35.8±8.13 cm^2) than wild-type (42.1±8.29 cm^2) (Figure 4.16D). Moreover, up-regulation of flowering genes was observed in transgenic Arabidopsis and tobacco plants. *SOC1* is a flowering pathway integrator gene and it is expressed in the very early stage of flowering. Higher expression of *SOC1* was detected both in overexpressing transgenic

Arabidopsis (14.5 to15.8 fold) and tobacco plants (4.2 fold) (Figure 4.27, 4.28). Besides, the expression of *AP1* (floral meristem identity gene) and *AG* (flower organ identity gene) were also up-regulated in transgenic Arabidopsis and tobacco plants (*AtAP1*: 1.5-1.8 fold, *AtAG*: 2.3-3.1 fold, *NtAP1a*: 3.4 fold, *NtAP1b*: 1.3 fold, *NtAG*: 2.8 fold) (Figure 4.27, 4.28). In contrast, expression of endogenous *LFY* gene in transgenic Arabidopsis plants were similar to wild-type Arabidopsis indicating that the up-regulation of flowering genes were caused by overexpression of Glyma.06G1636001.1 (Figure 4.27). However, up-regulation of tobacco *LFY* gene (*NFL1*) was detected in transgenic tobacco plants (Figure 4.25). This higher tobacco endogenous *LFY* gene can be explained by divergent roles of *NFL1* in tobacco flowering. In tobacco, *NFL1* is responsible for cell proliferation and identity in floral meristem (Ahearn et al., 2001) which ultimately forms the lateral structure such as leaves and branches. In addition, floral meristem development was regulated by *NFL1* by control of cell proliferation and identity (Ahearn et al., 2001). Since transgenic tobacco plants overexpressing Glyma.06G163600.1 exhibited early flowering phenotypes in this study, this result indicates earlier formation of floral meristem in transgenic plants than wild-type. Therefore, active cell proliferation and formation of floral organ primordia may increase the expression of endogenous tobacco *LFY* (*NFL1*) gene, resulting in a higher expression level of *NFL1* in transgenic tobacco plants. These early flowering phenotypes obtained from transgenic Arabidopsis and tobacco plants overexpressing Glyma.06G163600.1 indicate that Glyma.06G163600.1 regulates initiation of flowering via conserved flowering pathways.

Other functions of *LFY* gene is the regulation of floral development (Moyroud et al., 2010). In Angiosperm, the development of flower is regulated by combinational expressions of flowering homeotic genes, ABC genes (Bowman et al., 1991). Genes in class A is responsible for development of the outer layer of flower such as sepal. Formations of petal and stamen were controlled by the expression of class A and B genes. Pistil was formed by the expression of class B and C genes. In Arabidopsis, it has been identified that *LFY* gene plays a regulator role in flower development. *LFY* gene controls flower development by regulation of expressions of *AP1* (A class gene), *AP3* (B class gene) and *AG* (C class gene). In this study, *GUS* assay in transgenic tobacco harbouring 5' regulatory fragment of Glyma.06G163600.1 construct showed regulatory roles of Glyma.06G163600.1 in floral development (Figure 4.26). Strong *GUS* expression was detected on sepals of transgenic tobacco plants. In early developmental stages (-2 to 1 stages), *GUS* expression was detected on sepal and stamen and petal primordia (Figure 4.26). *GUS* expression became strong and confined in sepal during the later stage of flower development (stage 10 to 12) (Figure 4.26). Ectopic expression of Arabidopsis *LFY* in transgenic tobacco plants caused production of abnormally large terminal flowers and absence of inflorescence branches (Ahearn et al., 2001). Besides, constitutive expressions of tobacco *LFY* homologs (*NFL1*) in Arabidopsis caused production of precocious flowers (Ahearn et al., 2001). In contrast, a recent study on the functional analysis of jatropha *LFY* homolog (*JcLFY*) reported that an ectopic expression of *JcLFY* in Arabidopsis showed early flowering phenotypes without abnormal flower development. Moreover, overexpression of *JcLFY* in jatropha did not show any significant differences compared to wild-type (Tang et al., 2016). However, suppression of *JcLFY* in jatropha caused morphological differences in reproductive organs such as formation of impaired flowers and fruits (Tang et al., 2016). In this study, early flowering phenotypes were observed in Glyma.06G163600.1 overexpressing transgenic Arabidopsis and tobacco plants. However, no abnormal flowers and seeds were observed in transgenic lines. Since a strong expression of Glyma.06g163600.1 in transgenic tobacco flower development was detected in promoter analysis, further analysis of the roles of Glyma.06G163600.1 is needed. To solve this question, soybean *LFY*-RNA interference (RNAi) construct were prepared for the suppression experiments of soybean *LFY* homologs (Figure 4.29). RNAi is a biological process to repress gene expression or translation by binding to mRNA of target genes (Fire et al., 1998; Waterhouse et al., 1998; Helliwell and Waterhouse, 2003). The functional analysis of soybean *LFY* genes using transgenic plants harbouring soybean *LFY*-RNAi construct will provide new insight on the identification of soybean *LFY* gene functions in reproductive organ developments. In this study, it has been identified that Glyma.06G163600.1, one of soybean *LFY* homologs, is involved in the initiation of flowering. This result paves the way for the improvement of soybean as one of the most important crops in the world.

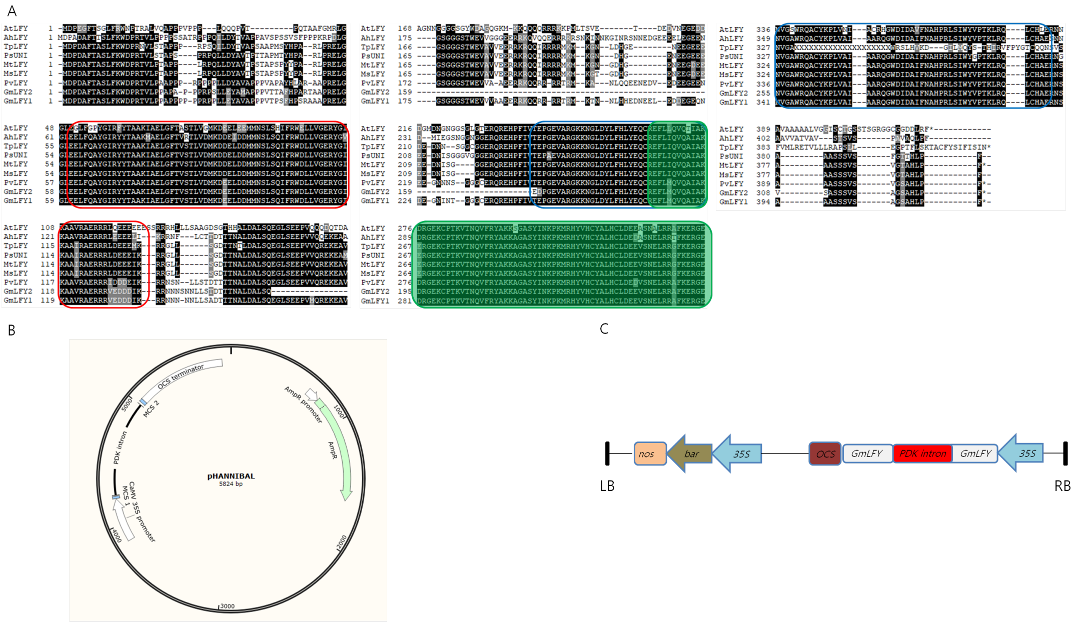


Figure 4. 29. Schematic diagram of RNAi-*GmLFY* construct.

(A) N-domain (red box) and C-domain (blue box) were highly conserved among legume species and Arabidopsis. A highly conserved region in the C-domains of the two soybean *LFY* homologs was designated as a target region for RNAi-*GmLFY* (green boxes). (B) The vector pHANNIBAL (plant RNAi vector) for gene silencing of *GmLFY* genes. The map was generated using a SnapGene® Viewer (www.snapgene.com). (C) A schematic diagram of RNAi-*GmLFY* construct. The conserved region of *GmLFY* genes (containing 76 amino acids in C-domain) was inserted between the 35S promoter and the OCS terminator. (LB: left border, *nos*: nopaline synthase terminator, *bar:* herbicide bialaphos resistant gene, 35S: CaMV 35S promoter, *GmLFY*: conserved region of *LFY* gene, *OCS*: octopine synthase terminator, RB: right border. *Medicago truncatula LFY* (MtLFY, Medtr3g098560.1), *Phaseolus vulgaris LFY* (PvLFY, Phvul.009g160900.1), *Trifolium paratense LFY* (TpLFY, Tp57577), *Arachis hypogaea LFY* (AhLFY, XP_025616417.1), *Pisum Sativum UNI* (PsUNI, AAB88139.1), *Medicago sativa LFY* (MsLFY, AEO06611.1), *Arabidopsis thaliana LFY* (AtLFY, AT5G61850.1), *Glycine max LFY* (GmLFY1, Glyma.06G163600.1; GmLFY2, Glyma.04G202000.1)

4.5 Conclusion

Soybean genome has two *LFY* homologs, Glyma.06G163600.1 and Glyma.04G202000.1. In sequence alignment analysis, two *LFY* homologs showed high similarity (96.27%) in peptide sequence levels. However, substitution of amino acids in two main domains (N-domain and C-domain) in soybean *LFY* genes were observed. These peptide substitutions suggest that differentiations of DNA-binding affinity in soybean *LFY* may have occurred. Under flowering inductive conditions, Glyma.06G163600.1 showed higher expression than Glyma.04G202000.1. In addition, spatial expressions of Glyma.06G163600.1 at SAM and floral meristem indicated that it may have roles in the flowering of soybean.

Ectopic expressions of Glyma.06G163600.1 in transgenic Arabidopsis and tobacco plants promoted initiation of flowering (7 DAG and 15 DAG respectively). However, these transgenic plants did not produce precocious flowers which are one of the main features characterizing overexpression of *LFY* gene in Arabidopsis. Moreover, all transgenic lines of Arabidopsis and tobacco plants with Glyma.06G163600.1 overexpression produced normal and fertile flowers. However, tissue-specific strong *GUS* expression in different flower developmental stages was detected in flowers of transgenic tobacco harbouring 5' regulatory fragment of Glyma.06G163600.1 (*LFY*::*GUS::OCS*) indicating that Glyma.06G163600.1 may have a different role in flower development.

In this study, it has been identified that Glyma.06G163600.1 influences regulation of flowering time. To further validate the roles of *LFY* homologs of soybean, analysis of ectopic and co-suppression of these genes in soybean is required.

Chapter 5. Functional analysis of *gma-miR156a* and *gma-miR172a*

Abstract

miR156 and miR172 are major components of a plant's ageing process and they are expressed sequentially to control the vegetative developments and the initiation of reproductive phase including flowering. In this study, investigations on the functions of *gma-miR156a* and *gma-miR172a* in transgenic tobacco plants (*Nicotiana tabacum* L.) harbouring overexpressed *gma-miR156a* and *gma-miR172a* was conducted. Expression patterns of *miR156* showed inverse patterns compared to expression patterns of *miR172* in soybean. Under flowering inductive conditions, expression of *miR156* was decreased whilst *miR172* showed increased expression levels. Ectopic expression of *gma-miR156a* and *gma-miR172a* altered leaf morphology in transgenic tobacco plants. Overexpression of *gma-miR156a* in T2 generation of transgenic tobacco plants produced round shape of leaves (L/W ratio: 1.8±0.18) with smallest leaf length (7.5±0.98 cm), width (4.2±0.45 cm) and leaf area (22.1±5.58 cm^2). On the other hand, overexpression of *gma-miR172a* in T2 generation of transgenic tobacco plants exhibited smaller leaf width (4.4±0.60 cm) than wild-type plants (5.2± 0.60 cm) which contribute to narrow shape of leaf (L/W ratio: 3.2±0.43). Moreover, chlorophyll contents and photosynthesis rate of leaves were reduced in *gma-miR156a* transgenic tobacco plants (34.4% and 15.1%). Ectopic expression of *gma-miR156a* and *gma-miR172a* in transgenic tobacco plants exhibited their effect on regulation of flowering time. Overexpression of *gma-miR156a* in T2 generation of transgenic tobacco plants showed extended vegetative phase with delayed time to first flower opening (89.9±8.22 days after germination: DAG). Whereas ectopic expression of *gma-miR172a* in T2 generation of transgenic tobacco plants flowered earlier (45.5±4.55 DAG) than wild-type (66.2±15.1 DAG). qRT-PCR analysis showed down-regulation of miR156 target genes, *NtSPL4a* (1.7 fold), *NtSPL5a* (70 fold), *NtSPL5b* (28.5 fold) and *NtSPL9* (4.7 fold), in *gma-miR156a* overexpressing transgenic tobacco plants. In addition, down-regulation of *AP2* (2.9 fold) was observed in *gma-miR172a* overexpressing transgenic tobacco plants. Ectopic expression of *gma-miR172a* in transgenic tobacco plants showed up-regulation of flowering time response genes. The expression levels of *NtAP1a* (3.4 fold), *NtAP1b* (13 fold), *NtLFY1*(7.4 fold) and *NtSOC1a* (4.2 fold) were up-regulated in the shoot apex of *gma-miR172a* transgenic tobacco plants.

These results indicated that *gma-miR156a* regulates vegetative development by repression of *SPL* genes and *gma-miR172a* is involved in flowering by regulation of a target gene (*AP2*) and flowering time response genes.

Key words: soybean, *gma-miR156a, gma-miR172a*, leaf morphology, flowering

5.1 Introduction

MicroRNAs (miRNAs) are the small non-coding RNAs consisting of 20 to 24 nucleotides (nt) which play central regulatory roles in gene expression in both plants and animals (Spanudakis and Jackson, 2014). miRNAs down-regulate the expression of their target genes by annealing to a mRNA of target genes (Bartel, 2004). Recently, it has been reported that miRNAs are involved in the regulation of gene networks which control various biological processes such as development of and stress-response in plants and animals (Moran et al., 2017). In plants, miRNAs play pivotal roles in their developmental processes (Zhang et al., 2006) such as leaf morphogenesis and polarity (Bao et al., 2004; Kim et al., 2005), floral patterning and development (Aukerman and Sakai, 2003; Chen, 2004), root development (Laufs et al., 2004; Guo et al., 2005), plant vascular system development (Kim et al., 2005), and phase change from vegetative to reproductive (Achard et al., 2004; Lauter et al., 2005). Furthermore, particular miRNAs have been shown to play major roles in various pathways which regulate the onset of flowering by acting in either negative or positive ways to initiate the reproductive phase. The miR156 and miR172 play important roles in phase transition and are major components of the ageing pathway. These miRNAs regulate the initiation of reproductive competency by sequential expressions (Wu et al., 2009; Yamaguchi and Abe, 2012). In early developmental stages, *miR156* is highly expressed and decreased during ageing process. In contrast, *miR172* is accumulated in the organs such as leaves and floral buds during the reproductive phase (Wu et al., 2009; Zhu and Helliwell, 2010). These temporal expression patterns show that these miRNAs play master roles in phase change (Huijser and Schimd, 2011; Yamaguchi and Abe, 2012).

miR156, one of the highly conserved plant miRNAs, plays master regulator roles in vegetative development in monocot and dicot plants such as Arabidopsis (Wu et al., 2009), rice (Xie et al., 2006; 2012) and tobacco (Feng et al., 2016) by repressing its target genes, *SQUAMOSA PROMOTER BINDING-LIKE* (*SPL*) transcription factors family (Feng et al., 2016). In Arabidopsis, *SPL* genes targeted by miR156 are 11 members of *SPL* transcription factors family (*SPL2, SPL3*, *SPL4, SPL5, SPL6, SPL9, SPL10, SPL11, SPL13* and *SPL15*) and have diverse roles in plant growth, development and flowering (Park et al., 2005; Huijser and Schmid 2011; Yamaguchi and Abe, 2012; Tripathi et al., 2018). The extended juvenile phase is one of the main characteristics of miR156 overexpressing transgenic plants. Constitutive expression of miR156 in rice had numerous small size leaves (Xie et al., 2006) and showed rapid leaf/tiller initiation speed with smaller size of SAM (shoot apical meristem) than wild-type (Xie et al., 2012). In Tobacco (*Nicotiana tabacum* L.), miR156a had an effect on the several aspects of plant development such as leaf morphology including leaf shape, numbers of leaf veins, size of epidermal cells, chlorophyll contents and photosynthesis rate, suggesting that miR156 has comprehensive regulatory roles in vegetative development (Wu et al., 2009).

miR172 plays pivotal roles in promoting adult epidermal identity by down-regulation of its target genes (Park et al., 2002; Jung et al., 2014). miR172 regulates the mRNA of the plant-specific transcription factor gene *APETALA2* (*AP2*) and a group of *AP2-like* gene, including *TARGET OF EAT* (*TOE1*), *TOE2*, *TOE3*, *SCHLAFMUTZE* (*SMZ*), *SCHNARCHZAPFEN* (*SNZ*) in Arabidopsis (Aukerman and Sakai, 2003; Schmid et al., 2003; Park et al., 2002; Chen 2004; Schwab et al., 2005). *AP2* is a floral organ identity gene (A class gene in ABC model of flower development). It is involved in floral patterning of sepal together with B class gene *APETALLA3* (*AP3*) in whorl 1 and petal identity working with C class gene *PISTILLATA* (*PI*) in whorl 2 (Bowman et al., 1991; Drews et al., 1991; Zhu and Helliwell, 2010). In Arabidopsis, *AP2* overexpression showed delayed initiation of flowering, while miR172 *resistant-AP2* (*rAP2*) transgenic plants exhibited severe floral disrupts (loss of floral determinacy with stamens-to-petals transformation) and enlarged floral meristems which are also found in miR172 overexpressing plants (Chen, 2004). These results indicated that miR172-mediated repression of *AP2* plays a crucial role in floral determinacy (Chen, 2004). In maize, *glossy15* (*gl15*) is an *AP2-like* gene and acts in regulation of epidermal cell traits (Wu et al., 2009; Yang et al., 2010; Feng et al., 2016). Increased levels of *gl15* caused increased number of leaves with juvenile epidermal traits and delayed initiation of reproductive phase (Lauter et al., 2005). Furthermore, the reversely relative expression pattern between maize miR172 and *gl15* was detected, suggesting that miR172 regulated phase transition in maize by repression of *gl15* (Lauter et al., 2005).

Arabidopsis has 10 miR156 loci (miR156a-j encoding 15 mature miR156) and 6 miR172 loci (miR172a-e encoding 8 mature miR172) (Aukerman and Sakai; 2003; Park et al., 2005; Xie et al., 2005; Lu et al., 2006; Jung et al., 2007; Moldovan et al., 2010; Huijser and Schmid, 2011; Yamaguchi and Abe, 2012). In Arabidopsis, miR156a overexpressing lines exhibited prolonged juvenile phases with more rosette leaves and a lack of abaxial trichomes (Wu et al., 2009). Whilst, overexpressing *miR172a* promoted flowering. (Aukerman and Sakai, 2003). Recent bioinformatics analysis on soybean miRNAs revealed that soybean has 28 loci for encoding 28 mature miR156 (MIR156 a-z and aa, ab) and 12 loci for encoding 15 mature miR172 (MIR172 a, b3p, b5p, c, d, e, f, g, h3p, h5p, i3p, i5p, j, k and l) (Subramanian et al., 2008; Kulcheski et al., 2011; Wong et al., 2011; Goettel et al., 2014; Zhao et al., 2015). However, studies on experimental validations of soybean miRNA156 and miRNA172 remain limited. In this regard, functions of *gma-miR156a* and *gma-miR172a* in vegetative and reproductive phases need to be investigated to understand soybean developmental phase transition mechanisms.

In this study, the roles of *gma-miR156a* and *gma-miR172a* in a model plant, tobacco plants, were investigated. The objectives of this chapter are as follows:

- Confirmation of expression patterns of *gma-miR156* and *gma-miR172* in soybean during flowering inductive conditions
- Investigation on functions of *gma-miR156a* and *gma-miR172a* on vegetative and reproductive growth of transgenic plants harbouring overexpressing *gma-miR156a* and *gma-miR172a*
- Analysis of miR156 and miR172 target genes in transgenic plants

5.2 Materials and methods

5.2.1 Sequence information of soybean miR156 and miR172 families

The precursor miRNA sequences and mature miRNA sequences of miR156 (family number: MIPF0000008) and miR172 (family number: MIPF0000035) families of soybean (*gma-miR156* and *gma-miR172),* Arabidopsis (ath-miR156 and ath-miR172) and tobacco (nta-miR156 and nta-miR172) were obtained from the miRbase database (Release 22, www.miRbase.org).

5.2.1.1 Phylogenetic analysis

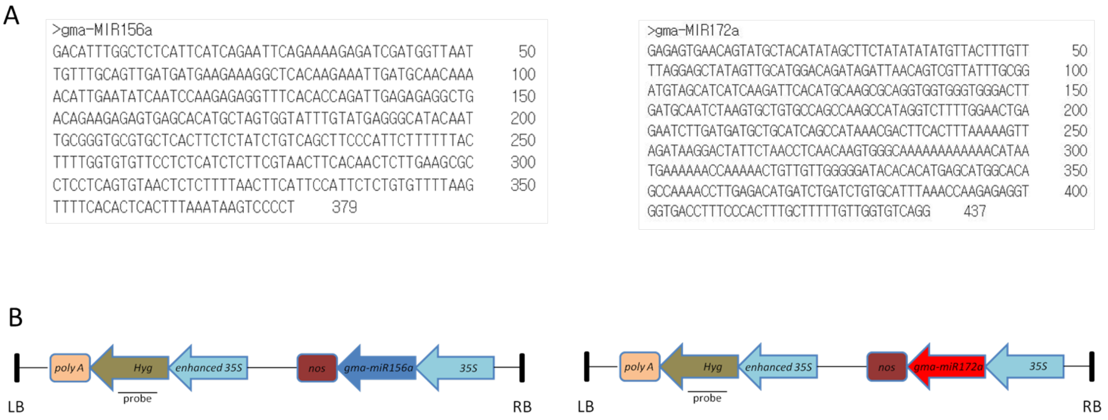
To compare the relationship between encoding loci of soybean miR156 and miR172 families, phylogenetic analysis was conducted using the hair-pin miRNA sequence of soybean miR156 and miR172. Phylogenetic tree was constructed using MEGA X with neighbour-joint-analysis, 100 replicates bootstrap. Sequence alignment analysis was conducted using multiple sequence alignment tool (CLUSTAL Omega) with default parameters.

5.2.2 Plant transformation and selection of transgenic plants

Tobacco transformation was conducted using leaf disc method (Horsch et al., 1985). Details of media composition, hormones and antibiotics stocks used in this study are provided in the appendices (Table A3).

5.2.2.1 Preparation of gma-miR156a overexpression and gma-miR172a overexpression constructs for plant transformation

Precursor sequences of *gma-miR156a* (accession number: MI0001784) and *gma-miR172a* (accession umber: MI0001780) were used for blast search to identify the *MIR156a* and *MIR172a* genes. The blast search was conducted using phytozome database (phytozome v.12) and NCBI soybean EST (Expressed sequence tag sequences) database. In blast search, Genebank ID: BE807821.1 (Glyma.17G074900.1) and BU084569 had sequences of precursor *gma-miR156a* and primary *gma-miR172a* respectively. Hence, BE807821.1 and BU084569 were used for overexpressing constructs. Sequences of *gma-miR156a* (379 bp encompassing precursor gma-miR156a) and *gma-miR172a* (437 bp containing precursor gma-miR172a) were amplified from the cDNA of soybean cv. Bragg (Figure 5.1A). Both amplicons of *gma-miR156a* and *gma-miR172a* were cloned into entry vector, pGEMT-easy (Promega, USA). These amplified DNA of *gma-imR156a* and *gma-miR172a* were recombined into binary vector pMDC-83 (which contains *hygromycin resistant gene* as a selective marker) between *CaMV35S* promoter and *nos* terminator (Figure 5.1B). The binary vectors containing *35S::gma-miR156a::nos* and *35S::gma-miR172a::nos* were transformed into *Agrobacterium tumefaciens* (cv. EHA105) for plant transformation.



