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석사학위 논문

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조선대학교 대학원

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ABBREVIATIONS

Agrobacterium	*Agrobacterium tumefaciences* GV3101 strain
Amp^R	Ampicillin resistance
AP3	*APETALA3*
Arabidopsis	*Arabidopsis thaliana* (L.) Heynh
BASTA	Glufosinate ammonium
CaMV	Cauliflower mosaic virus
cDNA	complementary DNA
Chl.	Chlorophyll
CI	Columella initial
CLV1	*CLAVATA1*
CLV3	*CLAVATA3*
COL	Columella
Col	Columbia-0
dag	day after germination
DEG2	degenerative primer 2
EDTA	Ethlylenediamine tetraacetic acid
EF1	*ELONGATION FACTOR 1*
EtBr	Ethidium bromide
gDNA	Genomic DNA
Gen^R	Gentamycin resistance
GFP	Green fluorescent protein

GL2	*GLABRA2*
GM	Genetically modified
grt-D	*green root-dominant*
GUS	β-glucuronidase
Kan^R	Kanamycin resistance
LB	Luria broth
LFY	*LEAFY*
LMO	Living modified organism
LRC	Lateral root cap
MADS	*MCM1, AGAMOUS, DEFICIENS, SRF* genes
Mb	Mega base pairs
MCS	Multiple cloning sites
MES	2-(N-morpholino)ehanesulfonic acid
mRNA	messenger RNA
MS	Murashige and skoog
NLS	Nuclear localization signal
PCR	Polymerase chain reaction
pga	*plant growth activator*
PI	*PISTILLATA*
PPCO	Polypropylene copolymer
QC	quiescent center
RT-PCR	Reverse transcription-PCR
SAM	Shoot apical meristem

SCR	*SCARECROW*
SDS	Sodium dodecyl sulphate
SHR	*SHORTROOT*
SGR	*STAYGREEN*
T-DNA	transferred DNA
TAIL-PCR	Thermal asymmetric interlaced-PCR
Tris-HCl	2-Amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride
Triton X-100	4-(1,1,3,3-Tetramethylbutyl)phenyl-polyethylene glycol
UAS	upstream activation sequence
UV	Ultra violet
Vis	Visible light
WOX5	*WUSCHEL-RELATED HOMEOBOX 5*
WT	Wild-type
WUS	*WUSCHEL*
X-gluc	β-D-glucuronide cyclohexylammonium salt

ABSTRACT

Induction of green root and petal development by the ectopic expression of *AtMYB115* and *AtMYB118* genes in Arabidopsis

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To screen for genes involved in the root development, several mutants displaying aberrant phenotypes have been isolated by using *GAL4/UAS* activation tagging system in the *Q2610* enhancer trap background. Among them, a dominant mutant developing the green-colored and thick root phenotypes was isolated and designated *green root-dominant* (*grt-D*). To understand the characteristics of the root of *grt-D*, the expression patterns of the above-ground tissue-specific reporters such as *CLAVATA3* (*CLV3*)*p:GUS* and *WUSCHEL* (*WUS*)*p:GUS*, the root-specific reporters such as *QC25* and *WUS-LIKE HOMEOBOX5* (*WOX5*)*p:GUS*, and root-radial patterning reporters such as *GLABRA2* (*GL2*)*p:GUS* and *SCARECROW* (*SCR*)*p:GUS* were examined after genetic crosses. As *CLV3p:GUS* and *WUSp:GUS* were ectopically expressed in the *grt-D* roots together with normal *WOX5p:GUS* and expanded *QC25* expression, the root of *grt-D* is seemed to possess both shoot and root identities. In addition, the

expression of *GL2p:GUS* and *SCRp:GUS* observed in the *grt-D* roots suggests that the radial patterning of the *grt-D* root might be at least partially maintained. When *grt-D* was germinated and grown in dark condition, the root color was not turned green indicating the green-root phenotype is light-dependent.

To identify the gene inducing the *grt-D* phenotype, thermal asymmetric interlaced-PCR was performed. The T-DNA containing *UAS* sequence was localized in 111 base pairs upstream of the start codon of *AtMYB115* (*MYB115*) (At5g40360). The reverse transcription-PCR results showed that *MYB115* is highly expressed in the root of *grt-D* background. To further confirm that the *grt-D* phenotype is derived from the ectopic expression of *MYB115*, *Q2610>>MYB115* transgenic lines were prepared. *Q2610>>MYB115* lines successfully phenocopied *grt-D* suggesting that *grt-D* phenotype is attributed to overexpression of the *MYB115*. In addition, the ectopic expression of *AtMYB118* (*MYB118*), the closest homologue of *MYB115*, driven by *Q2610* also induced green root phenotype, suggesting that *MYB115* and *MYB118* are functionally equivalent.

To develop green-petal flowers by the ectopic expression of *MYB115* and *MYB118* under the regulation of petal- and stamen-specific *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) promoters, *AP3>>MYB115*, *PI>>MYB115*, *AP3>>MYB118* and *PI>>MYB118* transgenic lines were prepared. Consequently, all combinations of overexpression lines developed the reduced and at least partially green-colored petals and sterile stamens. In addition, the chlorophyll a/b content in the green petals was highly increased as compared with that in the wild-type petals. Therefore, the ectopic expression of the *MYB115* and *MYB118* induced the development of chloroplast throughout the floral organ development in petals and stamens and inhibited the growth and maturation of petals and stamens.

국문초록

애기장대 *AtMYB115*와 *AtMYB118* 유전자의 과대발현에 의한 녹색 뿌리 및 화편 형성의 유도

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뿌리 발달에 관련된 유전자를 선별하기 위해서, *Q2610* 인핸서 트랩 배경에 *GAL4/UAS* 활성 표지 시스템을 사용하여 비정상적인 표현형을 나타내는 수 개의 돌연변이들을 선발하였다. 그중에서, 녹색의 두꺼운 뿌리 표현형을 나타내는 우성 돌연변이체가 선발되었고, 그 돌연변이체를 *green root-dominant* (*grt-D*)로 명명하였다. *grt-D* 뿌리의 특성을 이해하기 위해서, 지상부 조직-특이적 리포터 유전자인 *CLAVATA3* (*CLV3*)*p:GUS* 및 *WUSCHEL* (*WUS*)*p:GUS*, 뿌리-특이적 리포터인 *QC25* 및 *WUS-LIKE HOMEOBOX5* (*WOX5*)*p:GUS*, 뿌리-방사형 패터닝 리포터인 *GLABRA2* (*GL2*)*p:GUS* 및 *SCARECROW* (*SCR*)*p:GUS*의 발현 양상을 유전적 교배를 통하여 도입한 후에 조사하였다. *grt-D*의 뿌리에서 정상적인 *WOX5p:GUS* 발현 및 확대된 *QC25* 발현과 함께 *CLV3p:GUS* 및 *WUSp:GUS*가 이소적으로 발현됨으로써, *grt-D*의 뿌리는 지상부 및 뿌리 정체성을 모두 보유하는 것으로 보였다. 또한, *grt-D*의 뿌리에서 관찰된 *GL2p:GUS*와 *SCRp:GUS*의 발현은 *grt-D*의 뿌리에서 방사성 패터닝이 적어도 부분적으로 유

지된다는 것을 제시한다. *grt-D*가 발아하여 암조건에서 자랄 때, 뿌리 색상은 녹색으로 변하지 않았으며, 이는 녹색 뿌리 표현형이 빛에 의존적임을 제시한다.

grt-D 표현형을 유도하는 유전자를 확인하기 위해서 thermal asymmetric interlaced-PCR을 수행하였다. *UAS* 서열을 포함하는 T-DNA는 *AtMYB115* (*MYB115*) (At5g40360)의 개시코돈의 111 염기쌍 상류에 위치하였다. Reverse transcription-PCR의 결과는 *MYB115*가 *grt-D* 배경의 뿌리에서 높은 수준으로 발현된다는 것을 보여주었다.

grt-D 표현형이 *MYB115*의 이소적 발현으로부터 유도된다는 것을 재검정하기 위해서, *Q2610>>MYB115* 발현 형질전환체를 준비하였다. *Q2610>>MYB115*는 성공적으로 *grt-D* 표현형을 모사하였으며, 이는 *grt-D* 표현형이 *MYB115*의 과대발현으로 인한 것임을 제시한다. 또한 *MYB115*의 가장 가까운 동족체 *AtMYB118* (*MYB118*)의 *Q2610*에 의한 이소적 발현은 녹색 뿌리 표현형을 유도하였다.