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>gma-MIR156a
GACATTTGGCTCTCATTCATCAGAATTCAGAAAAGAGATCGATGGTTAAT     50
TGTTTGCAGTTGATGATGAAGAAAGGCTCACAAGAAATTGATGCAACAAA    100
ACATTGAATATCAATCCAAGAGAGGTTTCACACCAGATTGAGAGAGGCTG    150
ACAGAAGAGAGTGAGCACATGCTAGTGGTATTTGTATGAGGGCATACAAT    200
TGCGGGTGCGTGCTCACTTCTCTATCTGTCAGCTTCCCATTCTTTTTTAC    250
TTTTGGTGTGTTCCTCTCATCTCTTCGTAACTTCACAACTCTTGAAGCGC    300
CTCCTCAGTGTAACTCTCTTTTAACTTCATTCCATTCTCTGTGTTTTAAG    350
TTTTCACACTCACTTTAAATAAGTCCCCT     379
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>gma-MIR172a
GAGAGTGAACAGTATGCTACATATAGCTTCTATATATATGTTACTTTGTT     50
TTAGGAGCTATAGTTGCATGGACAGATAGATTAACAGTCGTTATTTGCGG    100
ATGTAGCATCATCAAGATTCACATGCAAGCGCAGGTGGTGGGTGGGACTT    150
GATGCAATCTAAGTGCTGTGCCAGCCAAGCCATAGGTCTTTTGGAACTGA    200
GAATCTTGATGATGCTGCATCAGCCATAAACGACTTCACTTTAAAAAGTT    250
AGATAAGGACTATTCTAACCTCAACAAGTGGGCAAAAAAAAAAAACATAA    300
TGAAAAAACCAAAAACTGTTGTTGGGGGATACACACATGAGCATGGCACA    350
GCCAAAACCTTGAGACATGATCTGATCTGTGCATTTAAACCAAGAGAGGT    400
GGTGACCTTTCCCACTTTGCTTTTTGTTGGTGTCAGG     437
```

Figure 5. 1. Schematic diagram of *gma-miR156a* and *gma-miR172a* overexpression gene constructs.

(A) Nucleotide sequences of *gma-miR156a* (left) and *gma-miR172a* (right) (B) T-DNA region of *35S::gma-miR156a::nos* (left) and *35S::gma-miR172a::nos* (right) in pMDC83 vector. LB: left border, *poly A*: CaMV poly A terminator, *Hyg: hygromycin resistant gene,* 35S: CaMV 35S promoter, *nos*: nos terminator, RB: right border, Probe: probe region for southern blot analysis

5.2.2.2 Seed sterilization

Seeds of tobacco (*Nicotiana tabacum* L. cv. RF2) were sterilized by shaking in 70% (v/v) ethanol for 1 min followed by shaking in hyperchloric acid solution (0.5 ml of 32% hyperchloric acid, 30 µl of Tween 20 in 10 ml of sterilized distilled water) for 10 min. Sterilized seeds were rinsed five times with sterilized distilled water and they were transferred onto sterilized filter papers to remove excessive water. For germination, 10 seeds were placed onto germination medium [Murashige and Skoog, (1962) basal medium (MS basal medium) containing 30 g/L sucrose, 8 g/L Agar, pH 5.8] in petri dishes and kept in a tissue culture room (150 μmol m^{-2} s^{-2}, 16/8 h, 25±1℃). After 2 weeks of germination, each plantlet was transferred into fresh MS basal medium in a tissue-culture cup and cultured until leaf size reached 3 to 5 cm long. Leaf segments of *in-vitro* cultured tobacco plants (*Nicotiana tabacum* L. cv.RF2) were used for genetic transformation.

5.2.2.3 Preparation of infection medium

Agrobacterium containing a binary vector with overexpressed gma-miR156a or gma-miR172a respectively was cultured on YEP medium (solid) with antibiotics [25 mg/L of rifampicin (Sigma, USA), 50 mg/L of kanamycin (Sigma, USA)] for 20 h (28℃, dark)]. A single colony was cultured in 5 ml of liquid YEP medium (liquid) with antibiotics for 20 h, shaking at 200 rpm (28℃, dark). 3 ml of *Agrobacterium* culture was re-suspended with 27 ml of MS liquid medium for infection without antibiotics.

5.2.2.4 Infection and co-cultivation

Leaf segments were dissected from leaves 3 to 5cm in length. Firstly, the main vein was removed and were cut into small pieces (1cm x 1cm). These explants were submerged in *Agrobacterium* suspension for infection for 2 min at room temperature. Infected explants were transferred to sterilized filter paper to remove excessive *Agrobacterium* culture and were placed adaxial side down on the co-cultivation medium [MS basal medium containing 30 g/L sucrose, 8 g/L Agar, pH 5.8, 0.1 mg 1-naphthyl-acetic acid (NAA; Sigma, USA), 1 mg/L 6-benzylamino purine (BAP; Sigma, USA)]. Explants were co-cultivated for 2 days (25℃, dark).

5.2.2.5 Selection of transgenic plants

After co-cultivation, explants were transferred to a selection medium [MS basal medium containing 30 g/L sucrose, 8 g/L Agar, pH 5.8, 0.1 mg 1-naphthyl-acetic acid, 1 mg/L 6-benzylamino purine, 30 mg/L of hygromycin (Astral Scientific, Australia), 400 mg/L of carbenicillin (GoldBio, USA)] in petri plates and incubated in a tissue culture room (150 µmol m^{-2} s^{-2}, 16/8 h, 25±1℃). Explants were subcultured every 2 weeks into fresh selection medium. After 6 weeks, fully developed shoots were excised from explants and were transferred to rooting medium (MS basal medium, 30 g/L sucrose, 8 g/L Agar, pH 5.8, 30 mg/L of hygromycin, 400 mg/L of carbenicillin).

5.2.2.6 Transfer of plants into glasshouse

The rooted plants were removed from the tissue culture containers and washed with tap water to remove tissue culture medium. Plants were transferred to pots containing sterilized commercial soil mix (Debco, Australia) and were placed in plastic containers. The container was sealed with plastic wrap to maintain high humidity for acclimatization and kept in a plant growth incubator (150 µmol m^{-2} s^{-2}, 16/8 h, 25±1℃). After 3 days, acclimatized plants were transferred to a glasshouse (250 µmol m^{-2} s^{-2}, 16/8 h, 25±1℃) and grown until maturity and seeds were collected.

5.2.3 Morphological analysis of transgenic tobacco plants harbouring *gma-miR156a* and *gma-miR172a*

5.2.3.1 Segregation analysis of transgenic plants

Seeds from transgenic plants were used for segregation analysis using hygromycin selection to examine the inheritance patterns of the gene. Sterilized seeds of transgenic and wild-type tobacco seeds were placed on MS basal medium containing 30 mg/L of hygromycin and were kept in a tissue culture room for germination (150 µmol m^{-2} s^{-2}, 16/8 h, 25±1℃). After 4 weeks of culture, total numbers of survived plantlet were counted and only survived plantlets were transferred to fresh medium with 30 mg/L of hygromycin for further growth. Survived plants (8 weeks after germination) were transferred into individual pots for further analysis. T1 and T2 generation of transgenic seeds were screened for hygromycin resistance to obtain homozygous transgenic lines.

The segregation ratio of hygromycin resistant and susceptible plants was calculated using the number of survived and non-survived plants, respectively.

5.2.3.2 Flowering time measurements and morphological analysis

For flowering time analysis, days until first flower opening were recorded. These days were calculated by counting from the day of transfer to glasshouse to first flower opening in T0 generation and from germination to first flowering opening in T1 and T2 generations.

Transgenic mature seeds which were derived from T0 generation of transgenic tobacco plants containing a single copy of gene (line 7, line 14 of *gma-miR156a* transformants and line 8, 14 of *gma-miR172a* transgenic plants) were sown on selection medium (SM) containing 30 mg/L of hygromycin. After 4 weeks of selection, survived plants were acclimatized on sterilized soil in pots and transferred to the glasshouse. For flowering time analysis, 20 plants of each line were randomly selected.

Plant height was measured from ground level to shoot apex. For analysis of leaf morphology, length and width of leaf and petiole of fully expanded leaves were measured. Leaf length was measured from leaf apex to a junction between leaf lamina and petiole, along the main vein of a leaf. Leaf width was measured end-to-end between the widest lamina perpendicular to the mid-rip. Leaf area of fully expanded leaves was measured using a LI-3100 leaf area meter (LI-COR, USA). An average of three measurements was used for leaf area analysis. Flower morphological parameters such as length and width of flowers, sepals and pedicles of fully opened flowers were measured. Width of flower was measured from petal to petal and length of flowers was recorded from top of flower to base of sepal.

5.2.3.3 Determination of chlorophyll concentration and photosynthesis rate

Three leaves from different locations (top, middle and bottom) of 8-week-old plants were used for chlorophyll and photosynthesis rate measurement. Measurements of chlorophyll content and photosynthesis rate were taken on two different positions of intact leaf lamina from leaves of transgenic plants. The amount of chlorophyll was quantified with a SPAD 502 Plus Chlorophyll Meter. Photosynthesis rate was measured between 10:30am and 12:30pm using MINI-PAM (Walz, Germany). Photosynthesis yield data (Y (Ⅱ) = (Fm’-F)/Fm) was recorded in MINI-PAM.

5.2.3.4 Pollen viability

Pollens were extracted from three preanthesis flowers of transgenic and wild-type plants. Pollens were placed on the slides with a drop of staining solution containing 40μl of 10% sucrose with 2 μl of Propidium iodide (1 mg/ml) and 2 μl of Fluorescein diacetate (FDA, 2 mg/ml). Pollen viability was observed under an optical microscope with UV light.

5.2.4 Molecular analysis of gene expression

5.2.4.1 Confirmation of putative transgenic plants (T0 generation)

5.2.4.1.1 Genomic DNA isolation

Genomic DNA was extracted from leaves of putative transgenic plants by the modified CTAB (cetyl trimethylammonium bromide) method (Doyle and Doyle, 1990). About 1 g of fully expanded leaves from transgenic tobacco plants were homogenized in liquid nitrogen using mortar and pestle. Homogenized leaves were mixed with 10 ml of pre-warmed (55℃) 2% CTAB solution (2 g of CTAB, 100 mM Tris-HCl pH8.0, 50 mM EDTA, 0.1% v/v β-mercapoethanol in 100 ml) in 50 ml falcon tubes and incubated at 55℃ in water bath for 15 min with gentle shake. The tubes were centrifuged at 4,500 rpm at room temperature for 10 min to precipitate debris. Supernatants were transferred to new 50 ml tubes and 5 ml of chloroform:isoamyl alcohol (24:1) was added. The tubes were then mixed by inverting and were centrifuged at 4,500 rpm at room temperature for 10 min. Only the upper phase was transferred to new 50 ml tubes and mixed with 1 ml of 10% CTAB solution (10 g of CTAB, 100 mM Tris-HCl pH 8 in 100 ml) by inverting. The tubes were incubated for 5 min at room temperature and mixed with 2 ml of chloroform:isoamyl alcohol (24:1). The mixtures were centrifuged at 4,500 rpm for 10 min. The upper phase was transferred to new 50 ml tubes and the 10% CTAB solution step (mixing with 1 ml of 10% CTAB solution followed by mixing with 2 ml of chloroform:isoamyl alcohol) was repeated. The mixtures were centrifuged at 4,500 rpm for 10 min. The upper phase was transferred to new 50 ml tubes and mixed with 2/3 volume of isopropanol (ice-cold) by inverting. The mixtures were kept on ice for 1 hour to precipitate genomic DNA. Tubes were centrifuged at 4,500 rpm for 10 min to precipitate DNA. The pellet was washed with 70% (v/v) ethanol and air dried for 5 min. Washed DNA was dissolved in TE buffer. DNA purity (260/280 nm) and concentration was measured using a nano-drop.

5.2.4.1.2 Southern blot analysis

To examine the gene insertion and gene copy numbers in each transgenic line, Southern blot analysis (Southern, 2006) was performed. A 682 bp fragment amplified hygromycin resistance gene (*Hyg*) from pMDC-83 vector was used as the probe for Southern blot analysis. The probe was amplified using DNA DIG-labelling kit (Roche, USA) at 94℃ for 3 min, then at 94℃ for 45 sec, then at 55℃ for 30 sec, then at 72℃ for 90 sec for 30 cycles and then at 72℃ for 10 min.

Ten micrograms of genomic DNA from transgenic tobacco plants were digested with 10 units of restriction enzyme, *BamHI (single-cutter of gene)* for 16 hours at 37℃. The digested DNA was concentrated using DNA concentrator to 20 µl and mixed with 6X DNA loading dye. The DNA was resolved on 0.8% agarose gel in 1X TAE buffer. The digested DNA samples were transferred to a positively charged nitrocellulose membrane (Roche, USA) by capillary transfer. The membrane was treated with short-wavelength ultraviolet light in the UV-crosslinker (120,000 µJ, 2 min) for immobilization of transferred DNA. Before hybridization with probes, membranes were pre-hybridized with 40 ml of hybridization solution [2.85 ml of 1 M $Na_2H_2PO_4$, 12.15 ml of 1 M Na_2HPO_4, 120 µl of 0.5 M EDTA pH 8.0, 30 ml of 20% SDS (Sodium dodecyl sulfate, Sigma, USA), 0.3 g of Blocking reagent (Roche, USA), 14.8 ml of sterilized distilled water in 60 ml] for 4 h with gentle shake. For hybridization, membranes were hybridized with 20 ml of hybridization solution containing 25 ng/ml of DIG-labelled probe for 16 h at 60℃. After hybridization, membranes were washed two times with washing buffer [0.1 x Sodium citrate (SSC), 0.1% SDS] to remove excessive probe from membranes. For gene detection, 1 ml of detection buffer (0.1 ml of 1 M Tri-HCl pH 9.5, 0.1 M NaCl in 1 ml) containing 10 µl of a chemiluminescent substrate [CDP-star (Roche, USA)] was applied on a membrane and was incubated for 5 min at room temperature. Chemiluminescent was detected in ChemiDoc MP imaging system (Biorad, USA).