꽃잎과 수술에 특이적인 활성을 가진 *APETALA3* (*AP3*) 및 *PISTILLATA* (*PI*) 프로모터의 조절하에 *MYB115*와 *MYB118*을 각각 이소적으로 발현시켜 녹색 꽃잎을 가진 꽃의 발달을 유도하고자, *AP3>>MYB115*, *PI>>*MYB115, AP3*>MYB118*과 *PI>>MYB118*을 발현하는 형질전환체 계통들을 준비하였다. 모든 발현 계통들은 작고, 적어도 부분적으로는 녹색인 꽃잎과 불염성의 수술을 형성시켰다. 또한, 녹색 꽃잎의 엽록소 a/b 함량은 야생형 꽃잎과 비교해서 매우 높았다. 따라서 *MYB115* 및 *MYB118*의 이소적 발현은 꽃 기관 발달의 전 과정 동안 꽃잎과 수술에서 엽록체의 발달을 유도하였고, 꽃잎과 수술의 생장과 성숙을 억제하였다.

Ⅰ. Introduction

1. Arabidopsis: the model for genetic studies in higher plants

Arabidopsis (*Arabidopsis thaliana* (L.) Heynh) is a small plant belonging to the Brassicaceae (Cruciferae) and has been used for a good model organism to understand the growth and development of plants through molecular genetic researches (Meinke et al., 1998). Arabidopsis has a genome of around 130 mega base pairs (Mb), containing about 25,000 to 30,000 genes, many of which have not been studied yet. To investigate the function of the uncharacterized genes, reverse genetic approaches have been introduced after whole genome sequencing of Arabidopsis was completed (Sessions et al., 2002).

Pools of transgenic plants possessing T-DNA fragments originated from *Agrobacterium* (*Agrobacterium tumefaciences*) have been massively sequenced and cataloged (Parinov and Sundaresan, 2000). Thereby, the function of any gene of interest could be easily understood by the observation of the loss-of-function phenotype of the corresponding mutant led by the T-DNA insertion as the insertion could inactivate or suppress the activity of any neighboring gene by interrupting proper transcription or translation. It is not easy to unravel the function of genes with overlapping functions through the conventional loss-of-function mutation screening methods. However, any multiple mutants losing the functions of the similar genes could be prepared by the genetic crosses between the T-DNA insertion mutants (Alonso et al., 2003).

The ultimate goal of the Arabidopsis genome research is to understand the

identity and function of all genes, and this large-scale insertion mutagenesis has opened up new possibilities in functional genomics of Arabidopsis (Parinov and Sundaresan, 2000).

2. Overexpression mutants screening by the activation tagging system

Although the reverse genetic method has been used as an alternative method to understand the functions of the uncharacterized genes, it is not effective for studying any gene family having extreme redundancy or any small gene having little chance to have any T-DNA insertion. Therefore, a new screening method that could overcome the functional redundancy has been required.

To solve the above-mentioned problems, an activation tagging screening system was developed to understand any gene function from its overexpression phenotypes by randomly introduced enhancers. The *35S* enhancer from cauliflower mosaic virus (CaMV) has been widely used as a molecular tool to detect the gain-of-function phenotypes of many genes in Arabidopsis (Weigel et al., 2000). However, when the *35S* enhancer activates negative regulators, it could induce embryo- or seedling-lethality and sterility due to its high level of transcriptional activity throughout the whole plant body and lifespan. Therefore, *GAL4/UAS* activation tagging system, which is a representative method for selecting new genes by the introduction of the tissue-specific enhancer, has been studied and utilized (Brand and Perrimon, 1993).

Yeast-derived *GAL4* transcription factors promote transcription of downstream genes by binding to *UAS* cis-elements. The *GAL4* transcription factor has been modified to show higher transcriptional activity by the addition of *VP16* activator (*GAL4-VP16*) which enhances transcription activity. In the context of *GAL4/UAS* expression, gene overexpression can be tissue-specifically restricted. Among the enhancer trap lines expressing *GAL4-VP16* in Arabidopsis, the *Q2610* exhibited very strong *GAL4* activity in a root-specific manner (Haseloff, 1998). Therefore, even if the root growth is very defective by the overexpressed gene, the growth of the above-ground organs could manage to survive and produce seeds (Waki et al., 2013).

3. Root development and structure in Arabidopsis

In Arabidopsis, there exist two types of stem cells residing in root and shoot meristem producing the above-ground and the underground organs, respectively (Benfey and Scheres, 2000). In addition, the genes that function in the above-ground or underground parts meristem regions are very important for plant development. *WUSCHEL* (*WUS*), which is involved in the development of the above-ground parts, function as a marker of organizing center (OC), and *CLAVATA3* (*CLV3*) act as a stem cell marker. The *GLABRA2* (*GL2*) involved in root development is epidermis-specific and *WUSCHEL-RELATED HOMEOBOX 5* (*WOX5*) is expressed quiescent center (QC)-specific. Previous reports have shown that Arabidopsis establishes the root meristem during embryogenesis from globular to late heart stage (Doerner, 1995).

Most plant root organs have a columnar structure, and its radial patterning is essential for plant development (Dolan et al., 1993). These radial patterns are initially determined by embryogenesis and persisted by the division of the root meristem (Scheres et al., 1994). The cross-section of the Arabidopsis root organ consists of layers of pericycle, endodermis, cortex and epidermis, which have concentric circles. Previously reported, the plant-specific transcription factors *SHORTROOT* (*SHR*) and *SCARECROW* (*SCR*) regulate the asymmetric division of cortex/endodermis initial (CEI) cells and endodermis cell fate (Di Laurenzio et al., 1996; Helariutta et al., 2000; Sena et al., 2004). SHR protein extends to cells in contact with stele, vascular tissue and pericycle. It then constructs a nuclear-localized protein through interaction with the SCR (Nakajima et al., 2001). Thus, the developed SHR-SCR protein complex regulates several genes, including SCR, to confer endothelial cell identity (Cui et al., 2007).

The root stem cells, also called as initials, surrounding QC produce all cell types of root through the regulation of cell differentiation and expansion. As the growth of the roots varies depending on the influence of cues from the internal and external environment, this process continues throughout the lifespan of a plant. The root meristem is protected from rhizosphere by the two types of root caps, the columella (COL) located in the center and lateral root cap (LRC) at the flank. There are columella initials (CIs), epidermis / LRC initials, stele initials, and CEIs in the adjacent part of QC, and these initial cells perform the role of stem cells and continuously produce undifferentiated cells (Mansfield and Briarty, 1991; Bennett et al., 2010).

4. Roles of *MYB* in the development of Arabidopsis

MYB proteins are present in most eukaryotes and have diverse functions (Romero et al., 1998; Riechmann et al., 2000). MYB domain consists of 4 incomplete amino acid sequence repeats (R) as about 50 amino acids. Furthermore, each has three alpha-helices structures (Ogata et al., 1992; Konig et al., 1996). In plant systems with the relatively high diversity of MYB proteins, they have four classes (R1, R2, R3 and R4) (Stracke et al., 2001). In addition, *MYB* family in plants increased selectively from the R2R3-MYB large family (Romero et al., 1998).

Some R2R3-MYB are involved in the control of flavonoid biosynthesis. It also performs the biosynthesis function of flavonol, anthocyanin, lignin, xylan, cellulose, and aliphatic/indolic glucosinolates (Jin et al., 2000; Preston et al., 2004; Zhong et al., 2007). In addition, it gives cell fate and identity, such as control of trichome initiation in shoot and root hair patterning, regulation of trichome extension and branching in combination, regulation of external seed coat differentiation, a putative regulator of early seed germination (Kirik et al., 2005; Jakoby et al., 2008; Kang et al., 2009; Li et al., 2009b).

MYB also functions in plant development. For example, it promotes the development of pollen and anther, regulates hypocotyl growth by reacting with blue light and far-infrared rays, accompanies embryogenesis, regulates root development through control of cell cycles at the root tip and the expression of auxin-inducible gene to control lateral root formation (Millar and Gubler, 2005; Shin et al., 2007; Mu et al., 2009; Wang et al., 2009; Yang et al., 2009).

In addition, MYB is involved in biotic and abiotic stress responses. For instance, it is possible to encode activators of hypersensitive cell death

programs, to regulate stomatal movement by ABA signaling cascade, to participate in cold stress resistance, to respond to salt and dehydration, regulate the induction of ABA by the gene responsible for the phosphorylation, and involve a response to phosphate deficiency (Abe et al., 2003; Cominelli et al., 2005; Agarwal et al., 2006; Devaiah et al., 2009; Li et al., 2009a).

Functional redundancy among related transcription factors have been established in Arabidopsis research and recent studies show that *AtMYB118* (*MYB118*) and *AtMYB115* (*MYB115*) are close relatives of the *MYB* family (Zhang et al., 2009). *MYB115* and *MYB118* share the series of transcriptional targets with transcriptional induction when the endodermis maturation stage is initiated. As known, MYB115 protein contains a predicted nuclear localization signal (NLS) in the N-terminal domain and acts as a potential transcriptional activator. Furthermore, *MYB*-related transcription factors are well known for regulating various branches of the flavonoid pathway from plants and generally play a broad role in the regulation of pharmacopropanoid metabolism (Tamagnone et al., 1998).

5. *AP3-PI* Floral homeotic genes

Arabidopsis flowers are composed of the four tissues: sepal, petal, stamen and carpel, which occur in the proliferation of cells originating from floral meristem (Hill et al., 1998). As reported previously, the ABCE model is essential for determining the floral organ characteristics of plants (Figure 1). The combination of four genetically independent classes of genes establishes the continuous development of flower buds, sepals, petals, stamens and carpels

(Bowman et al., 1993).

The function of A and the E specify the identity of the sepal together. The function of A, B and E bind to determine the identity of the petals, while the B, C and E collectively regulate the identity of the stamens, and the C and E together regulate carpel formation (Jing et al., 2014). In addition, most of the A, B, C and E are MADS-box genes, and their homologs are mainly present in the major lines of the herbaceous plants (Kim et al., 2005; Melzer et al., 2010). According to the ABCE model, B function MADS-box genes such as *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) play an important role in determining the development of petals and stamens (Hill et al., 1998; Zahn et al., 2006; Causier et al., 2010; Burgos-Rivera and Dawe, 2012). The genes necessary to designate these floral patterns are classified into three main classes: meristem identity, organ identity and cadastral (Jack et al., 1993).

The meristem identity is necessary for the normal formation of flower meristem and the cadastral regulates the expression boundary of the identity in the organ (Hill et al., 1998). When the majority of the plant's genes are deficient, they have serious defects in the early stages of development and cannot have normal phenotypes. (Burgos-Rivera and Dawe, 2012). Therefore, the presence of genes is essential for the development of normal flowers in the course of plant development. *AP3* and *PI* are homeotic genes of flower that are important for petal and stamen development of Arabidopsis, and each part of the promoters confer initial petal-specific expression (Krizek and Meyerowitz, 1996; Honma and Goto, 2000; Burgos-Rivera and Dawe, 2012).

The 288 bp of the *AP3* promoter fragment were considered to be petal-specific (Hill et al., 1998), but the 300 bp of the *PI* promoter fragment were

reported to induce the expression in both petals and stamens (Honma and Goto, 2000). In addition, *AP3* transcripts were first detected in the meristem, which produced the prototypes of petal and stamen and remained expressed in this region during the development of the organ (Jing et al., 2014). When the petals and stamens are primitive, *AP3* mRNA is present at a fairly high level in all cells of the organ. At the late flowering stage of the petal, *AP3* transcripts are continuously detected during stamen development, and in the envelope of the developing ovule until fertilization (Jack et al., 1992).

The regulation of *AP3* transcription is divided into two phases: the initiation of expression and the maintenance of expression. The correct initiation of expression of *AP3* is dependent on the activity of the tissue division *LEAFY* (*LFY*) and *APETALA1* (*AP1*). A decrease of *AP3* expression in the strong *lfy* allele indicates that *LFY* is a positive regulator of *AP3* transcription (Weigel and Meyerowitz, 1993). It has also been demonstrated that *AP3* can positively and automatically regulate its own expression based on overexpression under the control of the CaMV *35S* promoter (Jack et al., 1994).

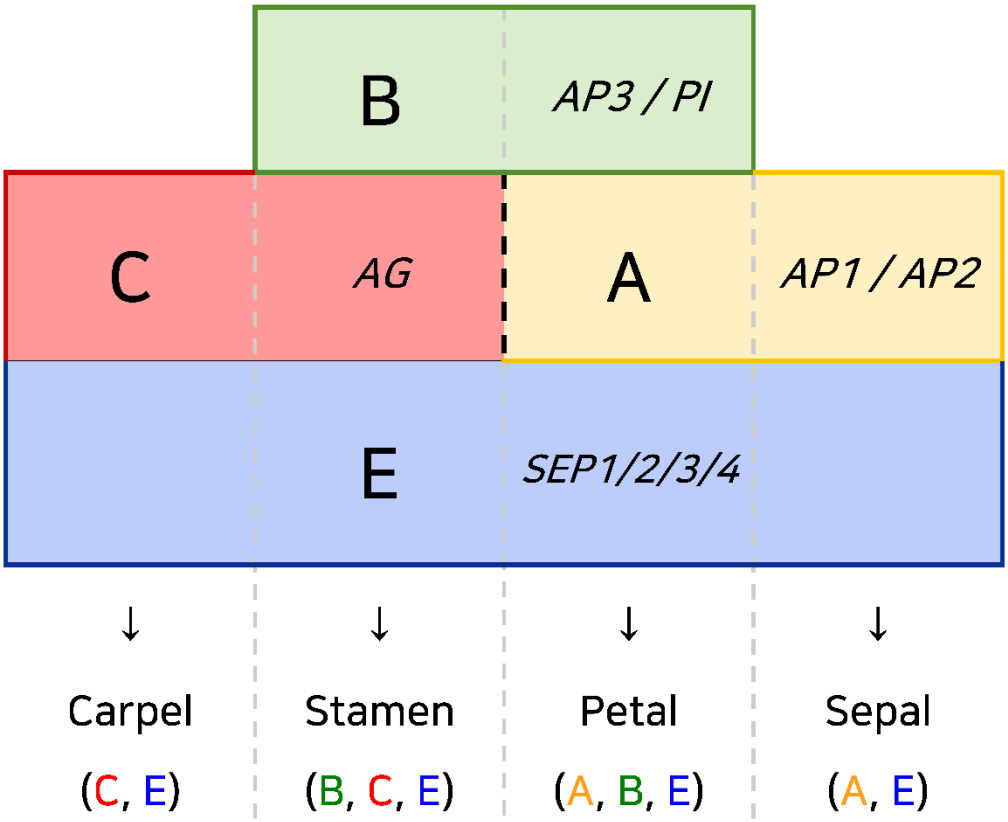


Figure 1. A diagram of the ABCE model in Arabidopsis.

In the plant, the ABCE model gives and controls the identity of the floral organ. The function of A gives the identity of sepal and petal. The function of B is accompanied by petal and stamen development. The function of C is involved in the carpel, stamen development. The function of E plays a role throughout the flower organ (Haughn and Somerville, 1988; Ma, 2005).

6. Applications of ectopic chloroplast biogenesis in plants

The chloroplast is a green plastid in which photosynthesis takes place. The biogenesis of chloroplast requires a coordinate representation of the nucleus and chloroplast genes. The nucleus and chloroplast genes are encoded in the nucleus and plastid, respectively, so chloroplast production requires a close coordination between the two genomes (Fitter et al., 2002; Zhou et al., 2011; Kobayashi et al., 2013). Despite many studies on the biogenesis of chloroplast, the genetic basis and molecular mechanism of chloroplast biogenesis and development have not yet been elucidated (Zhou et al., 2011). Therefore, it is expected that the commercialization of useful materials derived from plants and the production of ornamental plants will be increased by studying ectopic biogenesis of chloroplast in petal and root. If the process of chloroplast biogenesis is fully elucidated, it would be easy to manipulate the color of non-green organs such as roots and petals into green for the development of plants possessing commercially useful characteristics.

The flower industry occupies a very large market worldwide, and the color of flowers is one of the representative target traits in the botanical field (Lim et al., 2011). Previous reports showed that plants with flower color change increased decorations to attract pollinators and restricted the visitors unrelated to pollinators (Lamont, 1985; Weiss, 1995; Oberrath and Böhning-Gaese, 1999). However, in the above case, the plant changed the color of the flower itself for the pollinator.

Currently, botanists use traditional breeding methods or natural pigment synthesis methods by gene introduction to express colors that the flowers did not have (Ha et al., 2012). As a representative example, a blue rose expressed

using a flavonoid metabolic mechanism has been commercialized (Katsumoto et al., 2007).

Most of the roots of plants are usually heterotrophic organs that grow underground and rely on the energy produced from above-ground leaves, but some of the plants are able to change the root color into green for the photosynthesis (Aschan and Pfanz, 2003). Chloroplast development in Arabidopsis wild-type roots was substantially inhibited under light conditions, and chloroplast accumulation was observed only in the upper part of the primary root below the hypocotyl (Kobayashi et al., 2012). In plants, the *STAYGREEN* (*SGR*) has Mg-dechelatase, which catalyzes the conversion of chlorophyll a to pheophytin a, which destabilizes the protein-pigment complex and plays a key role in chlorophyll degradation (Hortensteiner, 2009; Sakuraba et al., 2012; Shimoda et al., 2016). Therefore, ectopic development of chloroplasts in Arabidopsis roots is not a usual phenotype. The application of these chloroplast ectopic develops opens up a new paradigm for the transformation of the traditional generalized leaf tissue culture field to the root-organ-culture sector for the production of secondary metabolites. Plant tissue culture methods require complex culture conditions to achieve cell growth and metabolism.