5.2.4.2 Total RNA and LMW RNA (Low Molecular Weight RNA) extraction

Shoot apex of transgenic and wild-type tobacco plants were used for total RNA isolation using TRIzol reagent (Invitrogen, USA). 100 mg of explants was homogenized in liquid nitrogen using homogenizer (Qiagen, Germany). 1 ml of TRIzol was added to each sample and mixed by inverting. The mixture was incubated for 5 min at room temperature and was centrifuged for 10 min at 4℃. Supernatant was transferred to new 1.5 ml tubes and mixed with 500 µl of chloroform by inverting. After 5 min of incubation at room temperature, the tubes were centrifuged for 10 min at 4℃. The upper phase was carefully transferred into new tubes and 750 µl of ice-cold isopropanol was added for precipitation of total RNA. The mixture was incubated for 10 min at room temperature. Afterward, total RNA was pelleted by centrifugation (13,200 rpm) for 25 min at 4℃. The pellets were washed with 750 µl of 70% (v/v) EtOH. After centrifugation for 5 min at 13,200 rpm at 4℃, supernatant was discarded and pellets were dried for 2 min at room temperature. The pellets were dissolved in TE buffer. Total RNA was treated with TURBO^{TM} DNase (thermos Fisher scientific, USA) following the manufacturer's instructions to remove possible genomic DNA contamination.

LMW RNA was extracted using LiCl method (Rosas-Cárdenas et al., 2011). 100 mg of homogenized frozen tissue was placed in a 1.5 ml tube and 500 µl of LiCl extraction buffer (100 mM Tris-HCl pH 9.0, 1% SDS, 100 mM LiCl, 10 mM EDTA pH 8.0) and 500 µl of phenol pH 8.0 was added. The tubes were mixed using vortex for 1min and were incubated for 5 min at 60℃. The mixtures were centrifuged for 10 min at 14,000 rpm at 4℃. The upper phase was transferred to new 1.5 ml tubes and the tubes were incubated for 15 min at 65℃. 50 µl of 5 M NaCl and 63 µl of 40% polyethylene glycol 8,000 were added and were mixed using vortex for 1min. The mixtures were incubated for 30 min on ice and were centrifuged for 10 min at 14,000 rpm at 4℃. The supernatant which contains LMW RNA was transferred to a new 1.5 ml tube and 500 µl of phenol-chloroform-isoamyl alcohol (25:24:1; v/v/v) was added. The tubes were mixed by inverting and were centrifuged for 10 min at 14,000 rpm at 4℃. The upper phase was transferred to new 1.5 ml tubes. LMW RNA was precipitated by adding 50 µl of 3 M sodium acetate, pH 5.2 and 1,200 µl of absolute ethanol. The mixtures were incubated overnight at -20℃. After centrifugation for 10 min at 14,000 rpm at 4℃, supernatant was discarded and the pellet was dried at room temperature for 2 min. The pellets were dissolved in 20 µl of RNase-free water.

5.2.4.3 Temporal expression analysis of gma-miR156 and gma-miR172

For temporal expression analysis of gma-miR156 and gma-miR172, soybean plants were grown under long day (LD) conditions (16/8h, 400 µmol/m^{-2}s^{-1}, 25±1℃) for 10 days and then transferred to short day (SD) conditions (10/14h, 400 µmol/m^{-2}s^{-1}, 25±1℃). Mature leaves were collected from different time points (SD 0 and 6) and were used for LMW RNA extraction. Stem-loop pulsed reverse transcription protocol (Varkonyi-Gasic et al., 2007) was used for cDNA synthesis. 500 ng of LMW RNA was used for cDNA synthesis. cDNA was synthesized with SUPERSCRIPT^{TM}III Reverse Transcriptase (Invitrogen, USA) using specific gma-miR156 and gma-miR172 RT primer (Table A3). After synthesis of cDNA, 40 µl of RNAse-free water was added to each cDNA template. qRT-PCR was performed with specific primer pairs for gma-miR156a, gma-miR172a (Table A3).

5.2.4.4 Gene expression analysis

To examine the expression level of genes of flowering pathway integrators and target genes of *miR156a* and *miR172a*, RT-PCR and qRT-PCR were performed. For mRNA analysis, 1 µg of total RNA was used for cDNA synthesis. cDNA was synthesized with Superscript^{TM}III Reverse Transcriptase (Invitrogen, USA) using oligo(dT) primer according to manufacturer's protocol. After synthesis of cDNA, 80 µl of RNase-free water was added to each cDNA template. *NtGAPDH* was used as an internal control. RT-

PCR analysis was conducted with 2 µl of cDNA template in a 25 µl reaction volume using *Taq* DNA Polymerase (Invitrogen, USA). Three biological replicates were performed and analysed on 3% agarose gel. For qRT-PCR analysis, 1 µl of cDNA template was used for 10 µl reaction volume. The reactions were pre-incubated at 95v for 3 min, followed by 40 cycles of denaturation at 95℃ for 20 sec, annealing at 60℃ for 20 sec, and extension at 72℃ for 20 sec, in the Agilent Mx3000p (Applied Biosystems) using Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent Technologies, USA). PCR specificity was confirmed by melting curve was analysed to confirm PCR specificity and the 2-ΔΔCt method was used for data analysis (Livak and Schmittgen, 2001). Two technical replicates were performed for each sample. Each run contained a no-template control, and RT-negative sample. Primers used for qRT-PCR and RT-PCR analysis were given in Table A3.

5.3 Results

5.3.1 Bioinformatic analysis of miR156 and miR172 in *Glycine max* L. (cv. Bragg)

Soybean is a palaeopolyploid plant which has multiple copies of the same gene due to duplication of genome (Schmutz et al., 2010). In miRBase data base, soybean has 28 loci for encoding 28 mature miR156 (MIR156 a-z and aa, ab) and 12 loci for encoding 15 mature miR172 (MIR172 a, b3p, b5p, c, d, e, f, g, h3p, h5p, i3p, i5p, j, k and l) (Subramanian et al., 2008; Kulcheski et al., 2011; Wong et al., 2011; Goettel et al., 2014; Zhao et al., 2015). Firstly, evolutionary relationship of miR156 and miR172 families was examined. Precursor miRNA sequences of soybean, Arabidopsis and tobacco were obtained from miRbase and phylogenetic analysis was conducted using MEGA X software. Phylogenetic analysis showed low sequence conservation in precursor miRNA levels among miR156 and miR172 families of soybean, Arabidopsis and tobacco (Figure 5.2). In soybean miR156 family, 28 loci were allocated into eight clades (Figure 5.2A). Twelve loci of soybean miR172 were composed of 4 clades in precursor miRNA sequence level (Figure 5.2B). To compare sequences conservation of mature gma-miR156 and gma-miR172, mature-miRNA sequences of gma-miR156 and gma-miR172 were grouped by sequence similarity. Twenty-eight mature gma-miR156 were categorized into 11 groups and mature gma-miR172 was comprised of 9 groups (Figure 5.3A, B).

5.3.2 Expression patterns of *gma-miR156a* and *gma-miR172a* during transition of soybean

To examine the expression patterns of *gma-miR156a* and *gma-miR172a* during plant development, expression levels of *gma-miR156a* and *gma-miR172a* in mature leaves were examined. For analysis of temporal expression of *gma-miR156a* and *gma-miR172a,* stem-loop qRT-PCR was performed to measure the level of mature miRNA expression in leaves on juvenile stage (10 days after germination: DAG, long day condition at this time point, growth condition was changed to flower inducing condition, short day) and adult stage (16 DAG). The expression level of *gma-miR156a* was higher (3.5 fold) than that of *gma-miR172a* at 10 DAG. The expression level of *gma-miR156a* decreased by 5.2 times at 16 DAG (Figure 5.4). In contrast, a higher expression level of *gma-miR172a* (4.4 fold) than that of *gma-miR156a* was detected at 16 DAG and the expression of *gma-miR172a* was increased by 2.9 fold from 10 DAG suggesting that this inverse expression pattern between *gma-miR156a* and *gma-miR172a* are involved in the vegetative and reproductive development, respectively (Figure 5.4).

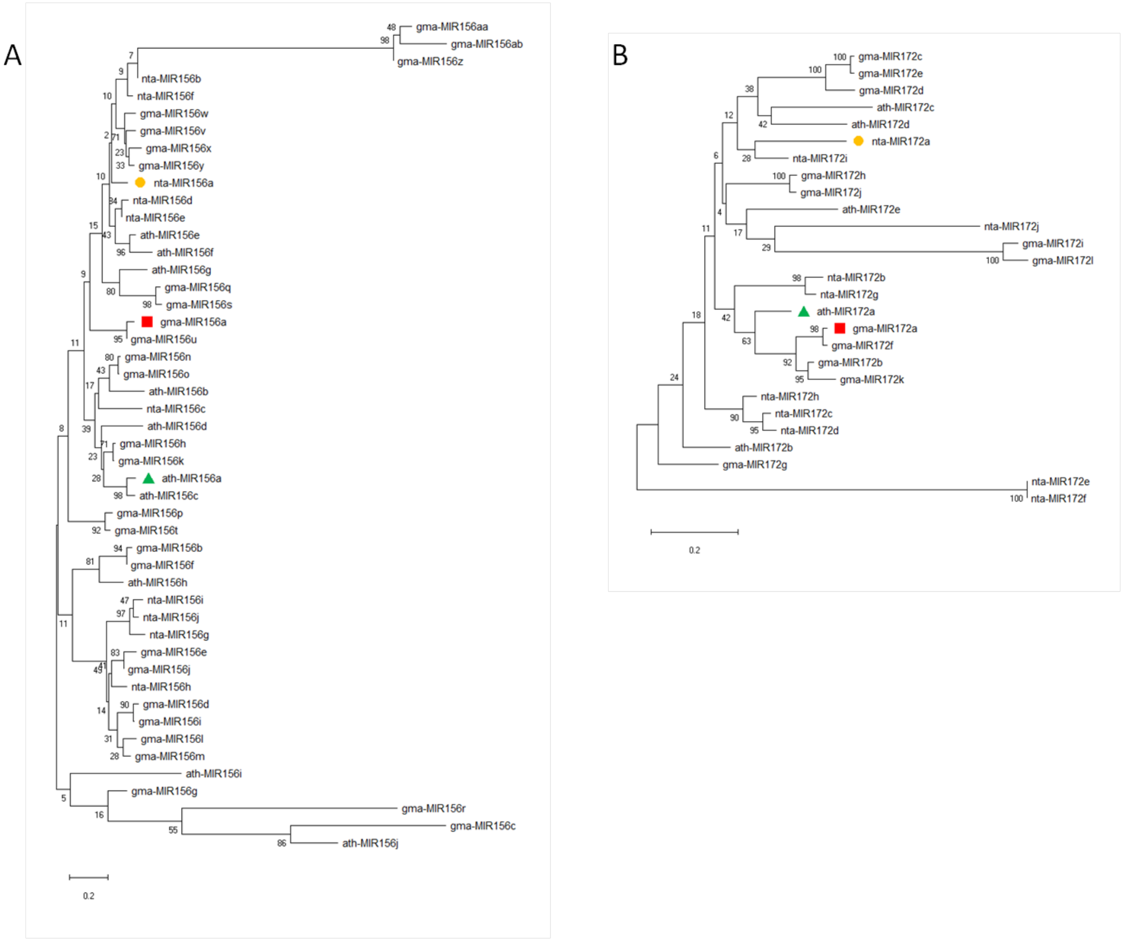
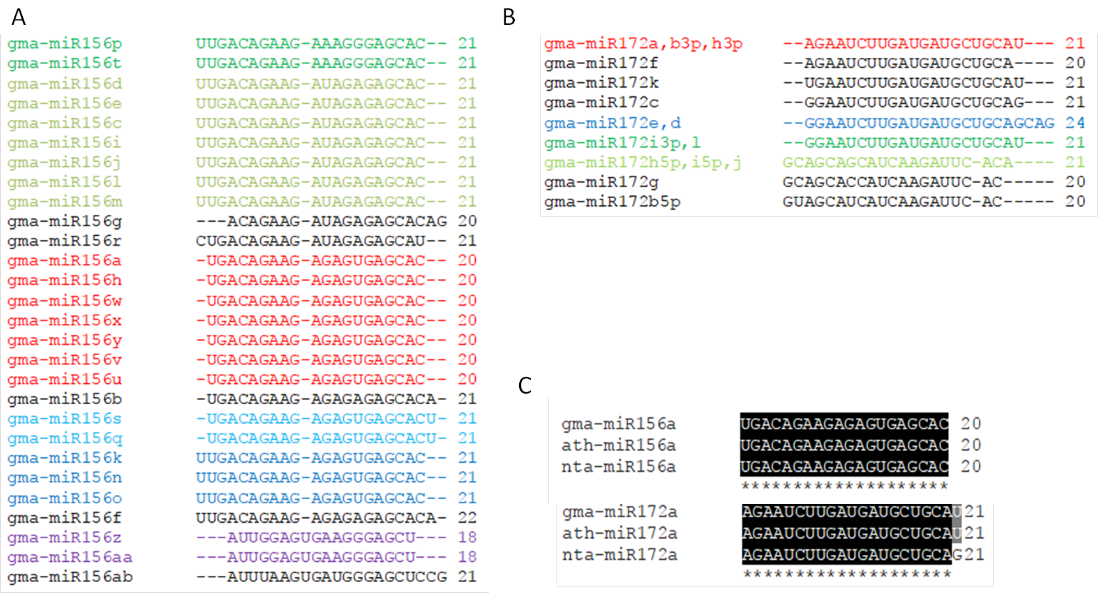


Figure 5. 2. Phylogenetic analysis of soybean miR156 and miR172.

Phylogenetic tree analysis of soybean miR156 (A) and miR172 (B) family with model species, Arabidopsis and tobacco. Soybean miR156 family are separated into 8 clades and total 12 soybean miR172 loci are allocated into 4 clades. Low conservation of precursor miRNA sequences was observed among soybean (*gma-miR156a*, *gma-miR172a*, red square), Arabidopsis (*ath-miR156a, ath-miR172a*, green triangle) and tobacco (*nta-miR156a, nta-miR172a*, yellow circle). Phylogenetic tree was constructed using MEGA 7.0 with neighbour-joint-analysis, 100 replicates bootstrap. Precursor miRNA sequences were obtained from miRbase (http://www.miRbase.org). The bootstrap values were shown at each node. The abbreviations: gma- soybean (*Glycine max* L.), ath- Arabidopsis (*Arabidopsis thaliana*), nta- tobacco (*Nicotiana tabacum* L.).



A

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gma-miR156p       UUGACAGAAG-AAAGGGAGCAC-- 21
gma-miR156t       UUGACAGAAG-AAAGGGAGCAC-- 21
gma-miR156d       UUGACAGAAG-AUAGAGAGCAC-- 21
gma-miR156e       UUGACAGAAG-AUAGAGAGCAC-- 21
gma-miR156c       UUGACAGAAG-AUAGAGAGCAC-- 21
gma-miR156i       UUGACAGAAG-AUAGAGAGCAC-- 21
gma-miR156j       UUGACAGAAG-AUAGAGAGCAC-- 21
gma-miR156l       UUGACAGAAG-AUAGAGAGCAC-- 21
gma-miR156m       UUGACAGAAG-AUAGAGAGCAC-- 21
gma-miR156g       ---ACAGAAG-AUAGAGAGCACAG 20
gma-miR156r       CUGACAGAAG-AUAGAGAGCAU-- 21
gma-miR156a       -UGACAGAAG-AGAGUGAGCAC-- 20
gma-miR156h       -UGACAGAAG-AGAGUGAGCAC-- 20
gma-miR156w       -UGACAGAAG-AGAGUGAGCAC-- 20
gma-miR156x       -UGACAGAAG-AGAGUGAGCAC-- 20
gma-miR156y       -UGACAGAAG-AGAGUGAGCAC-- 20
gma-miR156v       -UGACAGAAG-AGAGUGAGCAC-- 20
gma-miR156u       -UGACAGAAG-AGAGUGAGCAC-- 20
gma-miR156b       -UGACAGAAG-AGAGAGAGCACA- 21
gma-miR156s       -UGACAGAAG-AGAGUGAGCACU- 21
gma-miR156q       -UGACAGAAG-AGAGUGAGCACU- 21
gma-miR156k       UUGACAGAAG-AGAGUGAGCAC-- 21
gma-miR156n       UUGACAGAAG-AGAGUGAGCAC-- 21
gma-miR156o       UUGACAGAAG-AGAGUGAGCAC-- 21
gma-miR156f       UUGACAGAAG-AGAGAGAGCACA- 22
gma-miR156z       ---AUUGGAGUGAAGGGAGCU--- 18
gma-miR156aa      ---AUUGGAGUGAAGGGAGCU--- 18
gma-miR156ab      ---AUUUAAGUGAUGGGAGCUCCG 21
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B

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gma-miR172a,b3p,h3p    --AGAAUCUUGAUGAUGCUGCAU--- 21
gma-miR172f            --AGAAUCUUGAUGAUGCUGCA---- 20
gma-miR172k            --UGAAUCUUGAUGAUGCUGCAU--- 21
gma-miR172c            --GGAAUCUUGAUGAUGCUGCAG--- 21
gma-miR172e,d          --GGAAUCUUGAUGAUGCUGCAGCAG 24
gma-miR172i3p,l        --GGAAUCUUGAUGAUGCUGCAU--- 21
gma-miR172h5p,i5p,j    GCAGCAGCAUCAAGAUUC-ACA---- 21
gma-miR172g            GCAGCACCAUCAAGAUUC-AC----- 20
gma-miR172b5p          GUAGCAUCAUCAAGAUUC-AC----- 20
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C

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gma-miR156a       UGACAGAAGAGAGUGAGCAC 20
ath-miR156a       UGACAGAAGAGAGUGAGCAC 20
nta-miR156a       UGACAGAAGAGAGUGAGCAC 20
                  ********************

gma-miR172a       AGAAUCUUGAUGAUGCUGCAU21
ath-miR172a       AGAAUCUUGAUGAUGCUGCAU21
nta-miR172a       AGAAUCUUGAUGAUGCUGCAG21
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Figure 5. 3. Sequence comparison of soybean mature miR156 and miR172 family.

Mature miRNA sequences of gma-miR156 and gma-miR172 were grouped by sequence similarity. Colours represent each group with identical sequences. (A) Mature gma-miR156 sequences were categorized into 11 groups. (B) Mature gma-miR172 consists of 9 groups. (C) Sequence alignment analysis of mature-*miR156a* and *miR172a* of soybean, Arabidopsis and tobacco was conducted using Clustal Omega. Grey boxes represent sequence differences and the black boxes and asterisks show identical sequences.

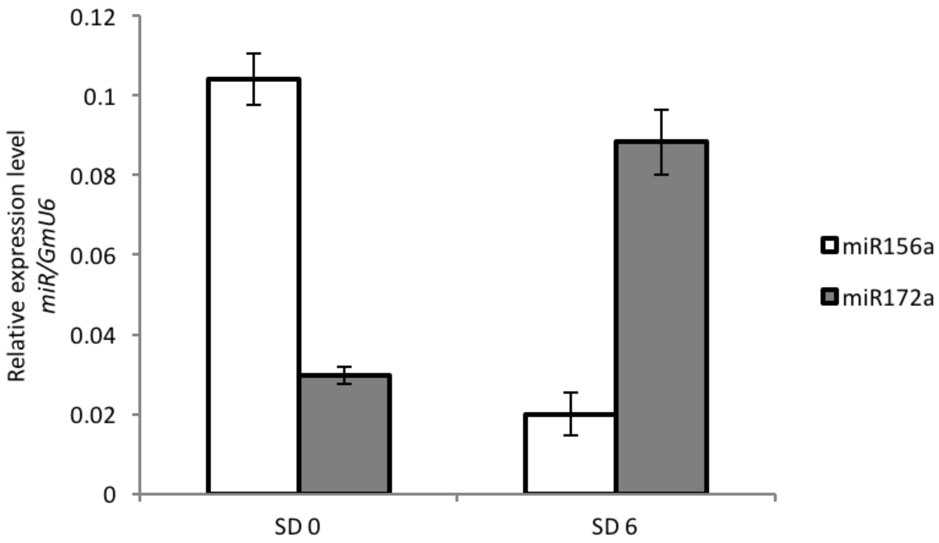


Figure 5. 4. Expression of *miR156a* and *miR172a* in soybean.

Expression of *miR156a* and *miR172a* was examined using qRT-PCR. Expression patterns of *miR156a* and *miR172a* were inversely related. Expression level of mature *miR156a* and *miR172a* were normalized by *GmU6*. Error bars: standard deviation (±SD). Data were from three independent biological replicates with two technical replicates (n=15).

5.3.3. Expression analysis of *gma-miR156a* and *gma-miR172a* in transgenic tobacco plants

5.3.3.1 Generation of transgenic plants