However, root organ culture is expected to be a new alternative in the biotechnology industry because relatively simple culture conditions are performed (Flores et al., 1987; Jeong and Park, 2006; Jeong and Park, 2017). In addition, it does not require hormones and is genetically stable (Giri and Narasu, 2000). Previous reports have shown that alkaloid production in root organ cultures is higher than callus tissue cultures. As a result, root cultured

under dark and light conditions produced higher yields of secondary metabolites under light conditions (Flores et al., 1987). From the above point of view, the root system of the plant has great biochemical potential. If the plant root-organ-culture is commercialized and widely distributed, it is considered to be the main medium for the production of secondary metabolites (Jeong and Park, 2017).

In this study, a *green root-dominant* (*grt-D*) mutant has been screened by using the *GAL4-UAS* activation tagging method, and the *MYB115* was found to be overexpressed in the root of the *grt-D* as reported previously (Wang et al., 2009). In the *grt-D* mutant, the green root phenotype was maintained without any inducer treatment. Therefore, I devised a method that can maintain the color of permanently altered flowers. To improve the characteristics of plants, Arabidopsis possesses green-petal flowers were developed by ectopically expressing *MYB115* and *MYB118* under the regulation of the petal- and anther-specific *AP3* and *PI* promoters.

Ⅱ. Materials and Methods

1. Plant materials and growth conditions

In this research, the Columbia-0 (Col-0) ecotype of Arabidopsis was used as the wild type. The *grt-D* was used as a mutant. The *GL2p:GUS* (Masucci et al., 1996), *QC25* (Aida et al., 2004), *SCRp:GUS* (Malamy and Benfey, 1997), *WUSp:GUS* (Gross-Hardt et al., 2002), *WOX5p:GUS* (Sarkar et al., 2007), *CLV3p:GUS* (Lenhard et al., 2002) lines were previously known.

In order to growth Arabidopsis in sterile conditions, the seeds surface were sterilized with a 0.7% sodium hypochlorite solution (Yuhan co.; Seoul, Korea) for 10 min and washed 6 times with sterile distilled water using a pasteur pipette. The sterilized seeds were sowed on half-strength Murashige and Skoog (MS) (Duchefa; Haarelm, Netherlands) plates containing 1% (w/v) sucrose, 0.02% MES (w/v) and 0.6% Gelrite (w/v). For cold stratified, the seeds incubated at 4℃ for 2 d. For germination, they grew vertically at 22℃ under the 24 h light. For combination of crossed, mutant and transgenic plants, seedling grown on MS plates were transferred to soli mixture (Sunshine Mix #5; Sungro, Agawam, MA, USA) and grown up to ripeness in growth chamber at 22℃ under the 24 h light.

2. Constructs and transformation

To produce transgenic lines of *UASp:MYB115*, *UASp:MYB118*, *MYB115* and *MYB118* coding region fragments were amplified by polymerase chain reaction (PCR). They were inserted into the pJET2.1 cloning vector (Thermo Fisher; Carlsbad, CA, USA). The *MYB115* and *MYB118* coding fragments inserted into the cloning vector were separated using restriction enzymes BamH I and Spe

Ⅰ. The insertion sites of *MYB115* and *MYB118* coding region were secured by using BamH Ⅰ and Spe Ⅰ in the multiple cloning sites (MCS) of the 5x *UAS*+pCB302+Tnos vector, which is a vector for overexpression. The coding region fragments of *MYB115* and *MYB118* were transferred into overexpression vector (Figure 2).

To produce transgenic lines of *AP3p:GAL4-VP16* and *PIp:GAL4-VP16*, *AP3* or *PI* promoters and *GAL4-VP16* fragment were amplified by PCR and they were inserted into the cloning vector. The *AP3* or *PI* promoters (EcoR Ⅰ and BamH Ⅰ) and *GAL4-VP16* (BamH Ⅰ and Xba Ⅰ) fragments inserted into the cloning vector were isolated using restriction enzymes. The insertion sites of *AP3* or *PI* promoters were secured by EcoR Ⅰ and BamH Ⅰ in the MCS of the pCB302+Tnos vector. The promoter fragments of *AP3* or *PI* were transferred into expression vector. Subsequently, the insertion sites of *GAL4-VP16* were obtained by using BamH Ⅰ and Xba Ⅰ in the expression vector into which the *AP3* or *PI* promoter. The fragment of *GAL4-VP16* was transferred to the expression vector (Figure 3).

Continually, constructs were introduced into *Agrobacterium tumefaciens* GV3101 strain (Hofgen and Willmitzer, 1988), respectively, and transformed into Col-0 plants using *Agrobacterium*-mediated floral dip method (Clough and Bent, 1998). To screen for transgenic plants, BASTA (glufosinate ammonium solution, Bayer crop sci.; Leverkusen, Germany), one of the plant herbicides, was sprayed onto T_1 plants or T_1 seeds sown on half-strength MS medium containing BASTA. The PCR was performed using premium-*pfu* polymerase kit (Nanohelix co.; Daejeon, Korea). The primers used for PCR were also displayed in table 1.

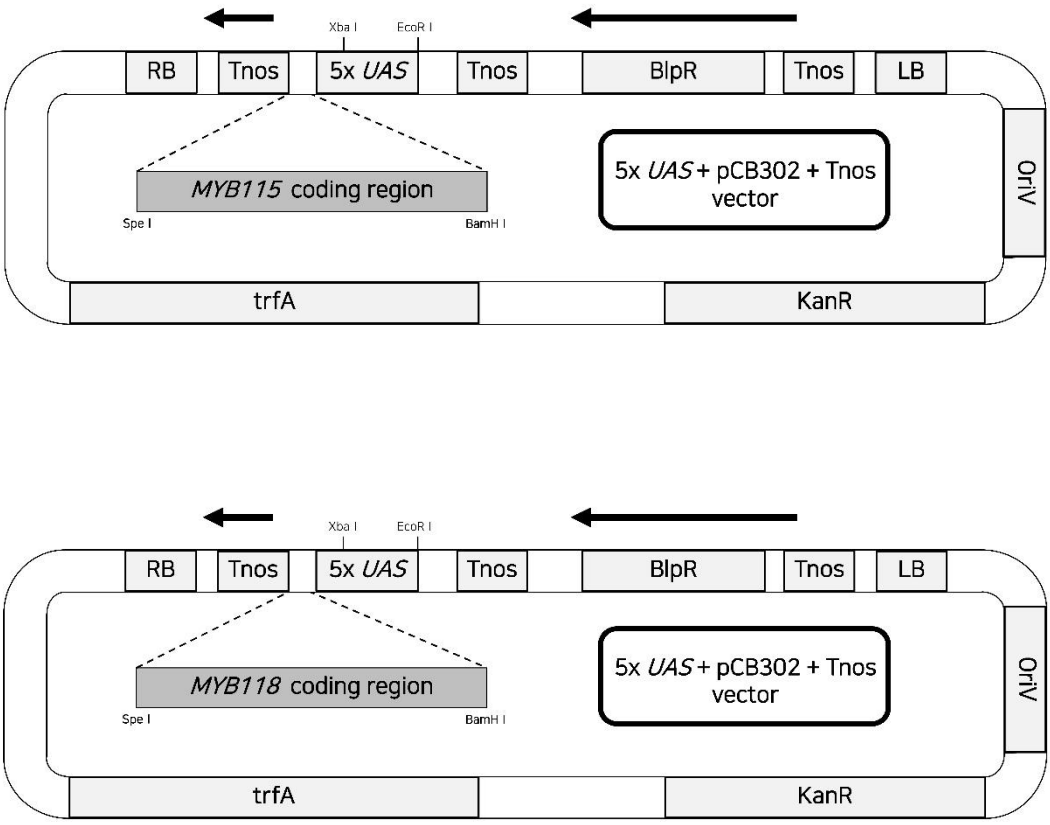


Figure 2. Diagrams of the expression vector constructs for *MYB115* and *MYB118* under the regulate of *UAS* promoter.

This diagram introduced *MYB115* and *MYB118* coding sequences into the 5x *UAS*+pCB302+Tnos vector. The Multiple cloning sites (MCS) region was cleaved with BamH I and Spe I. After that *MYB115* and *MYB118* were inserted.