Transgenic tobacco plants were produced using *Agrobacterium*-mediated transformation method. Total 45 putative *gma-miR156a* transgenic tobacco shoots and 37 putative *gma-miR172a* transgenic tobacco shoots were regenerated from infected explants and all putative regenerated shoots were transferred to rooting medium. Forty of 45 putative *gma-miR156a* transgenic plants and 34 of 37 putative *gma-miR172a* transgenic shoots produced roots after 4 weeks of culture on rooting medium (Figure 5.5). Twenty-two putative transgenic plants with fully developed roots were randomly selected and were transferred to soil for acclimatization. Total 18 and 14 putative transgenic plants survived in the glasshouse after acclimatization. None of the survived putative transgenic plants showed abnormal growth and morphological differences, compared to wild-type (germinated from seeds) and produced fertile flowers resulting in set seeds. The functions of *gma-miR156a* and *gma-miR172a* were examined in transgenic tobacco harbouring overexpressed *gma-miR156a* and overexpressed *gma-miR172a*, respectively.

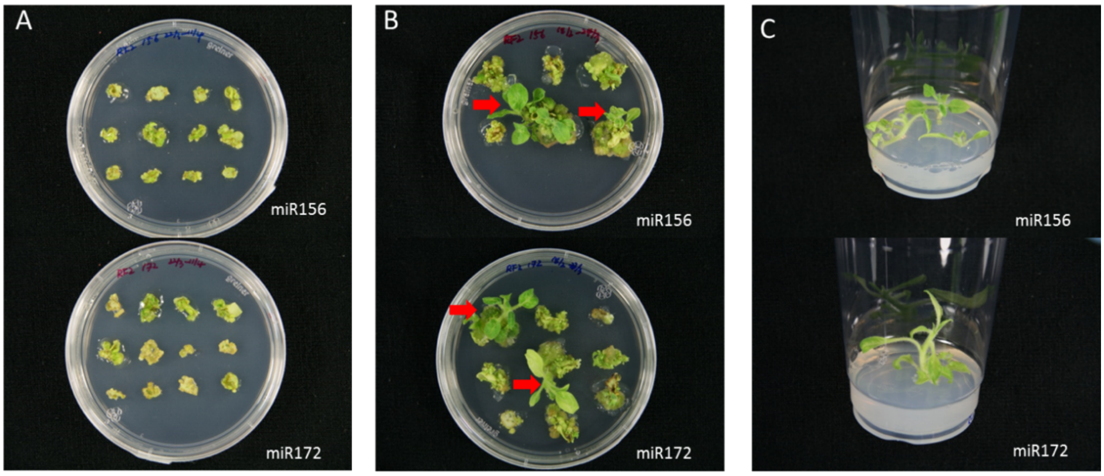
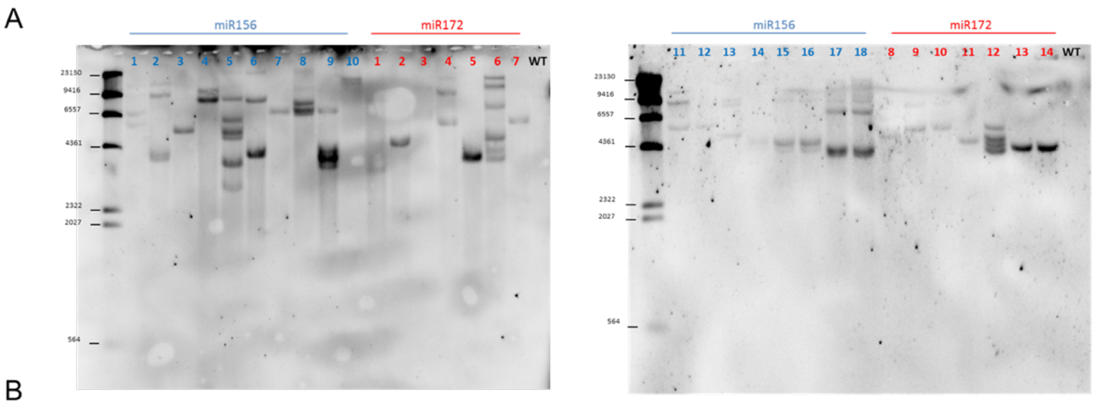


Figure 5. 5. Production of transgenic tobacco plants using *Agrobacterium*-mediated transformation.

(A) shoot primordia was regenerated from explants after 2 weeks of culture on selection medium containing 30 mg/L of hygromycin. (B) putative transgenic shoots (red arrows) were regenerated from explants on selection medium after 4 weeks of culture. (C) putative transgenic shoots were rooted on rooting medium.

5.3.3.2 Southern blot analysis

To confirm the gene insertion and copy numbers in transgenic plants, Southern blot analysis was conducted. Eighteen putative gma-miR156a transgenic tobacco plants and 14 putative transgenic tobacco plants in T0 generation were randomly selected and used for Southern blot analysis. Ten micrograms of genomic DNA of each line was digested with *BamHI* restriction enzyme for 16 hours at 37℃. DIG labelled-*Hyg* gene fragment was used as a probe for hybridization. In Southern blot analysis, single to multiple integration of T-DNA in transgenic lines was confirmed (Figure 5.6). Single copy gene insertion was detected in lanes 3, 7, 12 and 14 of putative *gam-miR156a* transgenic tobacco plants and lanes 1, 2, 5, 7, 8, 11, 13 and 14 in putative *gma-miR172a* transgenic tobacco plants. Multiple copies (2 to 7 copies) were identified in 14 lines of *gma-miR156a* and 7 lines of *gma-miR172a* transgenic tobacco plants (Figure 5.6). Fewer transgene copies (2 to 3 copies) were observed in lanes 1, 2, 4, 6, 8, 9, 10, 11, 13, 15 and 16 of *gma-miR156a* of transgenic plants and 3, 4, 8, 9 and 10 of *gma-miR172a* transgenic lines (Figure 5.6). Higher transgene copies (4 to 6 copies) were observed in lanes 5, 17 and 18 of *gma-miR156a* and lanes 6 and 12 of *gma-miR172a* (Figure 5.6). Transgenic lines with single gene insertion (lines 7 and 14 of *gma-miR156a* transgenic plants and lines 8 and 14 of *gma-miR172a* transgenic plants) were used for further analysis.



B

1 ATGAAAAAGCCTGAACTCACCGCGACGTCTGTCGAGAAGTTTCTGAT**CGAAAAGTTCGACAGCGTCTC**CGACCTGATGCAGCTCTCGGAGGGCGAAGAATCTCGTGCTTTCAGCTTCGATGTAGGAGGGCGTGGATATGTCCTGCGGGTA 150

151 AATAGCTGCGCCGATGGTTTCTACAAAGATCGTTATGTTTATCGGCACTTTGCATCGGCCGCGCTCCCGATTCCGGAAGTGCTTGACATTGGGGAGTTTAGCGAGAGCCTGACCTATTGCATCTCCCGCCGTGCACAGGGTGTCACGTTG 300

301 CAAGACCTGCCTGAAACCGAACTGCCCGCTGTTCTACAACCGGTCGCGGAGGCTATGGATGCGATCGCTGCGGCCGATCTTAGCCAGACGAGCGGGTTCGGCCCATTCGGACCGCAAGGAATCGGTCAATACACTACATGGCGTGATTTC 450

451 ATATGCGCGATTGCTGATCCCCATGTGTATCACTGGCAAACTGTGATGGACGACACCGTCAGTGCGTCCGTCGCGCAGGCTCTCGATGAGCTGATGCTTTGGGCCGAGGACTGCCCCGAAGTCCGGCACCTCGTGCACGCGGATTTCGGC 600

601 TCCAACAATGTCCTGACGGACAATGGCCGCATAACAGCGGTCATTGACTGGAGCGAGGCGATGTTCGGGGATTCCCAATACGAGGTCGCCAACATCTTCTTCTGGAGGC**CGTGGTTGGCTTGTATGGAGC**AGCAGACGCGCTACTTCGAG 750

751 CGGAGGCATCCGGAGCTTGCAGGATCGCCACGACTCCGGGCGTATATGCTCCGCATTGGTCTTGACCAACTCTATCAGAGCTTGGTTGACGGCAATTTCGATGATGCAGCTTGGGCGCAGGGTCGATGCGACGCAATCGTCCGATCCGGA 900

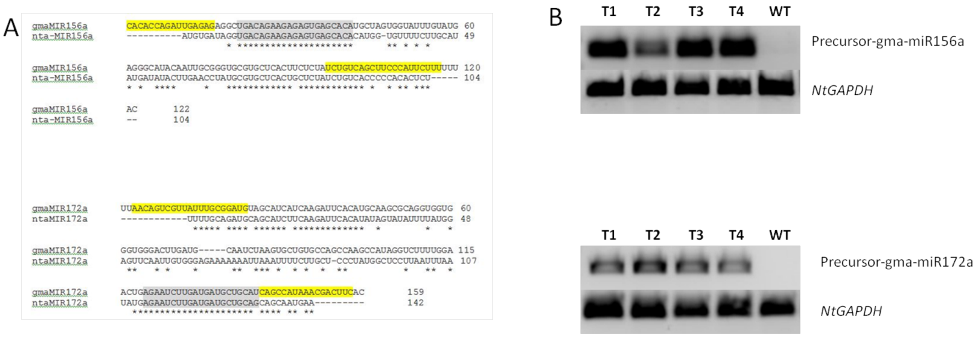
901 GCCGGGACTGTCGGGCGTACACAAATCGCCCGCAGAAGCGCGGCCGTCTGGACCGATGGCTGTGTAGAAGTACTCGCCGATAGTGGAAACCGACGCCCCAGCACTCGTCCGAGGGCAAAGAAATAG 1026

Figure 5. 6. Southern blots analysis of transgenic tobacco plants.

(A) *BamHI*-digested genomic DNA of putative *gma-miR156a* (miR156) transgenic tobacco plants and *gma-miR172a* (miR172) was hybridized with DIG-labelled *Hyg*-specific probe. Variable numbers of gene insertion (1-7 copies) were detected in transgenic plants. No insertion of gene was detected in non-transformed tobacco plants (wild-type). (B) The nucleotide sequence of *Hyg* gene: primer annealing regions for amplified DIG labelled *Hyg* probe for Southern blot analysis is indicated in red. miR156: putative *gma-miR156a* transgenic plants (1-18), miR172: putative *gma-miR172a* transgenic lines (1-14), WT: untransformed control. Ladder: DNA molecular weight marker II (Roche, USA).

5.3.3.3 Analysis of expression of *gma-miR156a* and *gma-miR172a* in transgenic tobacco plants

To examine the expression of *gma-miR156a* and *gma-miR172a* in transgenic plants, reverse-transcript PCR (RT-PCR) was performed. Since soybean mature *miR156a* and *miR172a* sequences showed 100% and 95% match with tobacco, integrated expression levels of miRNAs of both soybean and tobacco are detected in RT-PCR. For instance, it would be difficult to distinguish whether the expression level was from endogenous tobacco miRNAs (*nta-miR156a* and *nta-miR172a*) or derived from soybean miRNAs (*gma-miR156a* and *gma-miR172a*) when similar levels of expression was detected in transgenic and wild-type tobacco plants. To overcome this difficulty, precursor miRNA expression was examined. Sequence alignment analysis of precursor *miR156a* and *miR172a* between soybean and tobacco showed that sequences of precursor of *gma-miR156a* and *gma-miR172a* have low sequence similarity compared to tobacco (Figure 5.7A). Specific primer pairs to detect precursor *gma-miR156a* and *gma-miR172a* were designed and were used for RT-PCR analysis (Figure 5.7A, Table A3). cDNA of T2 generation of transgenic tobacco plants were used as templates and *NtGAPDH* gene was used as an internal control. In RT-PCR analysis, expression of precursor *gma-miR156a* and *gma-miR172a* were only detected in independent transgenic tobacco plants suggesting that soybean *miR156a* and *miR172a* were successfully introduced and expressed in transgenic tobacco genome (Figure 5.7B).



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gmaMIR156a      CACACCAGAUUGAGAGAGGCUGACAGAAGAGAGUGAGCACAUGCUAGUGGUAUUUGUAUG 60
nta-MIR156a     ----------AUGUGAUAGGUGACAGAAGAGAGUGAGCACACAUGG-UGUUUUCUUGCAU 49
                                  * *********************      ** * * *

gmaMIR156a      AGGGCAUACAAUUGCGGGUGCGUGCUCACUUCUCUAUCUGUCAGCUUCCCAUUCUUUUUU 120
nta-MIR156a     AUGAUAUACUUGAACCUAUGCGUGCUCACUGCUCUAUCUGUCACCCCCACACUCU----- 104
                * *  ****     *   ************ ************ *  * ** ***

gmaMIR156a      AC         122
nta-MIR156a     --         104


gmaMIR172a      UUAACAGUCGUUAUUUGCGGAUGUAGCAUCAUCAAGAUUCACAUGCAAGCGCAGGUGGUG 60
ntaMIR172a      ------------UUUUGCAGAUGCAGCAUCUUCAAGAUUCACAUAUAGUAUAUUUUAUGG 48
                             ***** **** ****** *************  *        *   *

gmaMIR172a      GGUGGGACUUGAUG-----CAAUCUAAGUGCUGUGCCAGCCAAGCCAUAGGUCUUUUGGA 115
ntaMIR172a      AGUUCAAUUGUGGGAGAAAAAAAUUAAAUUUUCUUGCU-CCCUAUGGCUCCUUAAUUUAA 107
                 **   * *    *      **  *** *  * *  *  **          *   **  *

gmaMIR172a      ACUGAGAAUCUUGAUGAUGCUGCAUCAGCCAUAAACGACUUCAC         159
ntaMIR172a      UAUGAGAAUCUUGAUGAUGCUGCAGCAGCAAUGAA---------         142
                  ********************** **** ** **
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Figure 5. 7. Analysis of expression of *gma-miR156a* and *gma-miR172a* in transgenic tobacco plants.

(A) Sequence alignment analysis of precursor *gma-miR156a* and *gma-miR172a* with tobacco precursor *miR156a* (*nta-miR156a*) and *miR172a* (*nta-miR172a*) showed low sequence similarity. Yellow indicates primer regions, grey shows mature miRNA sequence. Asterisks indicate identical sequences. (B) RT-PCR analysis of precursor *gma-miR156a* and *gma-miR172a* in transgenic tobacco plant. Amplified precursor of *gma-miR156a* and *gma-miR172a* were detected in transgenic tobacco plants. WT: wild-type plants. *NtGAPDH* was used as an internal control.

5.3.3.4 Segregation analysis

To examine inheritance, T1 lines with single T-DNA insertion (*gma-miR156a* lines 7 and 14 and *gma-miR172a* lines 8 and 14) and multiple T-DNA inserted lines (*gma-miR156a* lines 2, 4 and 9 and *gma-miR172a* lines 1 and 9) were tested by hygromycin selection. Mature seeds of T1 generation were placed on selection medium containing 30 mg/L of hygromycin. After 4 weeks, hygromycin resistance was examined. Hygromycin-susceptible seeds did germinate but showed stunted growth in successive culture (Figure 5.8). In contrast, hygromycin resistant seedlings exhibited normal growth similar to wild-type, grown in non-selection medium (Figure 5.8). In T1 generation, single T-DNA copy inserted lines (*gma-miR156a* lines 7 and 14 and *gma-miR172a* line 14) showed Mendelian inheritance patterns (3:1) (Table 5.1). However, *gma-miR172a* line 1 did not show the single gene insertion inheritance pattern (3:1) (Table 5.1). Other transgenic lines with multiple T-DNA insertions showed deviations from monogenic segregation. To discriminate homozygous transgenic lines, T2 progenies of independent T1 lines with single copy of T-DNA insertion (*gma-miR156a*: lines 7 and 14 and *gma-miR172a*: lines 8 and 14) were screened on selection medium. Total 4 homozygous lines (lines 2 and 15 of *gma-miR15*6a and lines 8 and 14 of *gma-miR172a*) were obtained (Table 5.2). T1 generation with single T-DNA insertion (*gma-miR156a* lines 7 and 14 and *gma-miR172a* lines 8 and 14) and homozygous T2 generation (*gma-miR156a*: lines 7-1 and 14-15 and *gma-miR172a*: lines 8-6 and 14-19) were used for analysis.

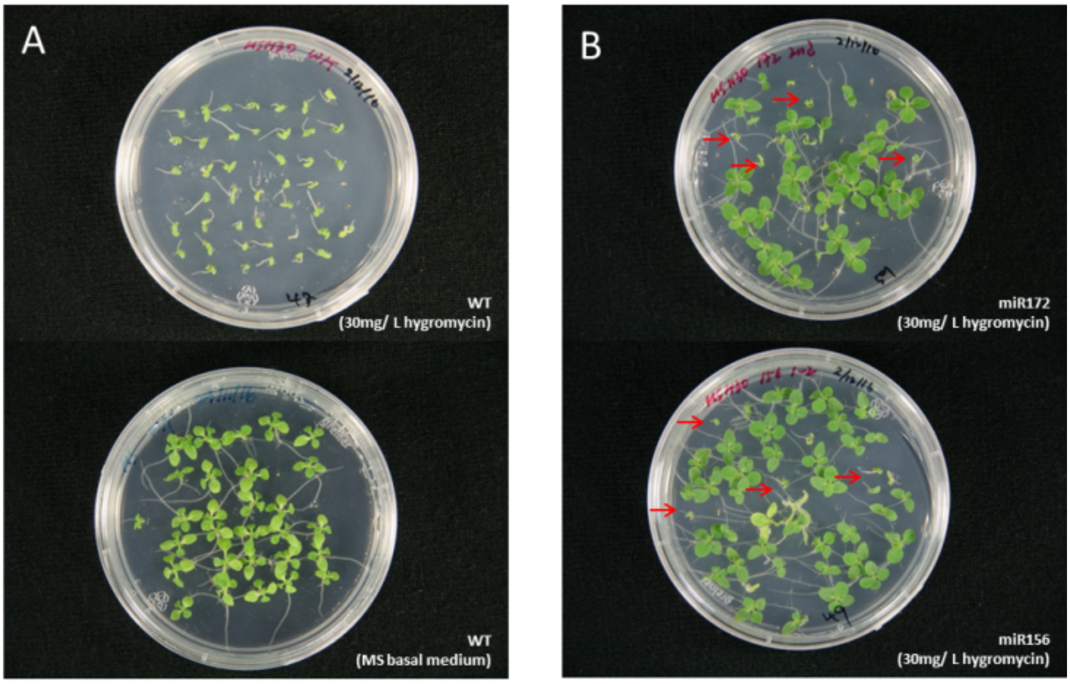

Figure 5. 8. Selection of T1 generation of transgenic tobacco plants harbouring *gma-iR156a* and *gma-miR172a*.

Hygromycin-resistant transgenic seedlings showed normal growth on selection medium (hygromycin 30 mg/L). Wild-type and Hygromycin-susceptible seedlings showed stunted growth on selection medium after 4 weeks of culture (Red arrow). (A) Wild-type seedling on selection medium (upper photo) and non-selection medium (lower photo). (B) Hygromycin-resistant seedlings on selection medium.

Table 5. 1. Analysis of segregation for Hygromycin-resistant gene in T1 generation of transgenic tobacco plants harbouring *gma-miR156a* and *gma-miR172a*.

Constructs	Lines	Copy numbers in T0	Total numbers of resistant plants (H^R)	Total numbers of susceptible plants (H^S)	Segregation ratio	χ^2 value (3:1)
miR156	2	3	63	37	1.7:1	7.68 $^{(h.s.)}$
	4	2	112	12	9.3:1	15.53 $^{(h.s.)}$
	7	1	88	19	4.6:1	2.99
	9	4	158	0	1:0	52.67 $^{(h.s.)}$
	14	1	126	40	3.2:1	0.07
miR172	1	2	87	15	5.8:1	5.76 $^{(h.s.)}$
	8	1	55	46	1.2:1	22.74 $^{(h.s.)}$
	9	2	38	7	5.4:1	2.14
	14	1	68	25	2.7:1	0.18

Data was obtained after 4 weeks of selection (30 mg/L of hygromycin). H^R: hygromycin-resistant plants showed normal growth. H^S: hygromycin-susceptible plants exhibited stunted growth. Lines 7 and 14 of *gma-miR156a* and 8 and 14 of *gma-miR172a* showed Mendelian inheritance patterns (3:1). χ^2 values for 1d*f*= 3.84 at 5% *P*-level. (H.S.): significant deviations from monogenic segregation

Table 5. 2. Genotype of homozygous T2 transgenic plants.

Constructs	T1 transgenic lines	T2 transgenic lines	Copy numbers in T0	Total numbers of resistant plants (H^R)	Total numbers of susceptible plants (H^S)	Segregation ratio	χ^2 value (1:0)
miR156	7	1	1	93	0	1:0	-
	7	10	1	55	10	5.5:1	1.54
	14	15	1	127	0	1:0	-
miR172	8	2	1	38	7	5.4:1	1.09
	8	4	1	106	12	8.8:1	1.22
	8	6	1	99	0	1:0	-
	8	10	1	102	0	1:0	-
	14	19	1	134	0	1:0	-
	14	20	1	90	5	18:1	0.26

Data was obtained after 4 weeks of selection (30 mg/L of hygromycin). H^R: hygromycin-resistant plants showed normal growth. H^S: hygromycin-susceptible plants exhibited stunted growth. Homozygous T2 generations were obtained from 2 lines of *gma-miR156a* (lines 1 and 15) and 3 lines of *gma-miR172a* (6, 10 and 19). χ^2 values for 1d*f*= 3.84 at 5% *P*-level.

5.3.4. Ectopic expression of *gma-miR156a* has a significant effect on leaf morphology