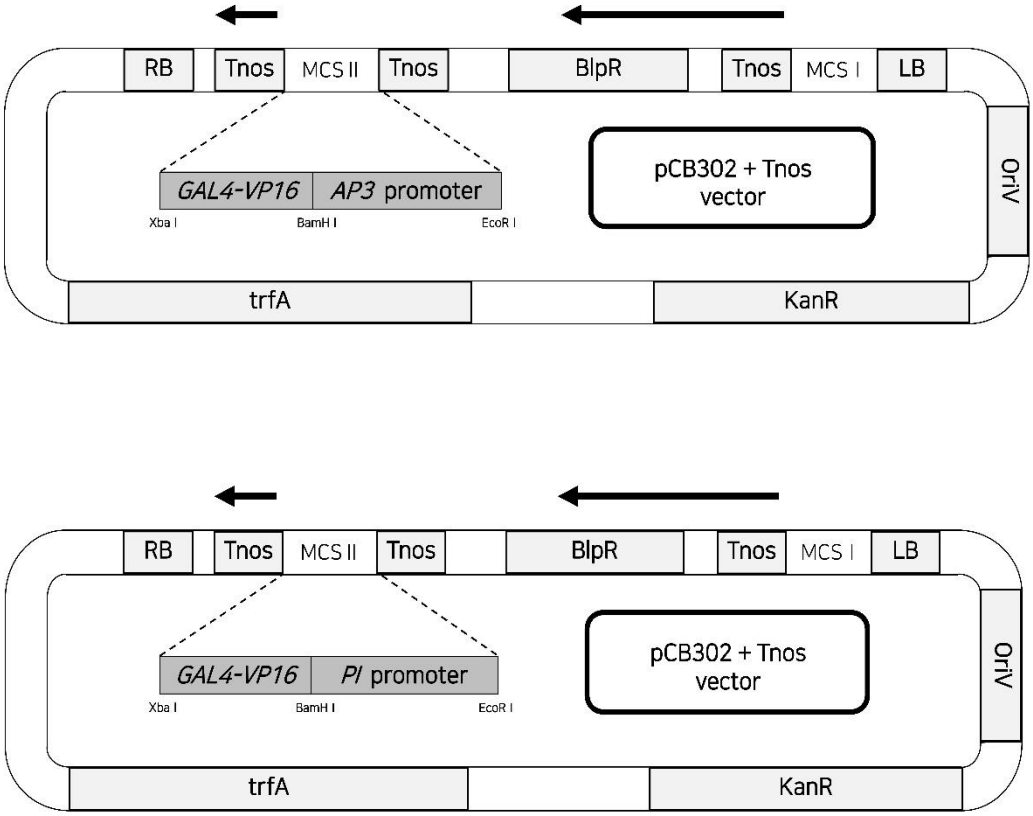


Figure 3. The schematic diagram of *AP3p:GAL4−VP16* and *PIp:GAL4−VP16*.

This diagram introduced *AP3p:GAL4−VP16* and *PIp:GAL4−VP16* into the pCB302+Tnos vector. The MCS region was cleaved with EcoR I and Xba I. After that *AP3p:GAL4−VP16* and *PIp:GAL4−VP16* were inserted.

3. Thermal asymmetric interlaced (TAIL)-PCR and integration site identification

TAIL-PCR was carried out by the previously reported three-step method (Liu and Whittier, 1995). The left border-specific (pBIB372, pBIB262 and pBIB172) primers and degenerative primer 2 (DEG2) were used each reaction of TAIL-PCR cycling. In addition, 20 ㎕ of the PCR product were prepared using *Taq*-plus PCR kit (Nanohelix co.; Daejeon, Korea). The primers used are displayed in table 1.

The cycling parameters for the primary reaction (pBIBLB372 / DEG2) of TAIL-PCR were as follows: 1 cycle of 93℃ for 1 min and 95℃ for 1 min; 5 cycles of 94℃ for 30 sec, 60℃ for 1 min and 72℃ for 2 min 30 sec; 1 cycle of 94℃ for 30 sec, 60℃ for 1 min, from 25℃ to 72℃ (0.2℃ / sec) for 3 min; 2 cycles of 94℃ for 30 sec, 60℃ for 1 min and 72℃ for 2 min 30 sec; 15 cycles of 94℃ for 30 sec, 44℃ for 1 min and 72℃ for 2 min 30 sec; 1 cycle of 72℃ for 5min.

The second-step reaction was prepared by using a 2% dilution of the first-step PCR product as a template and 20 ㎕ of PCR product using *Taq*-plus. The cycling parameters for the secondary reaction (pBIBLB262 / DEG2) were as follows: 2 cycles of 94℃ for 30 sec, 60℃ for 1 min and 72℃ for 2 min 30 sec; 12 cycles of 94℃ for 30 sec, 44℃ for 1 min and 72℃ for 2 min 30 sec; 1 cycle of 72℃ for 5 min.

The third-step reaction was performed using a 10% dilution of the second-step PCR product as a template and 50 ㎕ of PCR product using *Taq*-plus polymerase. The cycling parameters for the tertiary reaction (pBIBLB172 / DEG2) were as follows: 20 cycles of 94℃ for 30 sec, 44℃ for 1 min and 72℃

for 2 min 30 sec; 1 cycle of 72℃ for 5 min.

Subsequently, to identify the gene that induces the phenotypes of *grt-D*, the tertiary reaction product of TAIL-PCR was purified using Gel purification kit. The purified product was introduced into the pGEM-T-easy vector (Promega; Madison, WI, USA) and the corresponding sequence was analyzed.

Table 1. List of primers for PCR

Construct	Primer	Sequence (5' → 3')
MYB115 coding region	Forward	GGATCCATGTATCACCAAAATCTGATTTCA
	Reverse	ACTAGTTTAATTCCAACCATTCATGAGCA
MYB118 coding region	Forward	GGATCCATGGAGTTCGAGTCAGTGTTC
	Reverse	ACTAGTCTAAAGACGACCATGAGCAATC
AP3 promoter	Forward	CGAATTCAGTGTCTTGTAATTATACAA
	Reverse	GGGATCCATTCTTCTCTCTTTGTTTAA
PI promoter	Forward	CGAATTCTTATTACGTTACTTCAAGTT
	Reverse	GGGATCCCTTTCTCTCTCTATCTCTTT
GAL4-VP16	Forward	CGGATCCATGAAGCTCCTGTCCTCCATC
	Reverse	GTCTAGACTACCCACCGTACTCGTCAAT
pBIBLB372		GCAGCTGGCACGACAGGTTTC
pBIBLB262		GCTCGTATGTTGTGTGGAATTGT
pBIBLB172		GTCGACAGATCTCATGCCTGCA
DEG2		AWGCANGNCWGANATA

4. Microscopy

Root of seedlings at 3−5 day after germination (dag) and floral organs of *AP3>>MYB115*, *PI>>MYB115*, *AP3>>MYB118* and *PI>>MYB118* transgenic lines at two−weeks−old were observed under a Leica MZ10F stereomicroscope (Leica; Wetzlar, Germany). Root cross−sections of *grt−D* and control were also observed under an Axioskop20 optical microscope (Zeiss; Oberkochen, Germany). In this study, photographs of plants were taken with a microscope image detector module (Dixi sci., Deajeon, Korea).

5. Observation of root cross−section

The roots of Arabidopsis were solidified using Technovit® 7100 (Kulzer; Hanau, Germany). As manufacture protocol, thin sections of 2 ㎛ were obtained using a tungsten knife of the microtome (Leica; Wetzlar, Germany). Finally, these samples were observed after toluidine blue staining.

6. Histochemical GUS assay

The GUS solution used was 100 mM $NaPO_4$ (pH 7.4), 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 10 mM EDTA (pH 8.0), 0.1% Triton X−100 and 1 mM X−Gluc. 4 dag seedlings were stained with GUS solution for 2 − 10 h and then observed.

7. Reverse transcription (RT)−PCR

RNA was extracted using wild−type and mutant seedling using RNeasy plant mini kit (Qiagen; Hilden, Germany). The extracted RNA of *grt−D* and WT−

like sibling was measured and quantified with a Biophotometer (Eppendorf; Hamburg, Germany). Then, 1 ㎍ of RNA was treated with DNase I (Invitrogen; Carlsbad, CA, USA) at room temperature for 15 min to degrade gDNA. Subsequently, the EDTA solution was added to the tube containing the RNA and then the tubes were incubated at 65℃ for 10 min.

AccuScript High Fidelity First strand cDNA synthesis kit (Agilent; Santa Clara, CA, USA) was used for cDNA synthesis. The first strand cDNA was prepared by treating at 42℃ for 1 h. RT-PCR (94℃, 3 min - 72℃, 5 min) using the prepared cDNA as a template was carried out for 25 cycles.

8. Extraction of chlorophylls

The roots of *grt-D*, roots of WT-like sibling and petals of *AP3>>MYB115*, *PI>>MYB115*, *AP3>>MYB118* and *PI>>MYB118* transgenic plants were collected, respectively. 10 mg of the cut petals or roots were extracted in 1 mL of 80% ice-cold acetone with 2.5% sodium phosphate (pH 7.8) for 1 d at 4℃. This extraction method was carried out based on (Porra et al., 1989) study results.