Since morphological change on leaves is one of the factors distinguishing between vegetative and reproductive phases (Poethig, 1990; Feng et al., 2016), the morphological factors of leaves such as length and width of leaf and petiole, length-to-width ratio (L/W ratio) of leaf, total leaf numbers and leaf area were examined to investigate roles of *gma-miR156a* and *gma-miR172a*. In this study, distinct morphological alterations were observed. Ectopic expression of *gma-miR156a* in transgenic tobacco plants had a significant effect on leaf shape. *gma-miR156a* transgenic plants had round leaf shapes compared to wild-type, whereas *gma-miR172a* transgenic lines had narrower leaf shapes in T0, T1 and T2 generations (Figure 5.9). To analyse the leaf shape, L/W ratio of leaf was calculated by dividing the length of leaf by its width. Expected value of round shape is 1 and narrow shape has a value of ratio bigger than 1. *gma-miR156a* transgenic plants had smaller values (2.2±0.58) than wild-type (2.9±0.36), validating that leaves of *gma-miR156a* transgenic plants were rounder than those of wild-type. On the other hand, *gma-miR172a* transgenic plants showed bigger values (3.4±0.46) than wild-type in T0 generation (Figure 5.10A). Moreover, obvious differences on leaf shape were observed in T1 and T2 generations. T1 generation of *gma-miR156a* transgenic plants had rounder shaped leaves (1.5±0.15). However, L/W ratio between wild-type (2.0±0.24) and *gma-miR172a* transgenic plants (1.9±0.27) showed no significant difference (Figure 5.10B). Similarly, the L/W ratio of *gma-miR156a* (1.8±0.18) was smaller than those of wild-type (2.6±0.24) in T2 generation. On the other hand, leaves of *gma-miR172a* transgenic plants (3.2±0.43) were narrower than those of wild-type (2.6±0.24) (figure 5.10C).

The length and width of fully expanded leaves were measured. T0 generation of *gma-miR156a* transgenic plants had shortest leaf lengths (14.8±3.01 cm) while the leaf width of *gma-miR156a* transgenic plants (7.0±1.58 cm) was not significantly different from that of wild-type (6.6±1.34 cm). The leaf area of *gma-miR156a* (61.9±23.62 cm^2) was not significantly different to that of wild-type (67.8±25.20 cm^2). On the other hand, there was no significant difference on leaf length between T0 generation of *gma-miR172a* transgenic plants (16.9±2.92 cm) and wild-type (18.9±2.61 cm). However, the leaf width of *gma-miR172a* transgenic plants (5.1±1.28 cm) was the narrowest, resulting in smallest leaf area (47.1±17.49 cm^2). (Figure 5.11) Differences in leaf morphology were apparent in T1 and T2 generations. T1 generation of *gma-miR156a* transgenic plants had the shortest leaf length (7.1±1.44 cm) and width (5.0±1.00 cm) which contributed to formation of round shape of leaves with smallest leaf area (23.3±9.63 cm^2). In contrast, *gma-miR172a* transgenic lines had similar leaf length (10.9±2.52 cm) and width (5.7±1.35 cm) with wild-type (leaf length: 11.6±1.70 cm, leaf width: 6.0±1.24 cm). In addition, leaf area of *gma-miR172a* (40.4±18.15 cm^2) and wild-type (45.1±14.64 cm^2) showed no significant difference (Figure 5.12). Similar results were also observed in T2 generation. *gma-miR156a* plants had the smallest leaf area (22.1±5.58 cm^2) with shortest leaf length (7.5±0.98 cm) and width (4.2±0.45 cm). *gma-miR172a* transgenic plants had similar leaf length (13.5±1.37 cm) and leaf area (41.8±6.34 cm^2) while the width of *gma-miR172a* transgenic plants (4.4±0.60 cm) was smaller than wild-type (5.2± 0.60 cm), contributing to the formation of narrower shapes of leaf (Figure 5.13).

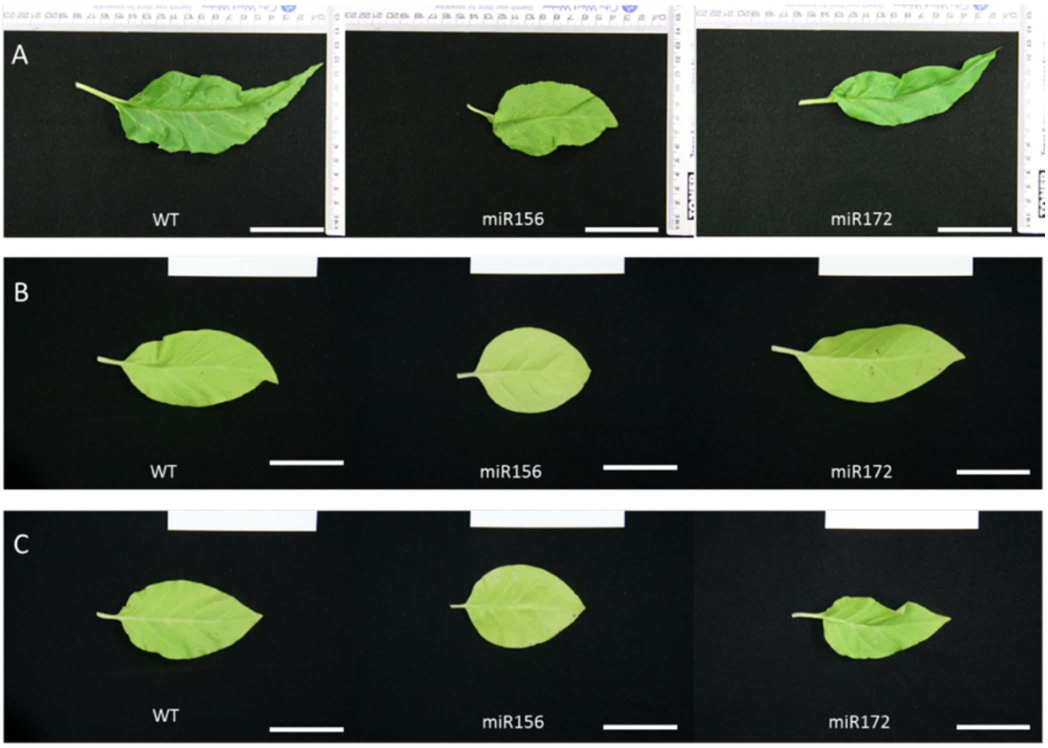

Figure 5. 9. Morphological differences in leaf shape of transgenic tobacco plants.

Representative fully expanded leaves of wild-type (WT), *gma-miR156a* (miR156) overexpressing and *gma-miR172a* (miR172) overexpressing transgenic tobacco plants of T0, T1 (line 7 of *gma-miR156a* and line 8 of *gma-miR172a*) and T2 (line 7-1 of *gma-miR156a* and line 8-6 of *gma-miR172a*) generations. Ectopic expression of *gma-miR156a* and *gma-miR172a* altered leaf shape. White bar: 5cm

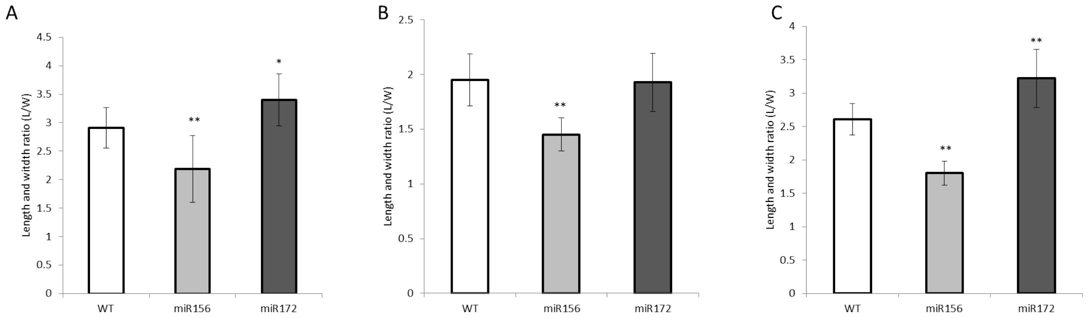

Figure 5. 10. Comparison of length and width ratio of tobacco leaves.

Length and width ratio (L/W ratio) was calculated on fully expanded leaves of wild-type plants (WT), *gma-miR156a* (miR156) and *gma-miR172a* (miR172) transgenic tobacco plants in T0, T1 and T2 generations. (A) L/W ratio of T0 generation, n=10 (WT), n=18 (miR156), n=14 (miR172). (B) L/W ratio of T1 generation, line 7 of *gma-miR156a* and line 8 of *gma-miR172a*, n=20, (C) L/W ratio of T2 generation, line 7-1 of *gma-miR156a* and line 8-6 of *gma-miR172a*, n=20. *gma-miR156a* transgenic plant had a round shape of leaf. Leaf shape of *gma-miR172a* transgenic tobacco plants were narrower than that of wild-type. Bar: standard deviation (±SD). The significant differences were calculated using student t-test. Asterisk: *P>0.05, **P>0.01.

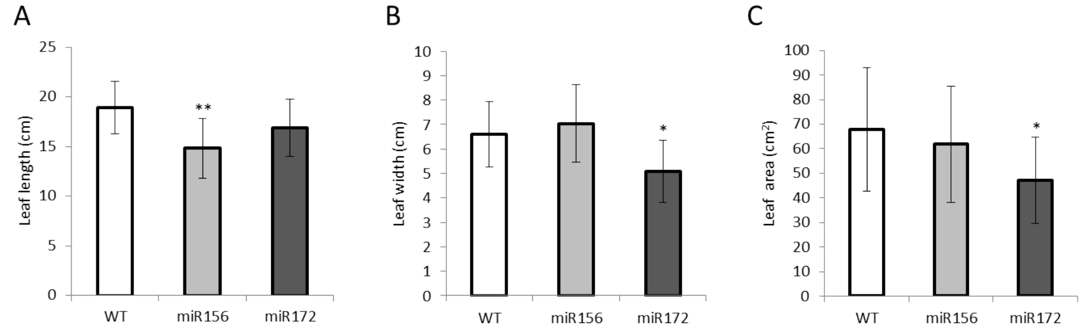

Figure 5. 11. Leaf morphology analysis of *gma-miR156a* and *gma-miR172a* transgenic tobacco plants (T0 generation).

(A) leaf length, (B) leaf width and (C) leaf area of fully expanded leaves. *gma-miR156a* transgenic plants had shortest but widest leaf. Leaf width of *gma-miR172a* transgenic plants was narrowest, resulting in smallest leaf area. T0 generation of wild-type (WT, 10 plants), *gma-miR156a* (miR156, 18 plants) and *gma-miR172a* (miR172, 14 plants) were used for analysis. Leaf area of fully expanded leaves was measured using Li-COR leaf area machine. Bar: standard deviation (±SD). Significant differences were calculated using student t-test. Asterisk: *P>0.05, **P>0.01.

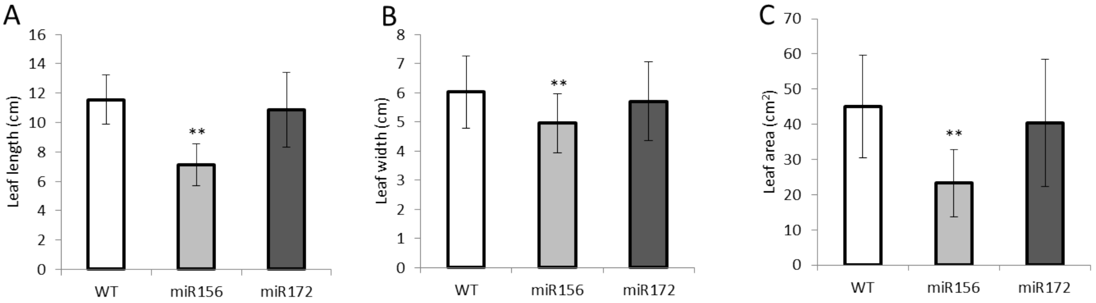

Figure 5. 12. Analysis of leaf length, width and area of *gma-miR156a* and *gma-miR172a* transgenic tobacco plants (T1 generation).

(A) leaf length, (B) leaf width and (C) leaf area of fully expanded leaves. *gma-miR156a* transgenic tobacco had the shortest leaf length and width, and the smallest leaf area. 7-week-old wild-type (WT), *gma-miR156a* (miR156, line 7) and *gma-miR172a* (miR172, line 8) were used for analysis. n=20, Bar: standard deviation (±SD). Significant differences were calculated using student t-test. Asterisk: *P>0.05, **P>0.01.

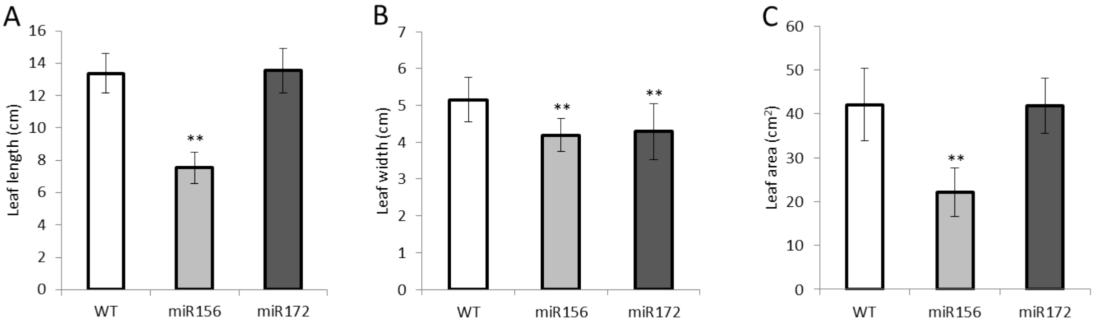

Figure 5. 13. Comparison of leaf morphology of *gma-miR156a* and *gma-miR172a* transgenic tobacco plants (T2 generation).

(A) leaf length, (B) leaf width and (C) leaf area of fully expanded leaves. *gma-miR156a* transgenic tobacco had the shortest leaf length and width, and smallest leaf area. 4-week-old wild-type (WT), *gma-miR156a* (miR156, line 7-1) and *gma-miR172a* (miR172, line 8-6) were used for analysis. n=20, Bar: standard deviation (±SD). Significant differences were calculated using student t-test. Asterisk: *P>0.05, **P>0.01.

5.3.5. Ectopic expression of *gma-miR156a* and *gma-miR172a* in transgenic plants alter chlorophyll content

Transgenic tobacco plants harbouring *gma-miR156a* had a slightly pale green colour in their leaves compared to wild-type and *gma-miR172a* transgenic tobacco plants. To examine whether these colour differences on leaves related to chlorophyll content, chlorophyll content was measured in leaves of *gma-miR156a* and *gma-miR172a* transgenic tobacco plants. Total chlorophyll content was measured on leaves from three locations of plants, apice (top), medial (middle) and basal (bottom) (Figure 5.14D). Reduction of total chlorophyll content was observed in *gma-miR156a* transgenic tobacco plants. T0 generation of *gma-miR156a* transgenic plants had the lowest total chlorophyll content (top: 27.2±4.83, middle: 22.4±3.96, bottom: 13.9±3.58). Similar results were observed in T1 and T2 generations. The total chlorophyll content of *gma-miR156a* transgenic plants was lower than that of wild-type in T1 (average 32.4%) and T2 (average 34.4%) generations of *gma-miR156a* transgenic plants. However, the total chlorophyll content of *gma-miR172a* plants was not significantly different from that of wild-type plants (Figure 5.14).

5.3.6. Ectopic expression of *gma-miR156a* and *gma-miR172a* in transgenic plants exhibited changes in photosynthesis rate

Photosynthesis rate was decreased in T1 and T2 generations of *gma-miR156a* transgenic plants. Overall photosynthesis rate of T1 *gma-miR156a* transgenic plants (17.5%) was lower than that of wild-type. However, a significant reduction of photosynthesis rate (28.3%) was observed on medial leaves (Figure 5.15B). T2 generation of *gma-miR156a* transgenic plants had a significantly lower photosynthesis rate (15.1%) than wild-type plants (Figure 5.15C). In contrast, *gma-miR172a* transgenic tobacco plants had a similar photosynthesis rate with wild-type except that basal leaves of T1 and T2 generations had a higher photosynthesis rate (T1: 22.6%, T2: 16.6%) (Figure 5.15).

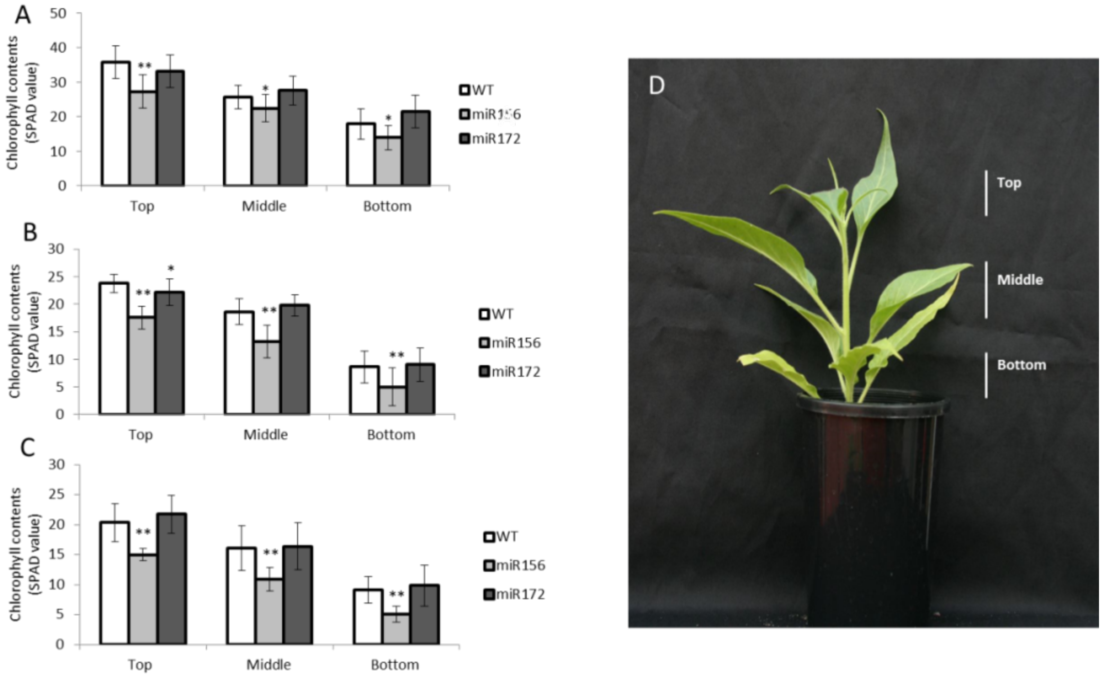

Figure 5. 14. Comparison of total chlorophyll content of transgenic tobacco plants (T0, T1 and T2 generation).

(A) Total chlorophyll content was measured with apical (top), medial (middle) and basal (bottom) leaves. (A) total chlorophyll content of T0 generation. Leaves were obtained from 10 plants of wild-type (WT), 18 plants overexpressing *gma-miR156a* (miR56) and 14 plants overexpressing *gma-miR172a* (miR172), (B) total chlorophyll contents of T1 generation, line 7 of *gma-miR156a* and line 8 of *gma-miR172a*, n=20 (C) total chlorophyll contents of T2 generation, line 7-1 of *gma-miR156a* and line 8-6 of *gma-miR172a*, n=20. Reduction in total chlorophyll content was observed in *gma-miR156a* transgenic plants. Bar: standard deviation (±SD). Asterisk: student t-test (*P>0.05, **P>0.01)

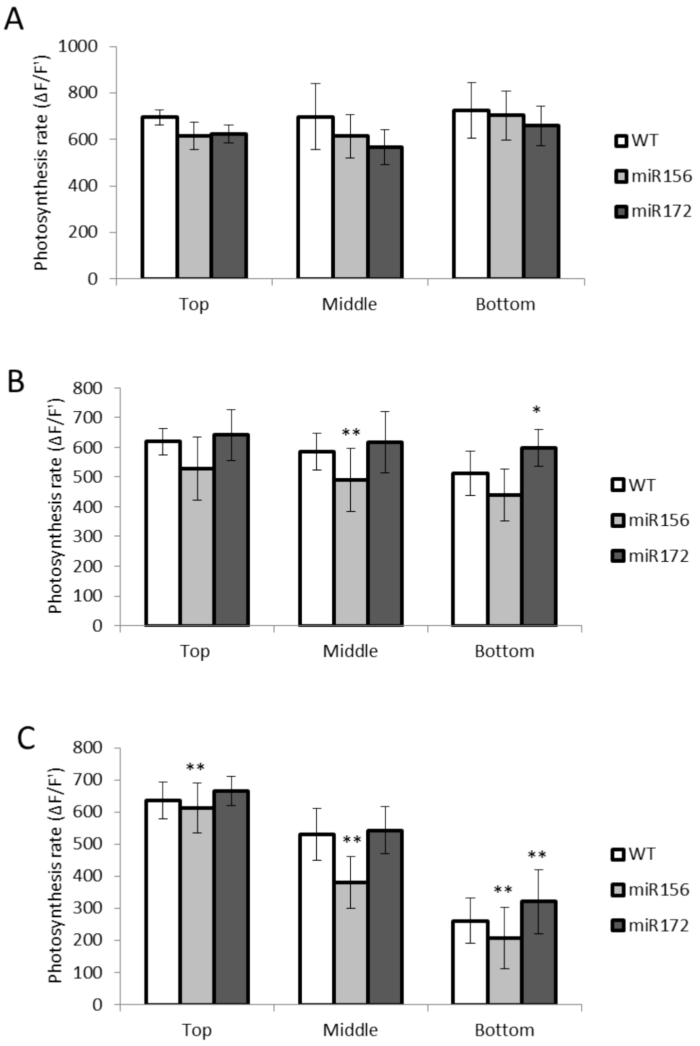

Figure 5. 15. Analysis of photosynthesis rate of transgenic tobacco plants (T0, T1 and T2 generations).

Photosynthesis rate was measured with apical (top), medial (middle) and basal (bottom) leaves. (A) photosynthesis rate of T0 generation, leaves were obtained from 10 plants of wild-type (WT), 18 plants overexpressing *gma-miR156a* (miR56) and 14 plants overexpressing *gma-miR172a* (miR172), (B) photosynthesis rate of T1 generation, line 7 of *gma-miR156a* and line 8 of *gma-miR172a*, n=20 (C) photosynthesis rate of T2 generation, line 7-1 of *gma-miR156a* and line 8-6 of *gma-miR172a*, n=20. Photosynthesis rate was low in *gma-miR156a* transgenic plants. Bar: standard deviation (±SD). Asterisk: student t-test (*P>0.05, **P>0.01)

5.3.7. Ectopic expression of *gma-miR156a* and *gma-miR172a* has an effect on floral initiation

To investigate whether *gma-miR156a* and *gma-miR172a* regulates flowering time in transgenic tobacco plants, days to first flower opening were measured in T0, T1 and T2 generations. *gma-miR156a* transgenic tobacco plants showed delayed flowering whilst *gma-miR172a* transgenic tobacco lines exhibited early flowering compared to wild-type. In T0 generation, wild-type plants started flowering at 47.7 (±3.23) days after transferring to the glasshouse. *gma-miR156a* transgenic plants showed significantly delayed flowering (62.5 ±13.2 days) with a prolonged vegetative phase. Constitutive expression of *gma-miR172a* in transgenic tobacco plants promoted only slightly earlier flowering (45±5.79 days) than wild-type (Figure 5.16). In T1 generation, transgenic mature seeds which were derived from T0 generation of transgenic tobacco plants containing a single copy of gene were sown on selection medium (SM) containing 30 mg/L of hygromycin. After 4 weeks of selection, survived plants were acclimatized on sterilized soil in pots and transferred to the glasshouse. For flowering time analysis, 20 plants of each line were randomly selected. Wild-type plants were flowering at 105.5 (±8.46) DAG (days after germination). *gma-miR156a* transgenic lines started flowering at 154.1 (±5.57) DAG, while *gma-miR172a* transgenic tobacco plants showed early flowering time (94.1±14.3 DAG) (Figure 5.17). In T2 generation, homozygous lines were selected and 20 plants of each construct were used for flowering time analysis. Wild-type tobacco plants started flowering at 66.2±15.1 DAG. Overexpressing *gma-miR156a* transgenic plants flowered at 89.9±8.22 DAG, whereas overexpressing *gma-miR172a* lines showed early flowering time (45.5±4.55 DAG) than wild-type (Figure 5.18). These delayed and promoted flowering times by ectopic expression of *gma-miR156a* and *gma-miR172a* indicated that soybean *miR156a* and *miR172a* play important roles in phase transition from vegetative to reproductive.

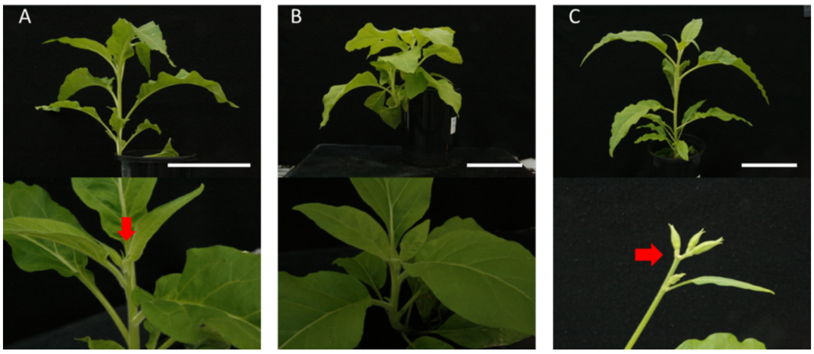

Figure 5. 16. Phenotypes of transgenic tobacco plants (T0 generation).