Chlorophyll (Chl.) contents were measured with the crude extracts from Arabidopsis root and petal tissues, respectively. The chlorophyll content was measured at 646.6 nm and 663.6 nm (red light), which is the maximum absorption wavelength, using a UV-Vis spectrophotometer (Klab co.; Daejeon, Korea). Then, the chlorophyll contents were calculated using the equations for chlorophyll concentrations (Table 2) (Porra et al., 1989; Porra, 2002).

Table 2. Equations for Chl. concentrations in plants

Equations for Chl. concentrations (㎍ / mL)
$[Chl.\ a] = 12.25\ Abs^{663.6} - 2.55\ Abs^{646.6}$
$[Chl.\ b] = 20.31\ Abs^{646.6} - 4.91\ Abs^{663.6}$
$[Chl.\ a + b] = 17.76\ Abs^{646.6} + 7.34\ Abs^{663.6}$

Abs means absorbance.

This equation refers to the results of the (Porra et al., 1989) study.

Ⅲ. Results

1. Screening of root defective mutants derived from the *GAL4-UAS* activation tagging system

The 5x *UAS*-repeat tags were introduced into the *Q2610* enhancer trap line possessing strong *GAL4-VP16* transcriptional activity mostly in the root tip in order to screen for genes involved in root development (Waki et al., 2013). About five thousands of T_1 seedlings were selected in the MS media containing the antibiotics, hygromycin B and the phenotypes of transgenic plants were observed. As a result, several transgenic plants with abnormal phenotypes were screened as compared with the wild type (Song, 2016). Among them, a mutant developing thick green root phenotype designated *green root-dominant* (*grt-D*). The root of *grt-D* showed autofluorescence emitted from chloroplasts was observed when observed through the Zeiss filter set 38 (EX BP 470/40, BS FT 495, EM BP 525/50) (Figure 4).

To examine the fine structure of the root, *grt-D* seedlings were embedded in resin, sectioned, and stained with toluidine blue. The root of *grt-D* displayed increase in the cortex and epidermal cell number. Furthermore, an additional epidermal layer developed in *grt-D*. In addition, it displayed defective root epidermal cell patterning by increased the layer of root epidermal cells and number of cortex (Figure 5B and 5D). On the other hands, wild-type root develop epidermis of a single layer and cortex consisting of eight cells. (Figure 5A and 5C). To examine the identity and patterning of root epidermis of *grt-*

D, *GL2p:GUS*, known to be expressed in non-hair cells was introduced in *grt-D*. *GL2p:GUS* expression was compromised but remained in the root epidermis of the mutant (Figure 6A). The expression patterns of *SCRp:GUS*, an endodermis marker and *WOX5p:GUS* in *grt-D* were consistent with those in WT indicating that the QC and endodermal identities in *grt-D* are maintained (Figure 6C and 6D). However, the expression of *QC25*, a *QC25*-specific enhancer trap line, was increased in *grt-D* as compared to that in the WT suggesting that the root meristem activity of *grt-D* might be increased (Figure 6B).

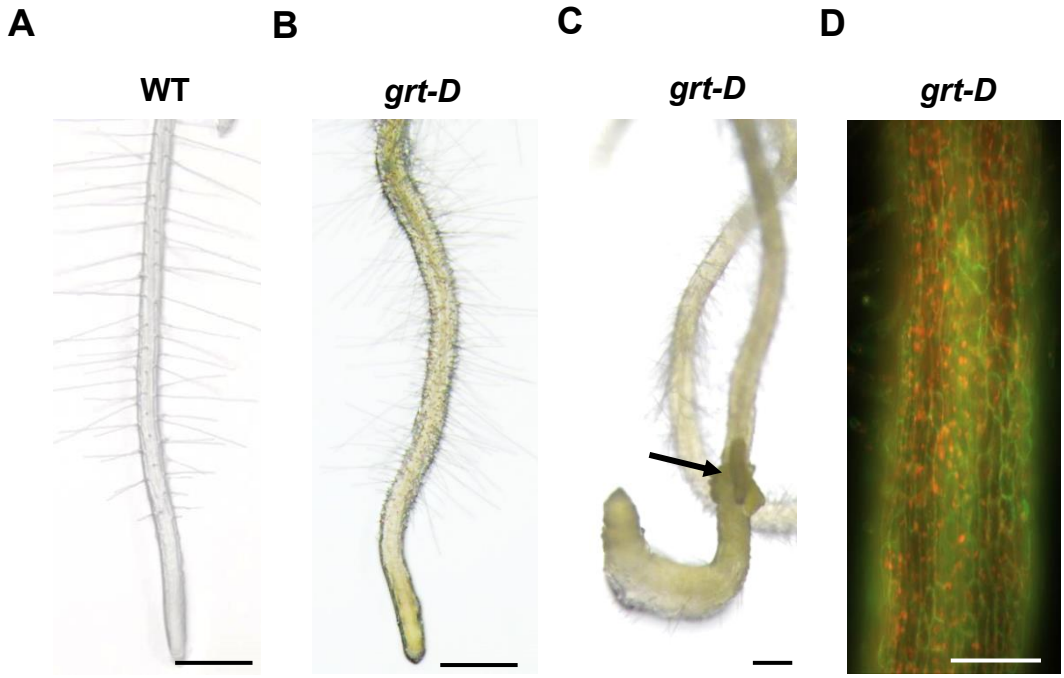


Figure 4. The phenotypes of *green root* (*grt*) *-D*

(A) Wild-type (*Q2610*) at 4 dag. (B) *grt-D* mutant at 4 dag. In the seedling, chloroplasts were expressed in the roots. (C) *grt-D* mutant at 10 dag. Somatic embryo-like tissue developed in the middle of the root (black arrow). (D) The autofluorescence of the chloroplasts in the roots was observed. Red dots indicate chloroplasts. Scale bars = 0.1 mm (A-C), 50 ㎛ (D).

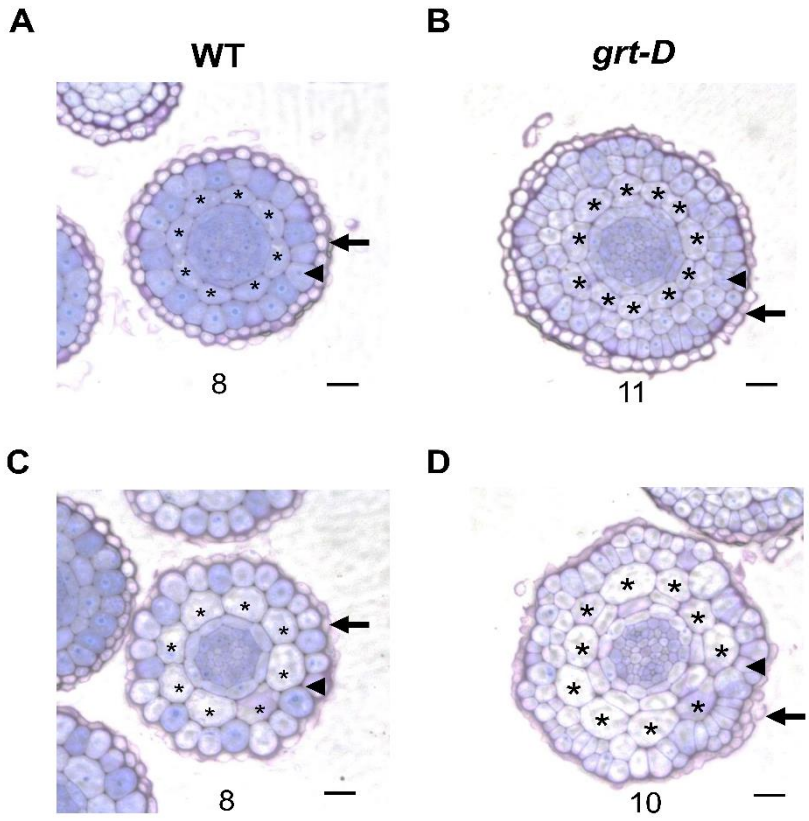


Figure 5. Expression of *grt-D* epidermal cell patterning.

(A) WT (*Q2610*) root type cross-section. The number of cortexes is 8, and the epidermis consists of a single layer. (B) *grt-D* root type cross-section. Unlike the WT, the number of the cortexes was 11, and the epidermis developed into 2-3 layers. (C) WT root middle part cross-section. The number of cortexes was 8, with a single layer of the epidermis. (D) *grt-D* root middle part. Unlike the results in the wild type, the number of cortexes was 10, and abnormal development of the epidermis was observed. The arrowhead indicates the epidermis. The arrow indicates the lateral root cap (LRC). The asterisk indicates cortex cell. scale bars = 50 ㎛.

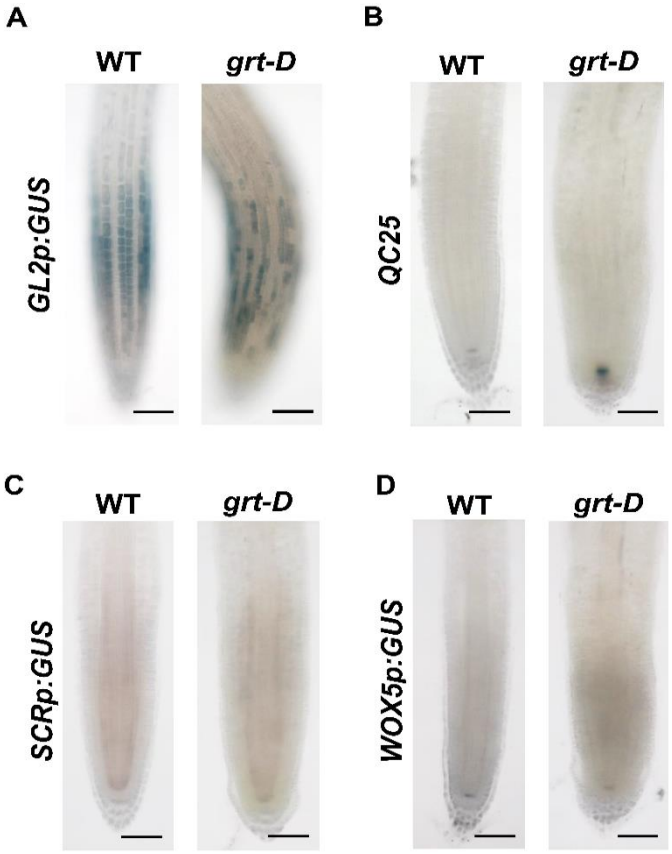


Figure 6. Expression patterns of reporter in the background of *grt−D* and WT.

(A) *GL2p:GUS* expression pattern in WT seedling (left) and *grt−D* (right). *GL2p:GUS* showed constant expression in root epidermis in wild type, and defective patterning in *grt−D*. (B) *QC25*. *QC25*, an enhancer trap line, has a specific expression in QC, where expression sites are further expanded in mutants. (C) *SCRp:GUS*. Endodermis−specific *SCRp:GUS* maintained endodermis identity in both backgrounds. (D) *WOX5p:GUS*. *WOX5p:GUS* specific for QC showed equal expression strength in QC. Seedlings at 3 dag were stained in X−gluc solution. Scale bars = 50 ㎛.

2. Phenotype of *grt-D* grown under dark conditions

In light conditions, the *grt-D* seedlings successfully developed chloroplasts in roots. To examine whether this process is light-dependent or not, grt-D seed was germinated and grown under dark conditions. roots of *grt-D* develop chloroplasts in the dark condition.

As a result, the hypocotyls of both *grt-D* and the WT were elongated without cotyledon expansion (Figure 7A and 7B). In addition, ectopic development of chloroplasts was not observed in the root of the *grt-D* (Figure 7A). The root length of the *grt-D* was reduced as compared with that of wild-type and root thickness of the *grt-D* was increased. In addition, the root hair of *grt-D* densely developed as compared with that of wild-type suggesting that root epidermal cell elongation might be suppressed. Together, it is confirmed that ectopic chloroplast development in the *grt-D* root is light-dependent.

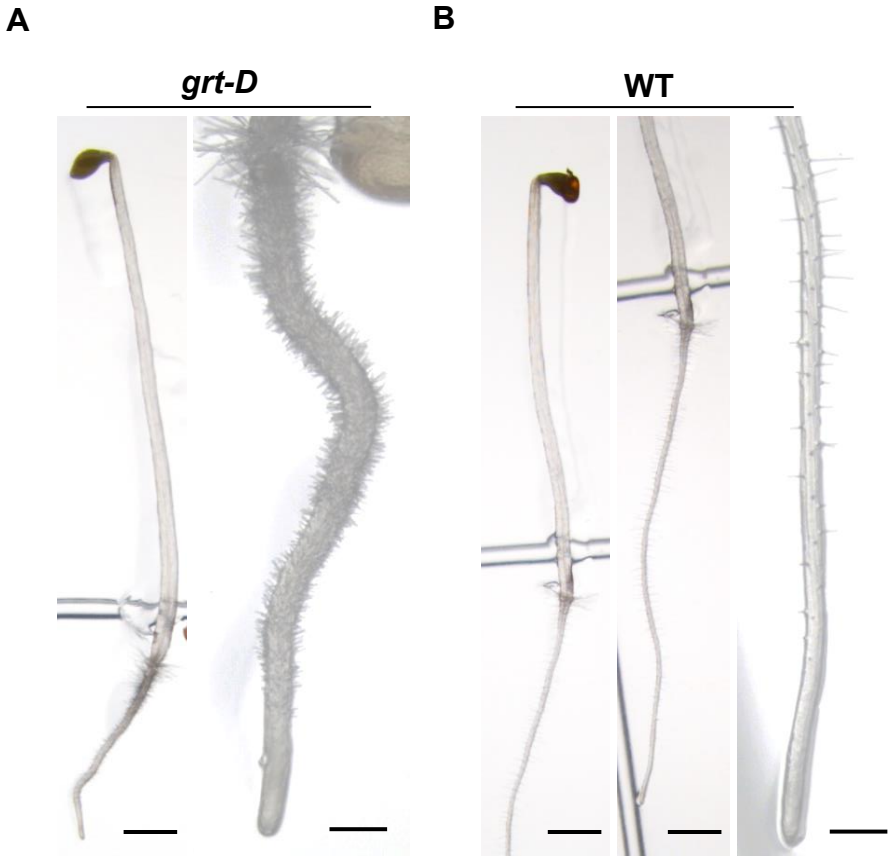


Figure 7. The phenotype of *grt-D* grown and wild-type seedlings in dark conditions.

(A, B) The 5 dag seedlings were used to observe the phenotypes of the *grt-D* and WT in dark conditions. In dark conditions, *grt-D* failed to develop chloroplast. Scale bars = 1 mm (The left panel in A, The leftmost panel and middle panel in B), 0.1 mm (The right panel in B and the rightmost panel in B).

3. Analysis of chlorophyll contents in the *grt−D* root

To verify the green root phenotype is led by the increased accumulation of chlorophylls in the *grt−D* root, the Chl. contents were measured. As a result, the amount of Chl. a in the *grt−D* roots was about 14.4 times higher than that in WT roots while the content of Chl. b in *grt−D* roots was about 22.4 times higher than that the control roots. The concentration of Chl. a+b content in *grt−D* roots was 16.6 times higher than that in the control. Therefore, it was confirmed that the green root of the *grt−D* was led by the ectopic synthesis of chlorophylls (Table 3 and Figure 8).

Table 3. Chlorophyll contents in the roots of *grt-D* mutant.

Genotypes	Concentrations of Chl. (μg / mL)		
	Chl. a	Chl. b	Chl. a+b
grt-D	1.443	0.83	2.273
WT	0.1	0.037	0.137

10 mg of mutant and wild-type roots were collected and chlorophylls were extracted.

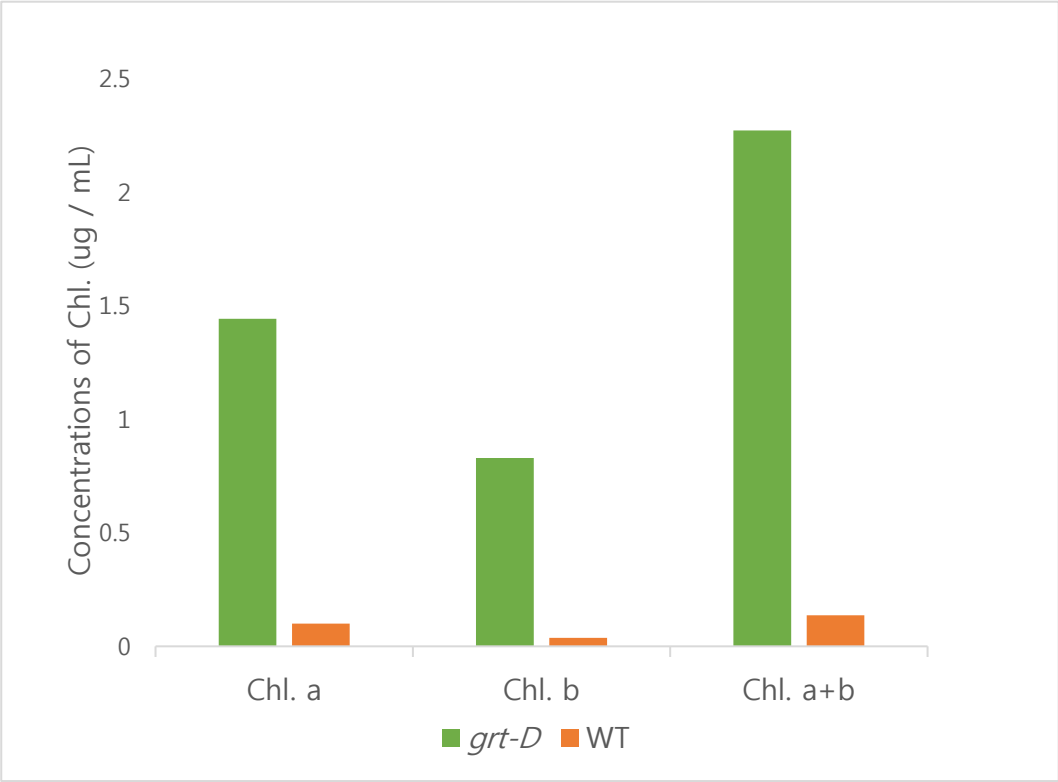


Figure 8. Concentrations of chlorophyll in roots of *grt−D* and control.