gma-miR156a and *gma-miR172a* transgenic tobacco plants after 2 weeks of acclimatization showed different phenotypes. (A) wild-type plant and floral bud primordia was formed at shoot apex (red arrow), (B) *gma-miR156a* (T0-line 7) transgenic tobacco plant and shoot apex, (C) *gma-miR172a* (T0-line 8) transgenic plants and presence of floral bud at shoot apex. White bar: 10cm

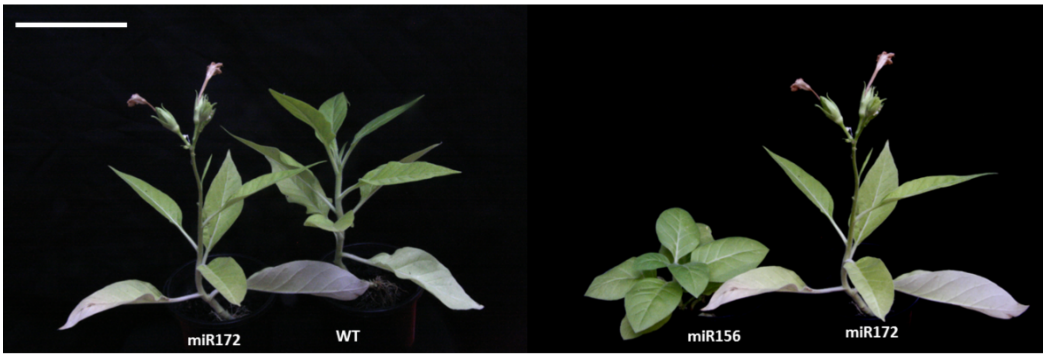

Figure 5. 17. T1 generation of *gma-miR156a* and *gma-miR172a* transgenic tobacco plants showed different pace in phase transition.

Representative transgenic plants of each construct (10 weeks old) were presented. *gma-miR172a* transgenic plant (miR172, T1-line 8) started flowering earlier than wild-type (WT). *gma-miR156a* transgenic T1-line 7 (miR156) exhibited prolonged vegetative phase. White bar: 10cm.

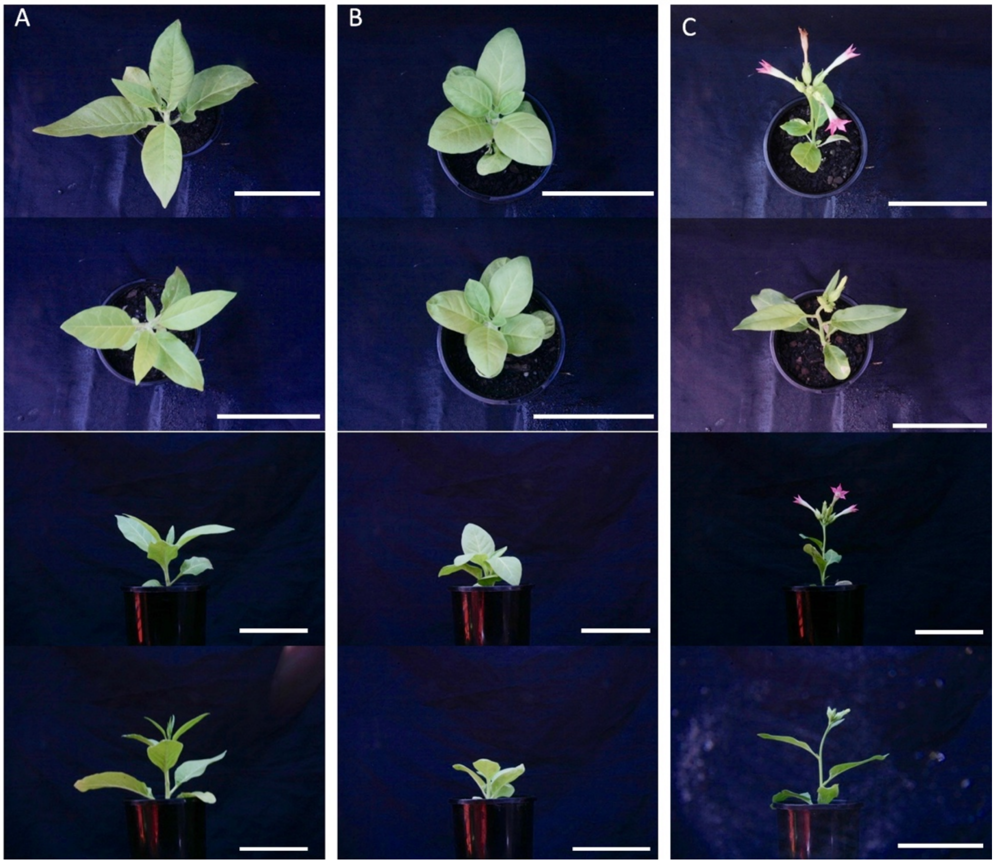

Figure 5. 18. Ectopic expression of *gma-miR156a* and *gma-miR172a* showed different phenotypes in T2 generation (homozygous transgenic plants).

Different phenotypes of wild-type, *gma-miR156a* and *gma-miR172a* (6-week-old plants after germination). (A) wild-type plants showed normal growth. (B) *gma-miR156a* (T2-line 7-1) transgenic plants showed extended vegetative growth with round shape of leaves. (C) ectopic expression of *gma-miR172a* (T2-line 8-6) caused early flowering. white bar: 10cm.

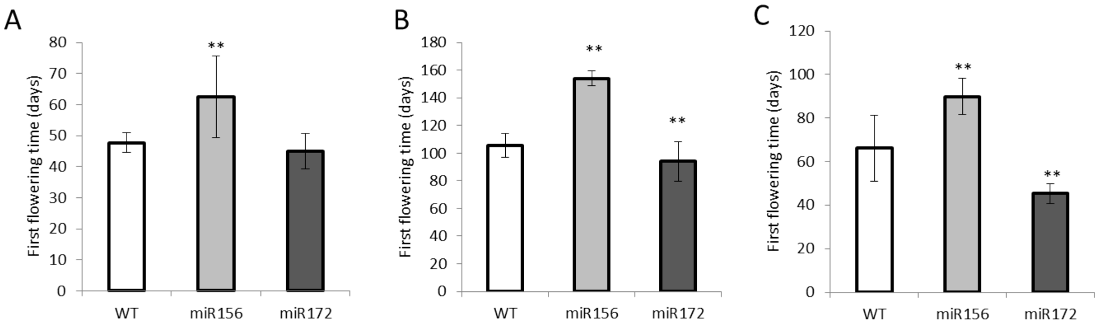

Figure 5. 19. Analysis of flowering time of ectopic expression of *gma-miR156a* and *gma-miR172a* in transgenic tobacco plants.

The date of first flower opening was recorded. (A) in T0 generation, days of flowering were calculated from transfer to glasshouse to first flower opening. 18 transgenic *gma-miR156a* tobacco plants (miR156), 14 *gma-miR172a* independent transgenic lines (miR172) and 10 wild-type plants (WT) were used for flowering time analysis. Flowering time was recorded from germination to emergence of first open flower in T1: line 7 of *gma-miR156a* and line 8 of *gma-miR172a* (B) and T2: line 7-1 of *gma-miR156a* and line 8-6 of *gma-miR172a* (C). n=20, Bar: standard deviation (±SD). Asterisk: student t-test (*P>0.05, **P>0.01)

5.3.8. Ectopic expression of *gma-miR156a* has an effect on flower size

Since overexpression of *gma-miR156a* and *gma-miR172a* in transgenic tobacco plants caused alterations on leaf morphology such as leaf shape and size (Figure 5.9), morphological traits of flowers such as length and width of flower, sepal and pedicle were measured using flowers from homozygous T2 generation. Flowers of *gma-miR156a* overexpressing transgenic plants were significantly smaller than those of wild-type in flower morphology (Figure 5.20). Flowers of *gma-miR156a* transgenic plants showed significantly shortest flower length (4.6±0.26 cm) and width (2.5±0.34 cm) than those of wild-type (length: 4.9±0.40 cm, width: 3.0±0.26 cm). The average length and width of sepal of overexpressing *gma-miR156a* lines were 1.7±0.15 cm and 0.6±0.11 cm while those of wild-type were 2.1±0.14 cm and 0.6±0.06 cm, respectively (Figure 5.20B, C). In contrast, flower morphology of *gma-miR172a* transgenic tobacco plants did not show significant differences from that of wild-type in width of flower (2.1±0.15 cm), length of sepal (2.1±0.15 cm) and width of sepal (0.6±0.09 cm), except length of flower (2.9±0.22 cm) (Figure 5.20). Significant reductions in pedicle width were observed in both *gma-miR156a* and *gma-miR172a* transgenic plants (Figure 5.20C, F). Pedicle width of *gma-miR156a* and *gma-miR172a* transgenic plants was 0.13±0.03 cm and 0.17±0.03 cm, respectively, whilst that of wild-type was 0.20±0.01 cm. However, there was no difference between wild-type and *gma-miR172a* transgenic plants in pedicle length while *gma-miR156a* transgenic plants had shorter pedicle length (0.13±0.03 cm) than wild-type (0.20±0.01 cm) and *gma-miR172a* transgenic plants (0.17±0.03 cm).

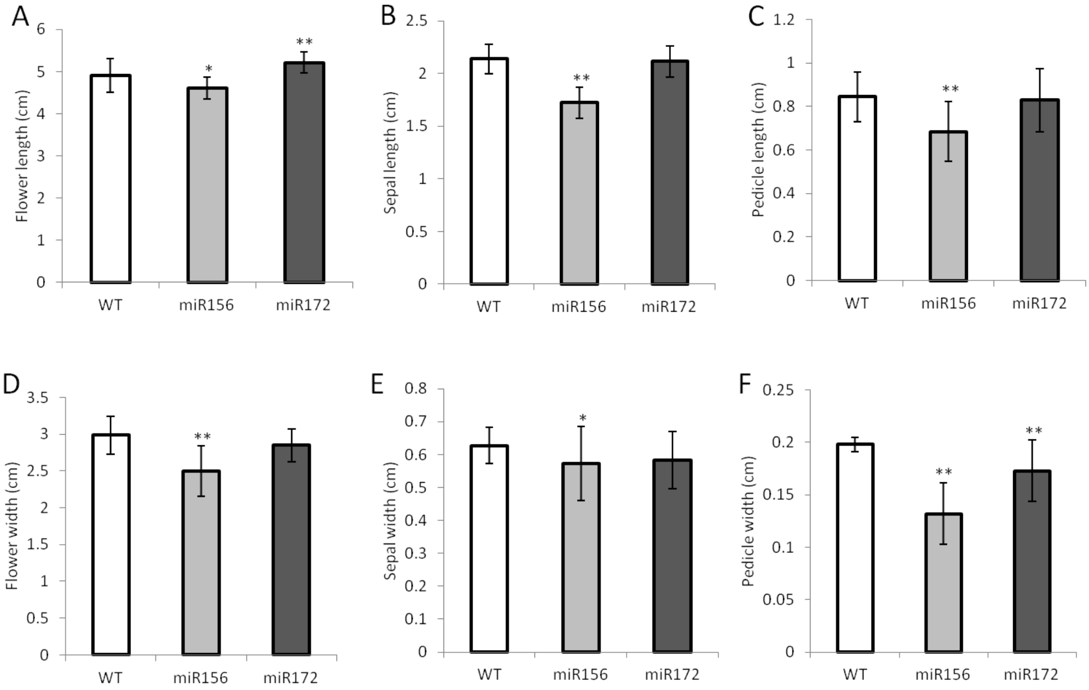

Figure 5. 20. Ectopic expression of *gma-miR156a* and *gma-miR172a* causes morphologic changes in flowers in T2 generation.

Differences in flower morphology such as length and width of flowers (A, D), sepals (B, E) and pedicle (C, E) were observed. Shortened length and width in all measured traits were observed in *gma-miR156a* transgenic tobacco plants. Ten fully opened flowers of wild-type (WT), *gma-imR156a* transgenic plants (miR156, line 7-1) and *gma-miR172a* transgenic plants (miR172, line 8-6) were randomly selected from 20 plants. Bar: ±SD. Significant differences were calculated using student t-test. Asterisk: *P>0.05, **P>0.01.

5.3.9. Overexpression of *gma-miR156a* and *gma-miR172a* does not affect pollen viability

In flower morphological analysis, ectopic expression of *gma-miR156a* and *gma-miR172a* in tobacco has an effect on flower size. To investigate pollen fertility of wild-type and transgenic plants, pollen viability was examined using the PI/FDA staining method. Fresh pollen grains were obtained from preanthesis flowers of overexpressing *gma-miR156a*, *gma-miR172a* and wild-type plants and stained with PI/FDA solution. After staining with PI/FDI solution, strong green fluorescence was detected in wild-type, *gma-miR156a* and *gma-miR172a* transgenic tobacco plants (Figure 5.21). Pollen viability of *gma-miR156a* (73.4±7.72%) and *gma-miR172a* (77.9±8.46%) transgenic plants showed high viability similar to wild-type (77.9±6.48%) in T1 generation (Figure 5.23A). Similar viability was observed in T2 generation showing high viability of wild-type (75.6±9.77%), *gma-miR156a* (73.6±7.78%) and *gma-miR172a* (75.9±8.53%) transgenic plants. (Figure 5.22 and 5.23B).

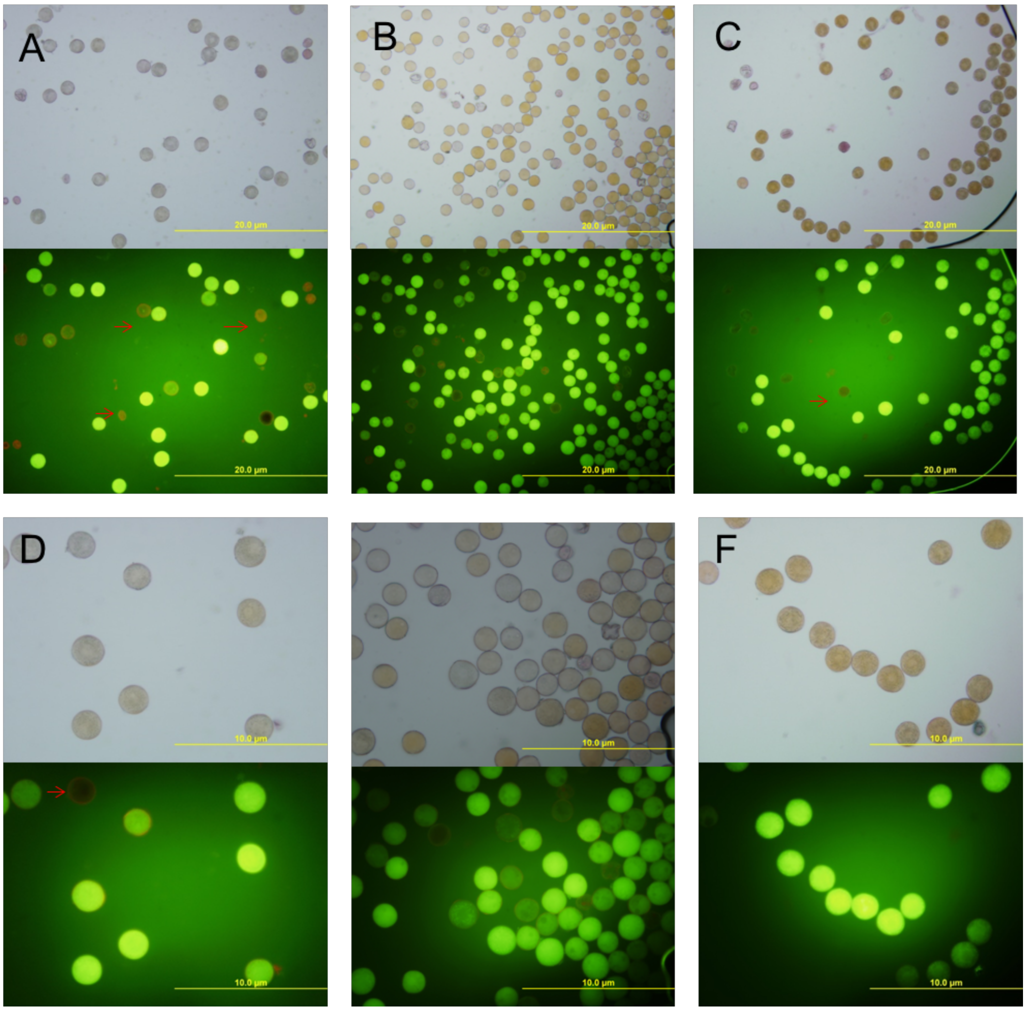

Figure 5. 21. Analysis of pollen viability of *gma-miR156a* and *gma-miR172a* transgenic tobacco plants (T1 generation).

Fresh pollen grains of wild-type (A), *gma-miR156a*: line 7 (B) and *gma-miR172a*: line 8 (C) transgenic tobacco plants were analysed for pollen viability using PI/FDI staining. Stained pollens were examined under microscope with white light (upper photos with white background) and UV light (below photos with green background). Green fluorescence indicates viable pollens. Red signal means non-viable pollens (Red arrows). Strong green fluorescence was detected in wild-type and transgenic plants. (A), (B) and (C): Yellow bar 20 µm, (D), (E) and (F): Yellow bar 10 µm.

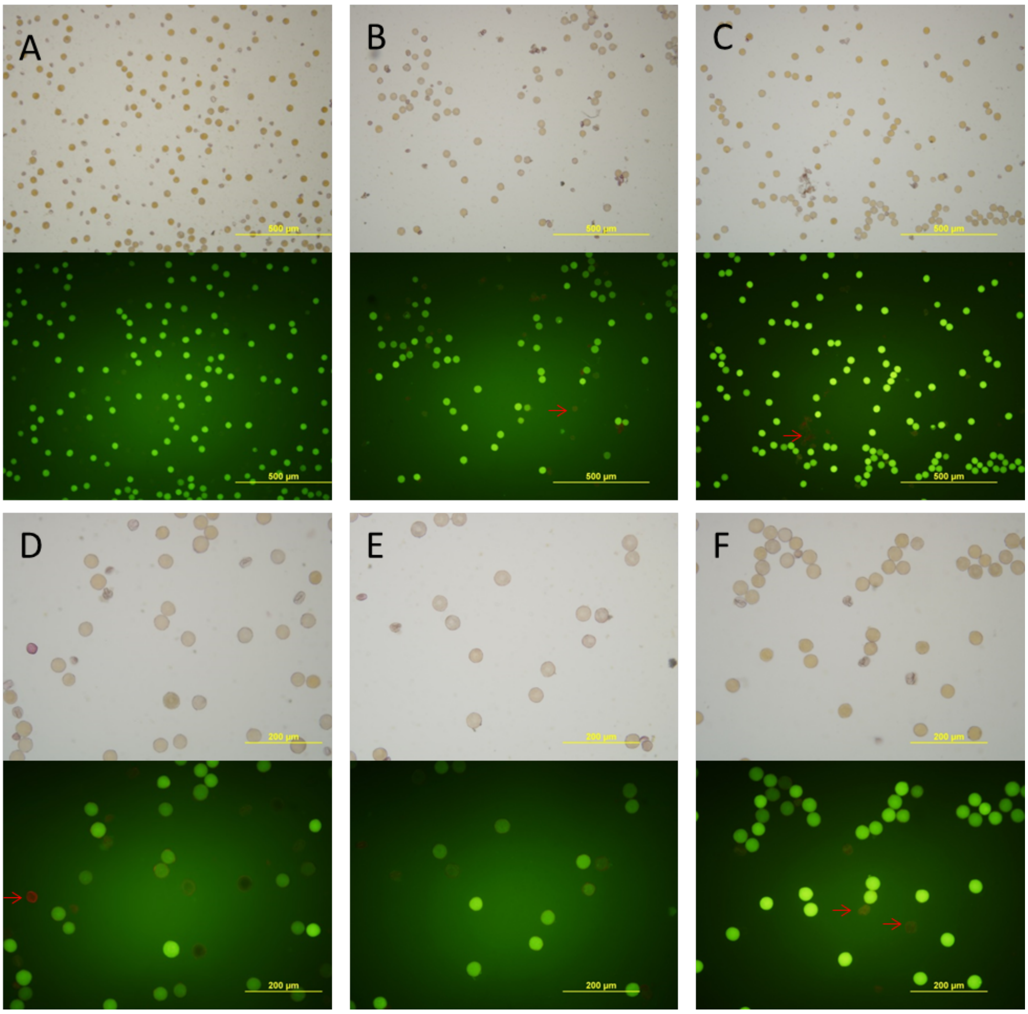

Figure 5. 22. Pollen viability of *gma-miR156a* and *gma-miR172a* transgenic tobacco plants (T2 generation).

Fresh pollen grains were stained with PI (propidium iodide, 2 mg/ml) and FDI (fluorescein diacetate, 5 mg/ml) solution. Green fluorescence indicates viable pollens. Red signal means non-viable pollens (red arrow). Strong fluorescence of fertile pollens was observed in wild-type (A), *gma-miR156a*: line 7-1 (B) and *gma-miR172a*: line 8-6 (C) transgenic plants indicated ectopic expression of *gma-miR156a* and *gma-miR172a* has no effect on pollen fertility**.** (A), (B) and (C): Yellow bar 500 µm. (D), (E) and (F): Yellow bar 200 µm.

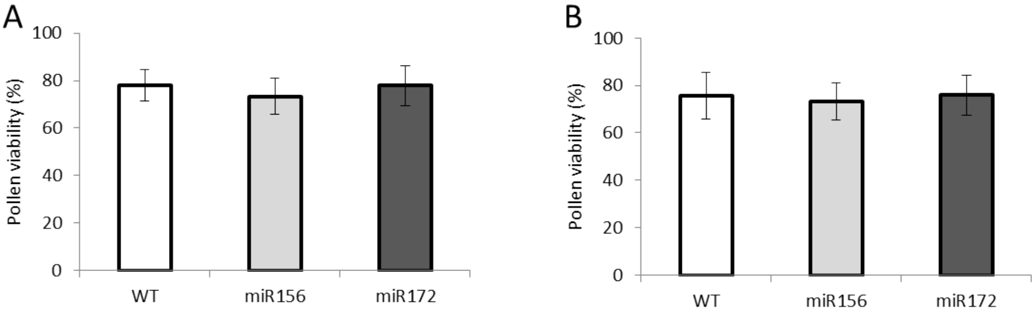

Figure 5. 23. Comparison of pollen viability of wild-type, transgenic *gma-miR156a* and *gma-miR172a* transgenic tobacco plants (T1 and T2 generations).

Fresh pollen grains from randomly selected 6 preanthesis flowers of wild-type (WT), *gma-miR156a* (miR156) and *gma-miR172a* (miR172) transgenic tobacco plants were used. T1 generation: 606 (WT), 542 (miR156, line 7), 541 (miR172, line 8) pollens and T2 generation: 578 (WT), 574(miR156, line 7-1), 573 (miR172, line 8-6) pollens were counted. Fertility rate was assessed by (number of sterile pollens/total pollens)x100

5.3.10. Ectopic expression of *gma-miR156a* and *gma-miR172a* has effects on overall plant morphology

Morphological analysis of *gma-miR156a* and *gma-miR172a* transgenic tobacco plants showed different phenotypes during vegetative growth. T2 generation of *gma-miR156a* transgenic tobacco plants had significantly short plant height (3.9±1.27 cm). On the other hand, the height of overexpressing *gma-miR172a* transgenic tobacco plants (6.7±2.57 cm) was not significantly different to that of wild-type (7.2±2.06 cm) (Figure 5.24). Moreover, ectopic expression of *gma-miR156a* in transgenic plants showed a significant effect on total leaf numbers. In contrast to plant height, *gma-miR156a* overexpressing transgenic tobacco plants produced significantly more leaves (16.0±2.47) than wild-type (9.9±1.97), whilst there were no significant differences observed in leaf numbers between *gma-miR172a* overexpressing transgenic plants (9.3±2.27) and wild-type (Figure 5.24). These results suggested that *gma-miR156a* plays an important role in vegetative growth whilst *gma-miR172a* has no effect on plant morphology during vegetative phase.

Enhanced effects of *gma-miR156a* on plant morphology were detected after flowering (Figure 5.25). Outgrowth of lateral shoots was observed in *gma-miR156a* transgenic plants. *gma-miR156a* transgenic tobacco plants had significantly more branches (5.4±1.27) than wild-type (2.5±0.53) and *gma-miR172a* transgenic plants

(2.3±0.68) (Figure 5.26A). Furthermore, *gma-miR156a* transgenic plants produced significantly more leaves (99.4±4.43) than wild-type (41.7±5.83). On the other hand, *gma-miR172a* transgenic plants had fewer leaves (28.3±4.67) than wild-type (Figure 5.26A). Similar to the number of total leaves, *gma-miR156a* transgenic plants produced more flowers (53.71±19.35) than wild-type (37.8±5.59) and *gma-miR172a* transgenic plants (35.4±2.65) (Figure 5.26C).

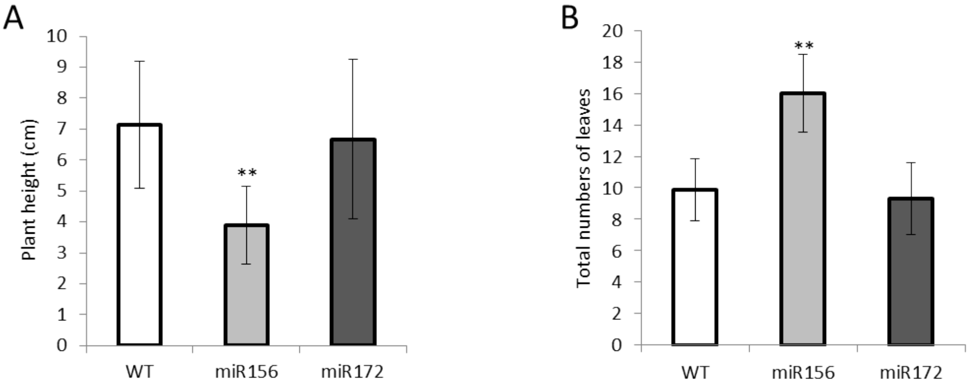

Figure 5. 24. Comparison of plant height and total leaf numbers in T2 generation of transgenic tobacco plants.

Plant height (A) and total leaf numbers (C) were measured from T2 generation of wild-type (WT), *gma-miR156a* (miR156, line 7-1) and *gma-miR172a* (miR172, line 8-6) transgenic plants. Six-week-old T2 plants were used for analysis. *gma-miR156a* overexpressing transgenic plants had significantly smaller height and more leaves than wild-type. n=20, Bar: ±SD. Significant differences were calculated using student t-test. Asterisk: *P>0.05, **P>0.01.

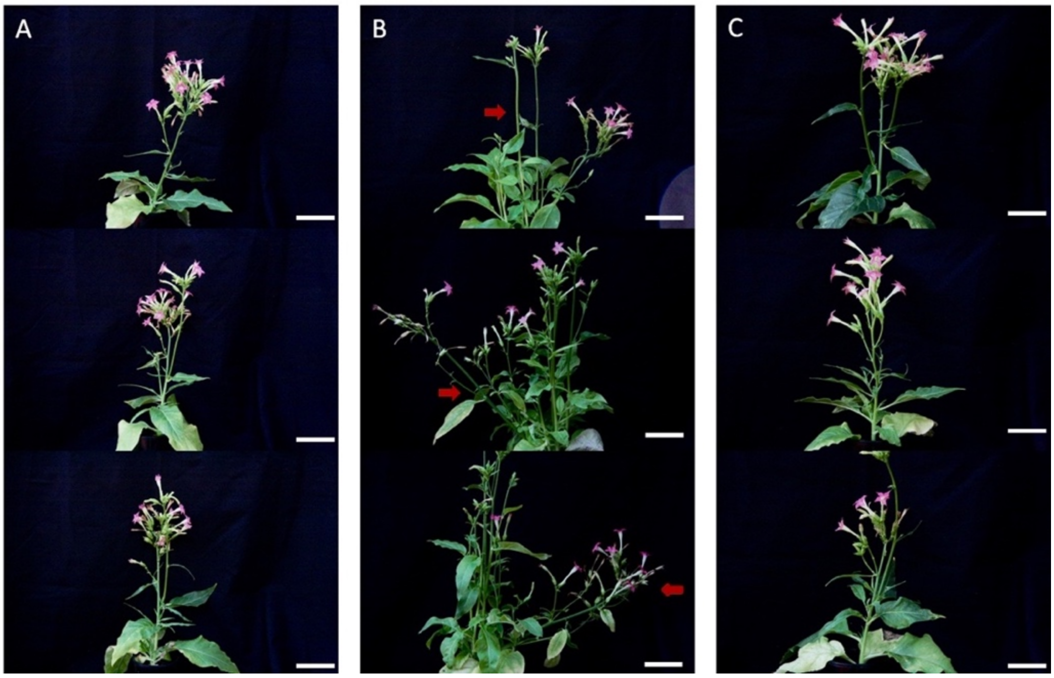

Figure 5. 25. Outgrowth of lateral shoots in *gma-miR156a* transgenic plants.

Morpholgocial differences were detected after flowering. T2 generation of 12-week-old wild-type (A), *gma-miR156a*: line 7-1 (B) and *gma-miR172a*: line 8-6 transgenic tobacco plants (C) were grown in the glasshouse. More lateral shoots growth from base of *gma-miR156a* transgenic tobacco plants and inflorescences (red arrows). White bar: 10cm

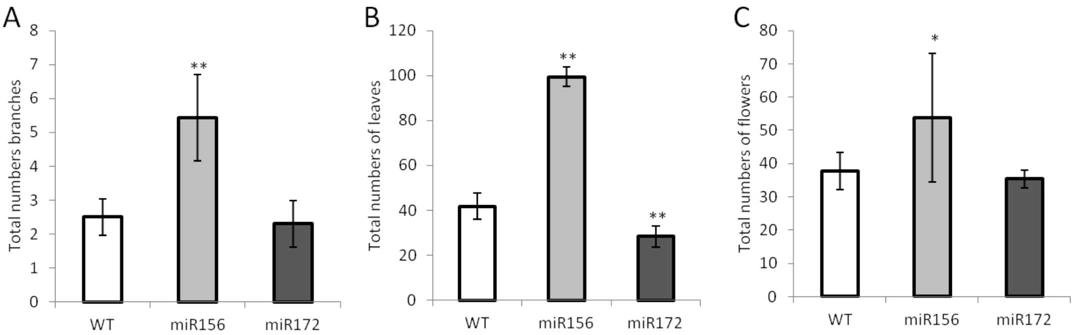

Figure 5. 26. Analysis of plant morpholgy of homozygous T2 generation, *gma-miR156a* and *gma-miR172a* transgenic tobacco plants during flowering.

Total number of branches (A), leaves (B) and flowers (C) were measured from 12-week-old wild-type (WT), *gma-miR156a* (miR156, line 7-1) and *gma-miR172a* (miR172, line 8-6) transgenic tobacco plants. *gma-miR156a* trangenic plants produced more branches resulting in more leaves and flowers. n=10, Bar means standard deviation (±SD). Significant differences were calculated using student t-test. Asterisk: *P>0.05, **P>0.01.

5.3.11. Analysis of expression patterns of miR156 and miR172 target genes and floral pathway integrator genes in transgenic tobacco plants

5.3.11.1. Ectopic expression of *gma-miR156a* and *gma-miR172a* regulates the expression of putative target genes in transgenic tobacco plants

To investigate the molecular mechanism of miR156- and miR172-mediated changes in plant development and phase transition from vegetative to reproductive, putative target genes of *gma-miR156a* and *gma-miR172a* in tobacco were searched using a blast search tool for identification of miRNA targets (http://plantgrn.noble.org/psRNATarget/). *NtSPL4a, NtSPL5a, NtSPL5b, NtSPL9* and *NtAP2a* were identified as target genes of *gma-miR156a* and *gma-miR172a* (Figure 5.27) and their expression levels were examined using qRT-PCR. In real-time qRT-PCR analysis, putative target genes of *gma-miR156a* were seen to be down-regulated in *gma-miR156a* transgenic tobacco plants. Expression levels of *NtSPL4a* (1.7 fold), *NtSPL5a* (70 fold), *NtSPL5b* (28.5 fold) and *NtSPL9* (4.7 fold) were decreased in shoot apex of *gma-miR156a* transgenic plants (Figure 5.28). In addition, ectopic expression of *gma-miR172a* in transgenic tobacco plants repressed the expression of *AP2a* (2.9 fold), a possible target gene of *gma-miR172a* (Figure 5.28E).

A

```
gma-miR156a  20   C A C G A G U G A G A G A A G A C A G U   1
                    : : : : :   : : : : : : : : : : : : :
NtaSPL4      715  U U G C U C U C U C C C U U C U G U C A   734

gma-miR156a  20   C A C G A G U G A G A G A A G A C A G U   1
                    : : : : :   : : : : : : : : : : : : :
NtaSPL5a     605  U U G C U C U C U C U C U U C U G U C A   624

gma-miR156a  20   C A C G A G U G A G A G A A G A C A G U   1
                    : : : :   : : : : : : : : :   : : : :
NtaSPL5b     488  A U G C U U A C U C U C U U C A G U C A   507

gma-miR156a  20   C A C G A G U G A G A G A A G A C A G U   1
                  : : : : : :   : : : : : : : : : : : : :
NtaSPL9      869  G U G C U C U C U C U C U U C U G U C A   888
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B

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gma-miR172a  21   U A C G U C G U A G U A G U U C U A A G A   1
                    : : : : : : : : : : : : :   : : : : :
NtaAP2 a     706  C U G C A G C A U C A U C A G G A U U C C   726
```

Figure 5. 27. Putative target genes of *gma-miR156a* and *gma-miR172a* in tobacco.

Putative target genes of *gma-miR156a* and *gma-miR172a* in tobacco genome. Target site was predicted using target gene search program (www.platgrn.noble.org/psRNATarget). (A) *NtSPL4, NtSPL5a, NtSPL5b* and *NtSPL9* were identified as putative target genes of *gma-miR156a*. (B) *NtAP2a* was identified as a possible target gene of *gma-miR172a*. Sequence alignment analysis of mature *gma-miR156a* and *gma-miR172a* with potential target genes in tobacco showed near perfect match.

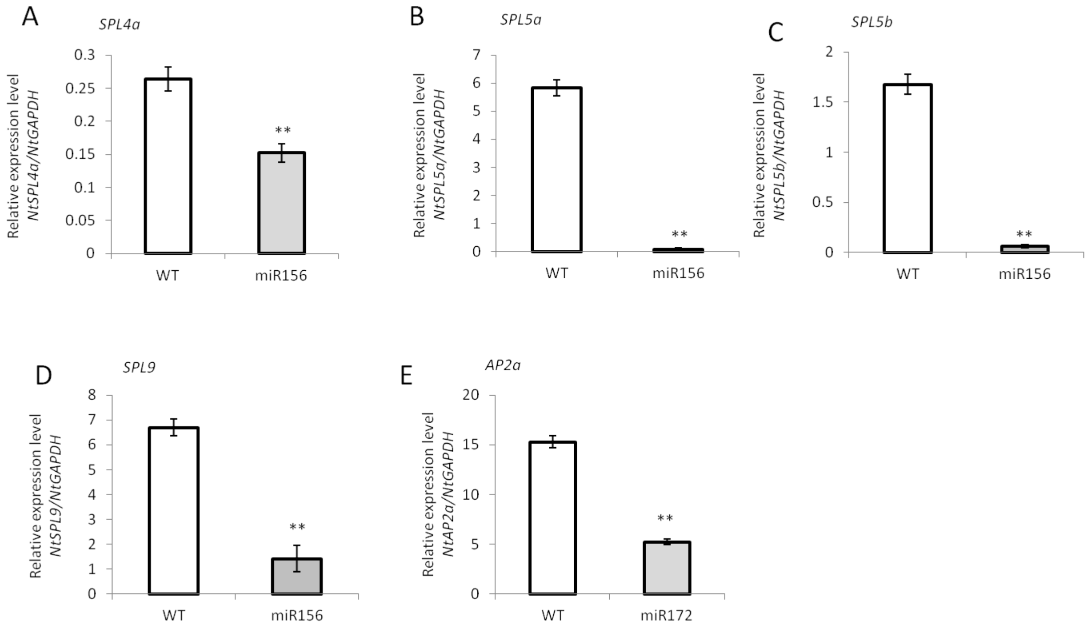

Figure 5. 28. Down-regulation of target genes of *gma-miR156a* and *gma-miR172a* in homozygous transgenic tobacco plants (T2 generation).

Expression levels of the four putative target genes of *gma-miR156a* and one gene of *gma-miR172a* were examined by real-time RT-PCR. Expressions of *NtSPL4a* (A), *NtSPL5a* (B), *NtSPL5b* (C), *NtSPL9* (D) and *NtAP2a* (E) were down-regulated in transgenic plants. The abundance of *NtSPL4a* (1.7 fold), *NtSPL5a* (70 fold), *NtSPL5b* (28.5 fold), *NtSPL9* (4.7 fold) were repressed in *gma-miR156a* transgenic tobacco plants (line 7-1). A putative target gene of *gma-miR172a* (*AP2a*) was down-regulated (2.9 fold) in *gma-miR172a* transgenic tobacco plants (line 8-6). 2-*ΔΔCt* method was used for real-time qRT-PCR analysis. Total RNA was extracted from 10-mm shoot apex of 4 weeks old plants grown in the glasshouse (n=9). N*tGAPDH* was used as the endogenous control. Bar: standard deviation (±SD) of three biological replicates. Asterisk: *P>0.05, **P>0.01.

5.3.11.2. Floral pathway integrator genes are up-regulated in overexpressing *gma-miR172a* transgenic tobacco plants

To investigate how expression of floral pathway integrator genes and floral meristem identity genes are affected by overexpression of *gma-miR156a* and *gma-miR172a* in transgenic tobacco plants, expression levels of floral meristem identity genes and floral pathway integrators (*NtAP1a, NtAP1b, NtLFY1, NtSOC1a*) were examined using real-time qRT-PCR. In real-time qRT-PCR analysis, ectopic expression of *gma-miR172a* in transgenic tobacco plants up-regulated transcription levels of floral pathway integrators. The expression levels of *NtAP1a* (3.4 fold), *NtAP1b* (13 fold), *NtLFY1* (7.4 fold), *NtSOC1a* (4.2 fold) were up-regulated in shoot apex of *gma-miR172a* transgenic tobacco plants (Figure 5.29). Interestingly, expression levels of *NtAP1b* (2.0 fold) and

NtLFY1 (2.2 fold) in gma-miR156a transgenic tobacco were higher than wild-type (Figure 5.29).

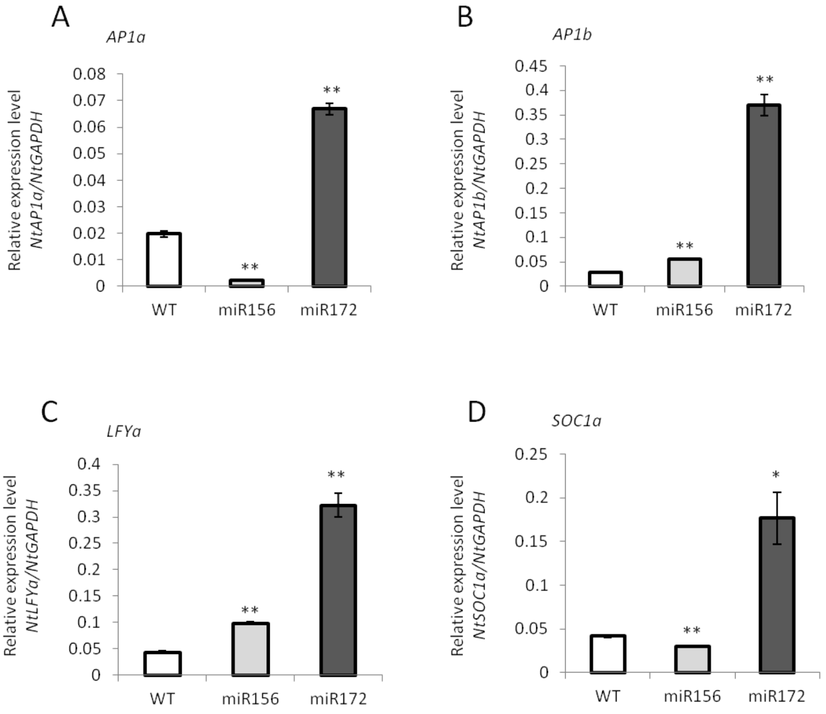

Figure 5. 29. Analysis of expression levels of flowering genes in *gma-miR172a* transgenic plants.

The expression abundances of *NtAP1a* (A), *NtAP1b* (B), *NtLFY1*(C) and *NtSOC1a* (D) were examined using real-time qRT-PCR. The expression levels of *NtAP1a* (3.4 fold), *NtAP1b* (13 fold), *NtLFY1* (7.4 fold) and *NtSOC1a* (4.2 fold) were up-regulated in the shoot apex of *gma-miR172a* transgenic tobacco plants (T2 generation, line 8-6). Total RNA was extracted from 10mm shoot apex of 4-week-old plants grown in the glasshouse (n=9). Transcription levels were normalized using *NtGAPDH* as a reference gene. 2-*ΔΔCt* method was used for data analysis. Bar: standard deviation (±SD) of three biological replicates. Asterisk: *P>0.05, **P>0.01.

5.4. Discussion

Life cycles of flowering plants consist of seed germination, vegetative growth and reproductive growth and setting of seeds. During the plant's life cycle, the timing of phase change (from vegetative to reproductive) plays an important role in reproductive success. The timing of this transition is regulated by endogenous and environmental cues derived from ageing, hormonal, photoperiod, vernalisation and ambient temperature pathways.

Of flowering pathways, it has been revealed that several miRNA families play important roles in regulation of ageing pathway by inhibition or promotion of phase transition. Among miRNA families, miR156 and miR172 are main regulators to control the ageing pathway. Studies on miRNAs in Arabidopsis, tobacco, rice and maize have revealed that miR156 regulates the juvenile phase by repressing their target genes*, SPLs*, whilst miR172 is responsible for initiation and development of reproductive phase by repression of *AP2* and *AP2-like* gene family (Lauter et al., 2005; Chuk et al., 2007; Wu et al., 2009; Feng et al., 2016). Recent studies on soybean miRNAs have identified 28 loci for encoding 28 mature miR156 and 12 loci for encoding 15 mature miR172 (Subramanian et al., 2008; Kulcheski et al., 2011; Wong et al., 2011; Goettel et al., 2014; Zhao et al., 2015). Phylogenetic analysis of precursor miR156 and miR172 family in soybean, Arabidopsis and tobacco showed an evolutionary diversification of soybean miR156 and miR172 family.

Expression patterns of soybean *miR156a* and *miR172a* showed inverse in nature in mature leaves. In SD 0, expression levels of *miR156a* were higher than that of *miR172a*. Expression of *miR156a* was decreased at SD 6 whilst *miR172a* showed increased expression levels higher than that of *miR156a*. In Arabidopsis, miR156 is highly expressed in early vegetative development and decreases with increasing plant age. In contrast, miR172 expression is accumulated in the organs such as leaves and inflorescences during reproductive phase (Wu et al., 2009; Nodine and Bartel, 2010). The temporally inverse expression patterns of *gma-miR156a* and *gma-miR172a* suggested that these miRNAs may play master roles in phase transition.

In this study, overexpression of *gma-miR156a* and *gma-miR172a* exhibited the effect on leaf morphology and physiology. Ectopic expression of *gma-miR156a* transgenic tobacco plants produced round shape of leaves (L/W ratio: 1.8±0.18) with smallest leaf length (7.5±0.98 cm) and width (4.2±0.45 cm). On the other hand, *gma-miR172a* transgenic tobacco plants had smaller leaf width (4.4±0.60 cm) and narrower shape of leaf (L/W ratio: 3.2±0.43) than wild-type plants (5.2± 0.60 cm; L/W ratio 2.6±0.24). In addition, reduction of chlorophyll contents (34.4%) and photosynthesis rate (15.1%) on leaves were observed in *gma-miR156a* transgenic tobacco plants (Figure 5.13, 5.14). Overexpression of miR156 in Arabidopsis transgenic plants produced more leaves with juvenile characteristics such as lack of trichomes and small and round leaf shapes

(Wu et al., 2006). Loss-of-function of chlorophyll B biogenesis mutant plants (*cao/chlorina1*) had a prolonged juvenile phase with small and round leaf shapes (Yu et al., 2013). In addition, this mutant plant showed a higher expression of miR156 than that of wild-type (Yu et al., 2013). Ectopic expression of miR156 in transgenic tobacco plants also caused a reduction in chlorophyll content and photosynthesis rate on leaves (Feng et al., 2016). In Arabidopsis, miR156 is shown to regulate vegetative development by repressing its target genes, *SPL* protein family, which has diverse functions in plant development and growth (Wu and Poethig, 2006; Wang et al., 2008; Wu et al., 2009; Tripathi et al., 2018). Ectopic expression of *SPL4* and *SPL5* in transgenic Arabidopsis plants accelerates flowering time and the production of adult traits (trichome on abaxial side) on leaves (Wu and Poethig, 2006; Gandikota et al., 2007). The loss of function of *SPL9* exhibited delayed production of adult traits on leaves and caused rounded leaf blade (Wu et al., 2009). However, constitutive expression of *SPL9* increases leaf size with a reduction of leaf initiation rate (Wang et al., 2008). In this study, down-regulation of miR156 target genes, *NtSPL4a* (1.7 fold), *NtSPL5a* (70 fold), *NtSPL5b* (28.5 fold) and *NtSPL9* (4.7 fold) was detected in *gma-miR156a* transgenic tobacco plants by qRT-PCR analysis, suggesting that *gma-miR156a* plays major roles in vegetative development similar to Arabidopsis.

In this study, ectopic expression of *gma-miR156a* and *gma-miR172a* in transgenic tobacco plants exhibited their effect on flowering time. Overexpression of *gma-miR156a* in transgenic tobacco plants caused an extended vegetative phase with late flowering time (89.9±8.22 DAG) than wild-type (66.2±15.10 DAG). Late flowering time by ectopic expression of *gma-miR156a* is coincident with results from other monocot (rice, switchgrass) and dicot (Arabidopsis, tobacco) plants (Xie et al., 2006; Wu et al., 2009; Fu et al., 2012; Feng et al., 2016). Overexpression of miR156 in dicot plants showed a prolonged vegetative phase (Wu et al., 2009; Feng et al., 2016). Transgenic Arabidopsis plants overexpressing *miR156a* exhibited a prolonged juvenile phase (12 fold delay in initiation of trichome, being one of the adult-specific traits in Arabidopsis) with more rosette leaves (90 rosette leaves) (Wu et al., 2009). In addition, overexpression of *miR156a* in transgenic tobacco plants caused delayed first flowering time (4 months later than wild-type) with numerous leaves, at five times higher than wild-type (Feng et al., 2016). Down-regulation of *miR156a* in transgenic tobacco (mimicry miR156) had fewer leaves (1.5 fold less) than wild-type and flowered early (1 month earlier than wild-type)

(Feng et al., 2016). Overexpression of miR156 in monocot plants also showed similar results. Transgenic rice with *Osa-miR156b* and *Osa-miR156h* overexpressed produced more tillers (10 to 15 times higher than wild-type) and showed dwarfism and late flowering time (7 to 10 days delayed flowering). Moreover, these transgenic lines showed morphological differences in reproductive organs. Transgenic rice had a reduction of number of spikelets (16 fold less) and grains per panicle (12 fold less), and secondary branches of panicle (3 fold less) with normal fertility (Xie et al., 2006). In transgenic switchgrass which contains rice miR156 (*Osa-miR156b*), different levels of overexpression of miR156 induced various morphological changes. A high level of expression of miR156 (120 to 250 fold higher than wild-type) showed dwarfism whilst moderate expression line (20 to 30 fold higher than wild-type) produced more tillers (4.8 to 6.4 fold) and biomass (58 to 63% increase) than that of wild-type. Transgenic switchgrass with a low level of miR156 (10 to 15 fold higher than wild-type) exhibited increased tiller numbers (1.6 to 2.1 times higher than wild-type) and biomass (58 to 63% increase) with normal growth (Fu et al., 2012). In this study, overexpressing *gma-miR156a* transgenic tobacco plants also produced more leaves (2.5 fold) with highly branched (2 fold) phenotype than wild-type. In contrast, the ectopic expression of *gma-miR172a* in transgenic plants promoted flowering and showed early flowering phenotypes (45.5±4.55 DAG). In Arabidopsis, overexpression of *miR172a-2* promoted flowering with fewer rosette leaves (5 fold less than wild-type) (Aukerman and Sakai, 2003). Moreover, transgenic Arabidopsis plants containing overexpressed *miR172b* produced trichomes 2- plastochron earlier than wild-type in non-flower inductive conditions (Wu et al., 2009). Ectopic expression of Arabidopsis *miR172a-1* in transgenic *Nicotiana benthamiana* plants promoted flowering 10 days earlier than empty-vector transformed plants (Mlotshwa et al., 2006). In monocots, transgenic rice with overexpression of *miR172d* induced flowering 9 to 30 days earlier than wild-type (Lee et al., 2014). Flowering is initiated by environmental and endogenous signals. The signals are integrated to activate flowering pathway integrator genes so that plants can flower (Mouradov et al., 2002). Ectopic expression of *gma-miR172a* in transgenic tobacco plants up-regulated the expression of flowering pathway integrators and floral meristem identity genes (*NtAP1a, NtAP1b, NtLFY1, NtSOC1a*). Flowering pathway integrator genes (*NtLFY1* and *NtSOC1a*) were up-regulated 2 to 6 fold than wild-type. Moreover, floral meristem identity genes (*NtAP1a* and *NtAP1b*) were expressed 3.5 to 12 fold higher than wild-type. This result suggested that *gma-miR172a* is involved in flowering genetic pathways. Although gma-miR156a transgenic tobacco plants exhibited extended vegetative developmental growth with delayed flowering initiation, up-regulations of two floral meristem identity genes (*NtAP1b*: 2.0 fold and *NtLFY1*: 2.2 fold) were detected. In tobacco, *NtLFY1* is responsible for floral meristem developments as well as growth of lateral structures such as leaves and branches (Ahearn et al., 2001). In this study, gma-miR156a transgenic tobacco plants showed extended vegetative developments resulting in more leaves and brunches. Therefore, longer vegetative developments might have effects on up-regulations of *NtLFY1* in gma-miR156a transgenic plant. Moreover, Jang and colleagues (2002) reported spatial expression patterns of *NtAP1b* (one of *AP1* homologs of tobacco). It was expressed not only in floral organs but also in vegetative organs, such as mature leaves and stems. Therefore, functional divergences of *NtAP1b* and *NtLFY1* may induce higher expression levels of these genes in gma-miR156a transgenic plants.

In this study, no abnormal flower development was detected in T0, T1 and T2 generation of *gma-miR156a* and *gma-miR172a* transgenic plants. Flowers of *gma-miR156a* and *172a* transgenic tobacco plants showed a high pollen viability and formed seeds. However, the ectopic expression of *miR172a-1* in Arabidopsis displayed a disruption in floral development. Overexpression of *miR172a-1* in transgenic Arabidopsis plants produced solitary gynoecium at the axil of cauline leaves and stigmatic papillae on cauline margins and (Aukerman and Sakai. 2003). In addition, transgenic *Nicotiana benthamiana* plants overexpressing Arabidopsis *miR172a-1* produced partial or complete sepal-to-petal transformed flowers at the frequency of 15 to 50% per plant (Mlotshwa et al., 2006). In Arabidopsis, *AP2* is one of the target genes of miR172 and is involved in sepal and petal development (Bowman et al., 1991; Drews et al., 1991). In putative target genes blast in the tobacco genome, the target site of *gma-miR172a* was identified in the coding region of *AP2* gene. To examine whether gma-miR172a represses the expression of the *AP2* gene in tobacco, qRT-PCR was performed. Although expression of *AP2* gene was down-regulated in *gma-miR172a* transgenic tobacco plants (3 fold less than that of wild-type), flowers in these transgenic tobacco plants exhibited normal developments. Therefore, it is a possible hypothesis that the *AP2* gene in tobacco has different roles in flower development.

In summary, this study showed that ectopic expression of *gma-miR156a* in tobacco plants exhibited prolonged vegetative phase with juvenile traits on leaves. On the other hand, *gma-miR172a* is involved in flowering initiation. Target genes of *gma-miR156a* and *gma-miR172a* were repressed in transgenic tobacco plants. These results suggested that *gma-miR156a* and *gma-miR172a* may have regulator roles in vegetative and reproductive phases. Although *gma-miR156a* and *gma-miR172a* exhibited their roles in vegetative phase and flowering in tobacco plants, functional analysis of these miRNAs in soybean plants are needed for further validation of their roles in vegetative development and flowering in soybean.

5.5. Conclusions

In this study, the roles of *gma-miR156a* and *gma-miR172a* in vegetative and reproductive development were confirmed. *gma-miR156a* and *gma-miR172a* expressed inversely during plant development and flowering. The expression of *gma-miR156a* was decreased whilst *gma-miR172a* exhibited increased expression levels under flowering inductive conditions, suggesting that two soybean *miR156a* and *miR172a* may be involved in the ageing process. Ectopic expression of *gma-miR156a* in transgenic tobacco plants induced the extended vegetative phase with numerous leaves, juvenile leaf traits such as round shape of leaves, smallest leaf length, width and leaf area. In contrast, the ectopic expression of *gma-miR172a* in transgenic tobacco plants exhibited narrow shapes of leaves with smaller leaf width than wild-type plants. Moreover, overexpression of *gma-miR156a* in transgenic tobacco plants delayed time taken until first flower opening, whilst the ectopic expression of *gma-miR172a* in transgenic tobacco plants promoted flowering. These results indicated that *gma-miR156a* and *gma-miR172a* are involved in vegetative and reproductive developmental phases. Furthermore, down-regulations of putative target genes of miR156 and miR172 in tobacco (miR156: *NtSPL4a, NtSPL5a, NtSPL5b, NtSPL9* and miR172: *AP2*) were observed in *gma-miR156a* and *gma-miR172a* transgenic tobacco plants, suggesting that soybean *miR156a* and *miR172a* have conserved roles in the controls of ageing process via repression of their target genes, respectively.

Chapter 6. General discussion

Flowering is a pivotal event in plant life cycles as it has a huge impact on reproductive success. Early flowering gives the plant an opportunity to secure its progeny in adverse flowering conditions. On the contrary, late flowering enables sufficient vegetative growth to produce more resources for reproductive developments, ultimately leading to increase of yield (Simpson and Dean, 2002; Komeda, 2004; Weinig and Schmitt, 2004). Therefore, a better understating on flowering pathways is the key to improving yield and fitness of plants. The progress in molecular genetics such as technical improvements in transcriptome analysis, comparative genomics and genome sequencing has opened a new era for understanding important plant developmental processes in plants, such as flowering. It has been identified that flowering is initiated via various genetic pathways (photoperiod, vernalisation, ambient temperature, autonomous, gibberellin and ageing pathways) in Arabidopsis (Roux et al., 2006; Gupta and Chakrabarty, 2013; Cheng et al., 2017). Over 180 genes in Arabidopsis flowering pathway have been identified and biological functions of genes have been examined via forward genetics (loss of function mutant plants) and reverse genetics (Fornara et al., 2010). Comparative analysis of flowering genes in Arabidopsis and important crops such as soybean have revealed conservation of flowering genes in soybean (Jung et al., 2012). However, the majority of functions of soybean flowering genes still remain unknown (Homrich et al., 2012; Liew et al., 2014). In this regard, the present study identified the functions of soybean flowering genes, a *LFY* homolog (Glyma.06G163600.1) and miR156-172 (*gma-miR156a-gma-miR172a*), via heterologous expression in model plants.

By comparing the amino acid compositions of soybean *LFY* homologs, the present study confirmed that two soybean *LFY* homologs (Glyma.04G202000.1 and Glyma.06G163600.1) may be involved in the regulation of flowering process. Two soybean *LFY* homologs exhibited high conservation in gene structures and DNA-binding sites. The genomic information of two soybean *LFY* homologs showed that they are made up of three exons, two introns and 3′UTR (Figure 4.2). This gene structure is coincident with *LFY* genes in other plant species. Analysis of *LFY* gene structures in Arabidopsis, cucumber, maize, isoetes, jatropha revealed that *LFY* gene has three exons, two introns and 3′UTR (Bomblies et al., 2003; Tang et al., 2016; Yang et al., 2017; Zhao et al., 2017). Moreover, a comparative analysis of two soybean *LFY* homologs exhibited high sequence conservations in two major DNA-binding domains. *LFY* gene is a plant-specific transcription factor and it plays regulatory roles in the onset of flowering and reproductive development (Moyroud et al., 2010, Tang et al., 2016). In Arabidopsis, *LFY* gene regulates the expression of target genes by binding to specific sequences (T/ANNNCCANT/GT/GNNNNT/A) in promoter regions of its downstream genes (Hamès et al., 2008). It has been reported that two highly conserved domains of *LFY* gene (N- and C-domain) play crucial roles in DNA-binding (Maizel et al., 2005; Hamès et al., 2008; Sayou et al., 2014; Wan et al., 2015; Sayou et al., 2016). C-domain is a helix-turn-helix motif and it is responsible for specific DNA-binding (Hamès et al., 2008). The other conserved domain, N-domain, is a Sterile Alpha Motif and is involved in the oligomerisation of the *LFY* gene (Sayou et al., 2016). However, differences in peptide compositions in conserved regions were also observed between two soybean *LFY* homologs (Figure 4.2). Especially, C-domain of Glyma.04G202000.1 showed peptide deletions due to shortened exon 2, suggesting functional divergence of Glyma.04G202000.1 (Figure 4.5). Since amino acid substitutions or deletions may have an impact on the formation of protein structure, they may lead to functional differences. Therefore, the structural differences of soybean *LFY* homologs suggest that two soybean *LFY* homologs may have undergone functional divergences during evolution.

In the present study, the result obtained from temporal and spatial expression analysis indicated that soybean *LFY* homologs may be involved in flowering evocation and emergence of floral meristem. In Arabidopsis, the expression of *LFY* gene is continuously elevated during plant growth and flowering is initiated when the expression level of *LFY* reaches a critical level (Blázquez et al., 1997, 2000). The expressions of soybean *LFY* homologs also showed patterns relative to flowering. Expression levels of soybean *LFY* homologs increased gradually under flowering inductive conditions (short day: SD) (Figure 4.6). In addition, Glyma.06G163600.1 showed higher expression levels (1.2 to 1.9 fold) than that of Glyma.04G202000.1 during SD, suggesting that Glyma.06G163600.1 plays overlapping roles with Glyma.04G202000.1 (Figure 4.6). In Arabidopsis, expression of *LFY* was detected on the flank region of shoot apical meristem during vegetative phase and an increased expression was detected on floral primordia (Blázquez et al., 1997). Similar expression patterns of Glyma.06G163600.1 was confirmed in soybean shoot apical meristems. It was expressed at the flank regions of vegetative meristem under flowering inductive conditions (between SD 0 to SD 6) (Figure

4.7). In SD 6, Glyma.06G163600.1 expanded its expression into the upper region of the shoot apical meristem. A strong expression of Glyma.06G163600.1 was detected on floral meristem (SD 10) (Figure 4.7). These spatial expression patterns of Glyma.06G163600.1 suggested that they may play regulating roles in the emergence of floral meristem.

In the present study, the regulatory roles of Glyma.06G163600.1 in flowering evocation was confirmed in transgenic Arabidopsis and tobacco plants. Ectopic expression of Glyma.06G163600.1 in Arabidopsis and tobacco caused early flowering. Constitutive expression of Glyma.06G163600.1 in transgenic Arabidopsis plants initiated flowering 7 days earlier than wild-type under flowering inductive conditions (Table 4.1). Similarly, ectopic expression of Glyma.06G163600.1 in transgenic tobacco plants also promoted early flowering, 15 days earlier than wild-type (Figure 4.22). Besides, expressions of floral pathway integrator genes and downstream genes of *LFY* were up-regulated in transgenic Arabidopsis and tobacco plants (Figure 4.27, 4.28). *LFY* gene, being a floral meristem identity gene, does not only play a crucial role in the initiation of flowering but also formulates floral identity by regulation of its downstream genes (Moyroud et al., 2010). In Arabidopsis, loss-of-function *lfy* mutant plants exhibited late flowering time (Weigel et al., 1992). In contrast, overexpressing endogenous *LFY* and *LFY* homologs from other plant species (aspen, cucumber, jatropha, rice and tobacco) in Arabidopsis promoted early flowering initiation with elevated expressions of flowering genes and downstream genes of *LFY* (Weigel and Nilsson, 1995; He et al., 2000; Tang et al., 2016; Zhao et al., 2018). These early flowering phenotypes with increased expressions of other flowering genes suggested that Glyma.06G163600.1 regulated flowering initiation via conserved pathways.

In this study, the roles of *GmLFY1* in flower developments were examined in transgenic tobacco plants containing *GmLFY::GUS.* Tissue-specific GUS expressions were detected on sepals of transgenic tobacco flowers, suggesting that Glyma.06G163600.1 is involved in expressions of flower homeotic genes in sepal formation (class A: *AP1* and Class B: *AP3*) (Figure 4.26). In Arabidopsis, *LFY* acts as a regulator in flowering developments via activation of downstream genes such as *AP1*, *AP3* and *AG*, which confer specific identities to floral organ primordia (Moyroud et al., 2010). It is reported that the ectopic expression of *LFY* causes abnormal floral organ developments such as shoot-like inflorescences and precocious flower developments in Arabidopsis and tobacco plants (Weigel et al., 1992; Ahearn et al., 2001). Although expressions of Glyma.06G163600.1 in floral organ developments were examined, no abnormal phenotypes during plant growth and flowering were observed. The overexpressed Glyma.06G163600.1 transgenic Arabidopsis and tobacco plants only promoted flowering evocation and exhibited insignificant morphological differences as against wild-type plants. This result may indicate functional diversification of Glyma.06G163600.1 in floral organ development. In other plant species, functional divergences of *LFY* gene were also reported (Ahearn et al., 2001). Tobacco has two *LFY* homologs in its genome and one of the tobacco *LFY* homologs, *NFL1*, is mainly responsible for the allocation of meristematic cells, rather than flowering initiation (Ahearn et al, 2001). In jatropha (*Jatropha cucurs*), defects on floral oragans were only detected in the *JtLFY* (a *LFY* homolog of jatropha) suppressed transgenic lines (Tang et al., 2016). In contrast, ectopic expression of *JtLFY* in Arabidopsis and jatropha only showed early flowering phenotypes with no morphological differences (Tang et al., 2016). In this regard, the analysis of biological functions of *GmLFYs* via transgenic soybean plants which harbor ectopically expressed (overexpression and suppression) two *GmLFY* genes respectively would provide new insight on the functions of soybean *LFY* homologs in flowering initiation and floral development.

The miR156-miR172 modules have recently been identified as main components in the ageing pathway and controllers of phase transition by way of inverse expressions (Wu et al., 2009; Yamaguchi and Abe, 2012). In Arabidopsis, *miR156a* and *miR172a* have been identified to play central roles in vegetative and reproductive development, respectively (Aukerman and Sakai, 2003; Mlotshawa et al., 2006; Wu et al., 2009). Recent studies on the identification of soybean miRNAs have revealed the presence of 28 loci in miR156 (encoding 28 mature miR156) and 15 loci in miR172 (encoding 5 mature miR172) in the soybean genome (Subramanian et al., 2008; Kulcheski et al., 2011; Wong et al., 2011; Goettel et al., 2014; Zhao et al., 2015). However, there are no reports on the functions of soybean miR156 and miR172. In the present study, the functions of soybean *miR156a* and *miR172a* (*gma-miR156a* and gma-*miR172a*) were investigated in tobacco plants. Extended juvenile phase-specific characteristics including round shape of leaves with small leaf area were observed in *gma-miR156a* transgenic tobacco plants (Figure 5.12, 13). On the other hand, shorthand juvenile phase was observed in *gma-*

miR172a transgenic tobacco plants. Constitutive expression of *gma-miR172a* in transgenic tobacco showed narrower shaped leaves than that of wild-type (Figure 5.12, 13). In addition, *gma-miR156a* showed late flowering phenotypes, as *gma-miR156a* transgenic lines started flowering at 89.9 DAG (days after germination), which was later than the flowering time of wild-type plants (66.2 DAG). *gma-miR172a* transgenic lines showed early flowering (45.5 DAG). These phenotypic differences such as prolonged juvenile developmental phase and early flowering are one of the main characteristics appearing due to the ectopic expression of miR156 and miR172 and the same has been reported in monocots (rice, swithgrass) and dicots (Arabidopsis, tobacco) (Xie et al., 2006; Wu et al., 2009; Fu et al., 2012; Feng et al., 2016). Therefore, morphological and phenotypical differences examined in the present study indicate that *gma-miR156a* and *gma-miR172a* are involved in the vegetative and reproductive developmental phases. miRNAs regulate the expression of their target genes by repression. In Arabidopsis, miR156 plays an important role in vegetative development via down-regulation of its target genes, *SPL* transcription factor family (Wu et al., 2009). In contrast, miR172 is responsible for reproductive developmental phase by repression of *AP2* and *AP2-like* genes (Aukerman and Sakai, 2003). Such regulatory mechanism of miR156-miR172 were also confirmed in the present study. *gma-miR156a* and *gma-miR172a* regulate vegetative and reproductive development via down-regulations of their target genes. The expressions of putative target genes *SPLs* (*NtSPL4a, NtSPL5a, NtSPL5b,* and *NtSPL9*) in *gma-miR156a* transgenic tobacco plants were repressed (Figure 5.28). In addition, the expression of *AP2a* (a putative target gene of *gma-miR172a*) was reduced in *gma-miR172a* transgenic lines suggesting that *gma-miR156a* and *gma-miR172a* may play regulating roles in vegetative and reproductive phases by repression of their target genes.

In this study, conserved roles of flowering genes in soybean (*GmLFY1* and *gma-miR156a-gma-miR172a*) were confirmed via heterologous expression in model plants (Figure 6.1). Soybean is a paleopolyploid plant and the genome of soybean has undergone two major duplications, resulting in about two-thirds of its genes having multiple copies and creating a possibility of functional diversifications (Schmutz et al., 2010; Jung et al., 2012). In this regard, further genetic investigations of these flowering genes in soybean are necessary in order to understand soybean flowering regulatory mechanisms. These analyses would provide pivotal information on functional diversifications of many homologs in soybean genome. In addition, genetic information of soybean gained from such analysis would provide deeper insights on understanding of genetic diversity of soybean which would be enable to use advances in soybean breeding. Molecular breeding using DNA-markers and manipulations of flowering times by plant biotechnology (genetic modifications) would accelerate the development of superior soybean varieties. Furthermore, these breeding systems would not only overcome maximum soybean yield barriers not previously broken-through using conventional breeding systems but also maximise soybean yield by control of vegetative developmental periods and expansions of cultivation area. Developments of stable soybean transformation systems could be an important tool to accelerate the improvement soybean yield. Although many studies on improvements of soybean transformation efficiency have been conducted, stable soybean transformations are only available in limited soybean cultivars (especially ergonomically inferior varieties) (Artif et al., 2013; Song et al., 2013). In recent studies on soybean transformation, Zhang and colleague (2016) reported that pathogen-defence systems of soybean have significant effects on genetic transformation of soybean. Susceptibility to *Agrobacterium* is related with the defence systems and this trait lead to cultivar-specific transformations in soybean. In this regard, studies on pathogen-defence systems of soybean would provide keys for developments of successful soybean transformation systems. In this study, additions of adenine hemisufate and PVP 40,000 improved shoot elongation rate during genetic transformation. Therefore, this improved shoot elongation will contribute to increase transgenic soybean shoots resulting in improvements of transformation efficiency. Diverse genetic information with stable genetic transformation systems in soybean would provide new tools and deeper insights in improvements soybean yield.

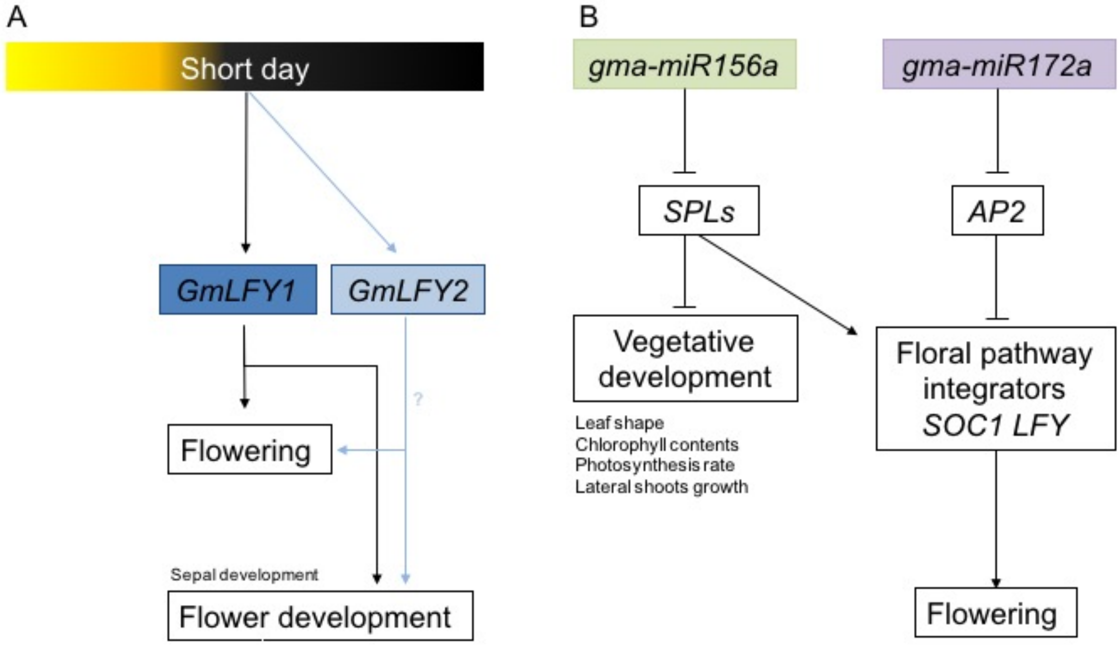

Figure 6. 1. Proposed roles of *GmLFY1*, *gma-miR156a and gma-miR172a*

(A) *GmLFY1* has conserved roles in flowering initiation. Expression of *GmLFY1* was elevated under flowering inductive condition (short day: SD) resulting in flowering evocation. Ectopic expression of *GmLFY1* in Arabidopsis and tobacco accelerated flowering initiation. Sepal-specific expressions of *GmLFY1* was observed during flower developments. *GmLFY2* has amino acid substitutions or deletions in a conserved DNA-binding domain indicating that functional divergences of *GmLFY2* may have occurred. (B) Soybean miR156-172 modules (*gma-miR156a* and *gma-miR172a*) plays crucial roles in vegetative and reproductive developments. *gma-miR156a* supressed expressions of *SPLs* to regulate vegetative developmental phase. *gma-miR172a* is responsible for reproductive developments via down-regulations of *AP2* gene. Ectopic expression of *gma-miR156a* in tobacco delayed flowering with prolonged vegetative development. Transgenic tobacco plants overexpressing *gma-miR156a* produced round shape of leaves with decreased chlorophyll contents and photosynthesis rate. After flowering *gma-miR156a* transgenic tobacco promoted lateral shoots growth with more leaves and flowers. *gma-miR172a* transgenic plants exhibited early flowering initiation via up-regulations of floral pathway integrator genes.

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Appendices

Table A1. Composition of medium for soybean transformation (Paz et al., 2006).

Medium	Compositions	Hormones	Additives	Antibiotics	Selection reagent
YEP medium (solid)	Yeast extract 10 g/L + Peptone 10 g/L + NaCl 5 g/L, Bacto Agar 15 g/L, pH 7.0	-	-	Kanamycin 50 mg/L Rifampicin 25 mg/L	-
YEP medium (liquid)	Yeast extract 10 g/L + Peptone 10 g/L + NaCl 5 g/L, pH 7.0	-	-	Kanamycin 50 mg/L Rifampicin 25 mg/L	-
Germination medium	B5 Gamborg's basal medium + 30 g/L of sucrose, Agar 8 g/L, pH 5.7	1.67 mg/L of BAP	-	-	-
Infection medium (IM)	0.1 x B5 Gamborg's basal medium + 3 g/L of sucrose + 3.9 g of MES, pH 5.4	1.67 mg/L of BAP + 0.25 mg/L of GA_3	40 mg/L of acetosyringone	-	-
Co-cultivation medium (CCM)	0.1 x B5 Gamborg's basal medium + 3 g/L of sucrose + 3.9 g of MES + 4.25 g/L of Agar, pH 5.4	1.67 mg/L of BAP + 0.25 mg/L of GA_3	40 mg/L of acetosyringone + 400 mg /L of L-cysteine + 154.2 mg/L of DTT + 157 mg/L of sodium thiolsulfate.	-	-
Washing medium (WM)	B5 Gamborg's basal medium + 0.59 g of MES + 30 g/L of sucrose, pH 5.7	1.11 mg/L of BAP	-	Cefotaxime 100 mg/L Timentin 100 mg/L Vancomycin 50 mg/L	-
Shoot Induction medium (SIM)	B5 Gamborg's basal medium+ 0.59 g of MES + 30 g/L of sucrose + 7 g of Agar, pH 5.7	1.11 mg/L of BAP	-	-	-
Shoot Induction medium 1 (SIM1)	B5 Gamborg's basal medium+ 0.59 g of MES + 30 g/L of sucrose + 7 g of Agar, pH 5.7	1.11 mg/L of BAP	-	Cefotaxime 100 mg/L Timentin 100 mg/L Vancomycin 50 mg/L	-
Shoot induction	B5 Gamborg's basal medium + 0.59 g of MES	1.11 mg/L of BAP	-	Cefotaxime 100 mg/L	Glufosinate 8 mg/L

medium 2 (SIM2)	+ 30 g/L of sucrose +7 g of Agar, pH 5.7			Timentin 50 mg/L Vancomycin 50 mg/L	
Shoot elongation medium (SEM)	MS basal medium + B5 vitamin + 0.59 g of MES + 30 g/L of sucrose + 7 g of Agar, pH 5.7	1 mg/L of zeatin + 0.5 mg/L of GA_3 + 0.1 mg/L of IAA	50 mg/L of L-asparagine + 100 mg/L of pyloglutamic acid	-	-
Shoot elongation medium containing AD and PVP (SEMAP)	MS basal medium + B5 vitamin + 0.59 g of MES + 30 g/L of sucrose + 7 g of Agar, pH 5.7	1 mg/L of zeatin + 0.5 mg/L of GA_3 + 0.1 mg/L of IAA	50 mg/L of L-asparagine + 100 mg/L of pyloglutamic acid 40 mg/L of Adenine hemisulfate + 500 mg/L of PVP 40,000	-	-
Shoot elongation medium 1 (SEM1)	MS basal medium + B5 vitamin + 0.59 g of MES + 30 g/L of sucrose + 7 g of Agar, pH 5.7	1 mg/L of zeatin + 0.5 mg/L of GA_3 + 0.1 mg/L of IAA	50 mg/L of L-asparagine + 100 mg/L of pyloglutamic acid	Cefotaxime 100 mg/L Timentin 50 mg/L Vancomycin 50 mg/L	Glufosinate 4 mg/L
Shoot elongation medium 2 (SEM2)	MS basal medium + B5 vitamin + 0.59 g of MES + 30 g/L of sucrose + 7 g of Agar, pH 5.7	1 mg/L of zeatin + 0.5 mg/L of GA_3 + 0.1 mg/L of IAA	50 mg/L of L-asparagine + 100 mg/L of pyloglutamic acid	Cefotaxime 100 mg/L Timentin 50 mg/L Vancomycin 50 mg/L	Glufosinate 4 mg/L
Rooting medium (RM)	0.5 x MS basal medium + B5 vitamin + 0.59 g of MES + 30 g/L of sucrose + 7 g of Agar, pH 5.7	1 mg/L of IBA	-	Cefotaxime 50 mg/L Timentin 25 mg/L Vancomycin 25 mg/L	--

BAP: 6-benzylaminopurine, GA_3: Gibberellic acid, IAA: Indole-3-acetic acid, IBA: Indole-3-butryic acid.

Table A2. Composition of medium for tobacco transformation (Horsch et al., 1985)

Medium	Compositions	Antibiotics	Selection reagent
YEP medium (solid)	Yeast extract 10 g/L + Peptone 10 g/L + NaCl 5g/L, Bacto Agar 15 g/L, pH 7.0	Kanamycin 50 mg/L + Rifampicin 25 mg/L	
YEP medium (liquid)	Yeast extract 10 g/L + Peptone 10 g/L + NaCl 5 g/L, pH 7.0	Kanamycin 50 mg/L + Rifampicin 25 mg/L	
Infection medium	MS basal medium + Sucrose 30 g/L + Agar 7 g, pH 5.8	-	
Co-cultivation medium (CCM)	MS basal medium + Sucrose 30 g/L, pH 5.8	-	
Shoot regeneration medium (SRM)	MS basal medium + Sucrose 30 g/L + Agar 7 g, pH 5.8	Carbenicillin 400 mg/L	Hygromycin 30 mg/L
Rooting medium (RM)	MS basal medium + Sucrose 30 g/L + Agar 7 g, pH 5.8	Carbenicillin 400 mg/L	Hygromycin 30 mg/L
Selection medium (SM)	MS basal medium + Sucrose 30 g/L + Agar 7 g, pH 5.8	Carbenicillin 400 mg/L	Hygromycin 30 mg/L

Table A3. Primer list used in this study

Name		Sequences (5’→3’)	Use	Remarks
GmLFY1	F	ATGGATCCAGACGCATTC	Cloning	Overexpressing GmLFY1 construct
GmLFY1	R	TTAGAAGGGAAGGTGAGCAC		
GmLFY1 pro	F	GGTCGGATCCCTATGAGTCATTCACCCTAC	Cloning	Promoter analysis
GmLFY1 pro	R	GTCGCCATGGGAGGGTTGGAAAGTGAGGAG		
GmLFY1-S	F	GCTTGTCGGAGGAGCCGGTGATGC	qRT-PCR	Detection of GmLFY1 expression
GmLFY1-S	R	GTCACGATGAAAGGATGCTCCC		
GmLFY2-S	F	TACCGTCCTCCCACCCGCTCCGG		Detection of GmLFY2 expression
GmLFY2-S	R	TCCGTGGAGAGGAGGTTG		
GmACT	F	ATCATGTTTGAGACCTTCAATGTG		
GmACT	R	CTCGAGTTCTTGCTCATAATCTAGG		
AtLFY	F	TGATGCTCTCTCCCAAGAAGGG		
AtLFY	R	TCAGTCTGGTCTTGTTGCTGCAC		
AtAP1	F	AGGGAAAAAATTCTTAGGGCTCAACAG		
AtAP1	R	GCGGCGAAGCAGCCAAGGTTGCAGTTG		
AtSOC1	F	ATAGGAACATGCTCAATCGAGGAGCTG		
AtSOC1	R	TTTCTTGAAGAACAAGGTAACCCAATG		

AtAG	F	TCACCAGCACAACCTTACCTTCC		
AtAG	R	TGGTACGCCGTGATTGCTGTTG		
AtTUB	F	GAGCCTTACAACGCTACTCTGTCTGTC		
AtTUB	R	ACACCAGACATAGTAGCAGAAATCAAG		
NtaAP1a	F	TTCACCTCGGGGAAGCGTA		
NtaAP1a	R	GCGGCATCACAGTGTTTTGT		
NtaAP1b	F	CTTTATATGTTGCATCTGAAGG		
NtaAP1b	R	CCTCCACATAAAGCAGACGAG		
NtaLFY1	F	GCCATTCTGAACGAAGCAACGC		
NtaLFY1	R	ATCACCAACACCACCAGAAACCG		
NtaSOC1a	F	TCTGCAGTCCTGTTCCCTTGTAG		
NtaSOC1a	R	TTCGTGCTCGGATGGTACTGAC		
NtaAP2a	F	TGTAGGAGAAATTTCGCTGTC		
NtaAP2a	R	GGATTGCCATTGTGAAGAAGC		
NtaGAPDH	F	GGTGTCCACAGACTTCGTGG		
NtaGAPDH	R	GACTCCTCACAGCAGCACCA		
Bar	F	GTACCGGCAGGCTGAAGTCC	Probe (Southern blot)	
Bar	R	CGGTCTGCACCATCGTCAAC		
HPT	F	CGAAAAGTTCGACAGCGTCTC		
HPT	R	GCTCCATACAAGCCAACCAC		
Pri-miR156a-F-BamHI	F	CCGGATCCGACATTTGGCTCTCATTCATCA	Cloning	
Pri-miR156a-R-KpnI	R	CCGGTACCAGGGGACTTATTTAAAGTGAGTGTG		
Pri-miR172a-F-BamHI	F	CCGGATCCGAGAGTGAACAGTATGCTACATATAGC	Cloning	
Pri-miR172a-R-KpnI	R	CCGGTACCTGACACCAACAAAAAGCA		
StemLoop-R	R	GTGCAGGGTCCGAGGT	Stem-loop qRT-PCR	
miR156a-ST	F	GCGGCGGTGACAGAAGAGAGT		
miR156a-ST	R	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGTGCT		
miR172a-ST	F	GCGGCGGCTAGAATCTTGATGATG		
miR172a-ST	R	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACATGCAG		
Pri-miR156a	F	CAACAAAACATTGAATATCAATCCAAG	RT-PCR	
Pri-miR156a	R	AAAGAATGGGAAGCTGACAGA		
Pri-miR172a	F	AACAGTCGTTATTTGCGGATG		

Pri-miR172a	R	GAAGTCGTTTATGGCTGATGC		
NtaSPL9	F	CCATAATGAGCGTCGGAGGAA	qRT-PCR	Feng et al. (2016)
NtaSPL9	R	CAGAACAAGGTCGGATGTAGGA		Feng et al. (2016)
NtaSPL4a	F	ACGAAAGGCGAAGAAAGA		Feng et al. (2016)
NtaSPL4a	R	TCAGGAATAGAACTTGGAGAG		Feng et al. (2016)
NtaSPL5a	F	GACAGACACTCATCAGAT		Feng et al. (2016)
NtaSPL5a	R	GAGCAAGCATATCGTATC		Feng et al. (2016)
NtaSPL5b	F	CATCTTTCGTATCCCTTTCTG		Feng et al. (2016)
NtaSPL5b	R	TTCCTTACAAGCCGTGAA		Feng et al. (2016)
Nta5SRNA	F	AGGCGTAGAGGAACCACAC		Feng et al. (2016)
Nta5SRNA	R	CTCCCCTACAGTATCGTCACC		Feng et al. (2016)