Mutant and wild type roots were extracted in aqueous 80% acetone solution at 4℃ for 24 h. Compared with the wild type, the chlorophyll a+b content in the roots of *grt−D* was about 16.6 times higher. This chart is based on the measurements in table 3.

4. The *grt-D* root possessed the shoot identity

To determine whether the *grt-D* root of possesses the shoot identity, the expression of shoot-specific reporters such as *CLV3p:GUS* and *WUSp:GUS* were examined in the *grt-D* roots.

In the root of *grt-D*, the expression of *CLV3p:GUS* was mostly observed in the stele rarely in the epidermis (Figure 9A). The *WUSp:GUS* expression was mostly found in subepidermal layers (Figure 9B), which is well-correlated with the cross section images of the *grt-D* root possessing additional suvepidermal layers (Figure 5B and 5D). The ectopic expression of shoot-specific *CLV3* and *WUS* in the *grt-D* suggest that *grt-D* roots possess the shoot identities. However, it is likely that the feedback regulation between *CLV3* and *WUS* (Schoof et al., 2000) is not functional in the *grt-D* roots as they are independently expressed in different tissues.

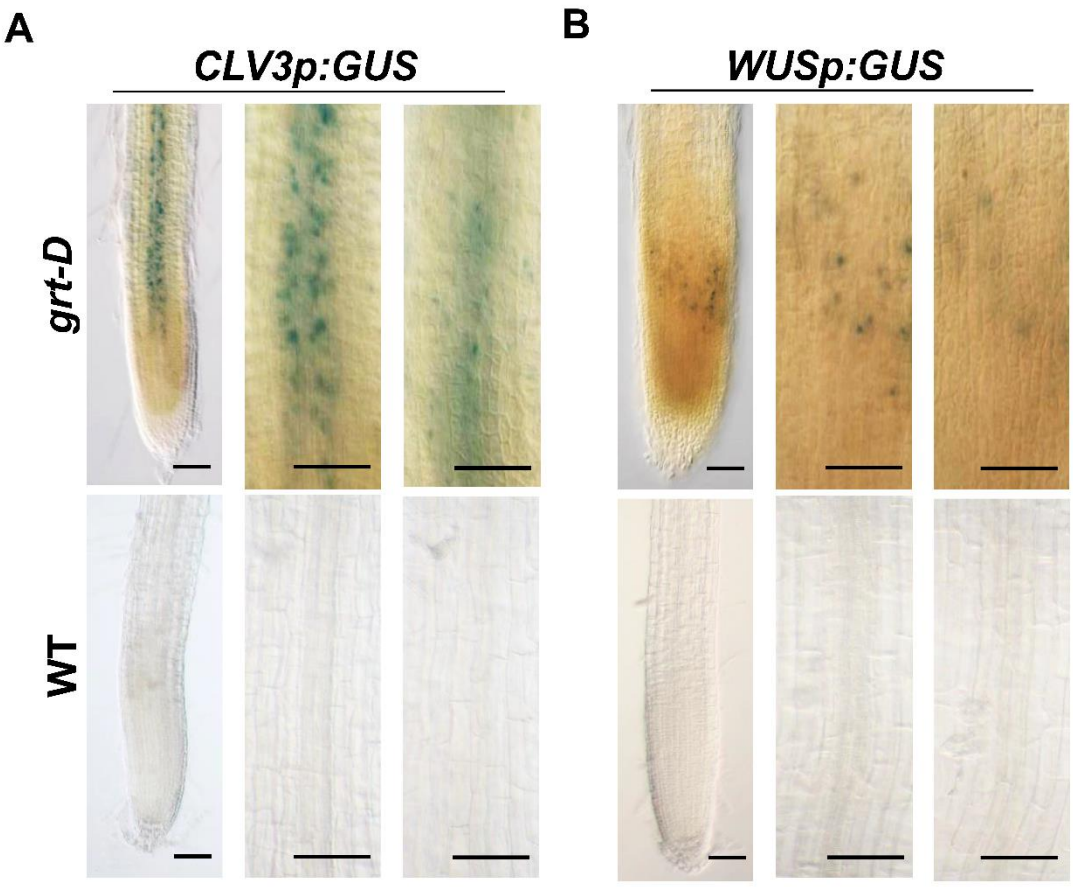


Figure 9. Expression patterns of *CLV3p:GUS* and *WUSp:GUS* in the root of *grt-D* and WT.

(A) *CLV3p:GUS* reporter expression was observed in the stele and epidermis regions of roots. (B) *WUSp:GUS* reporter expression was weakly observed in the root cortex and epidermis regions. Two reporter lines showed no expression in wild-type roots, whereas *CLV3* in the *grt-D* root was expressed in the stele and epidermis regions, while *WUS* was expressed in the cortex and epidermis regions. Scale bars = 50 ㎛.

5. Determination of the T-DNA insertion site by TAIL-PCR analysis

To determine the T-DNA insertion site inducing the *grt-D* phenotype TAIL-PCR was performed with left border primers (LB372, LB262, LB172) and DEG2 using *grt-D* gDNA as a template. In the respective reactions using LB372, LB262 and LB172, the size of the tertiary reaction product was reduced compared with the secondary reaction products. The size of the third reaction product was about 700 bp (Figure 10).

The sequencing analysis of the tertiary PCR product revealed the presence of 5x *UAS* repeats at the 111 bp upstream of the start codon of the *MYB115* (At5g40360) (Figure 11).

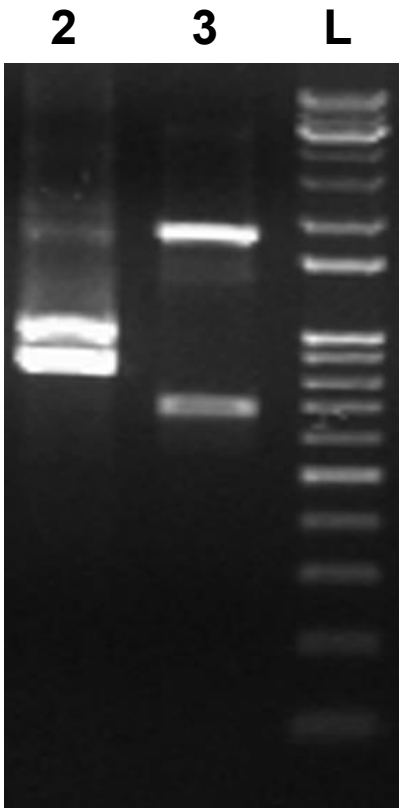
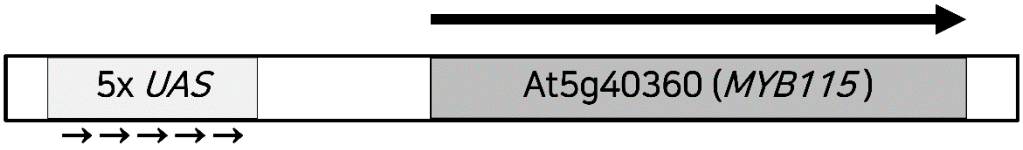


Figure 10. Gel electrophoresis results of TAIL-PCR for the identification of gene that induced the phenotype of *grt-D*.

In the TAIL-PCR third reaction, the product showed a lower bp size than the second reaction product. DNA was amplified by using a left border primer and a degenerated primer. The rightmost band used 1 kb + DNA ladder. In addition, the DNA was visualized using EtBr.



TGTCGACAGATCTCATGCCTGCAGGTCGGAGTACTGTCCTCCGAGCGGAG
TACTGTCCTCCGAGCGGAGTACTGTCCTCCGAGCGGAGTACTGTCCTCCG
AGCGGAGTACTGTCCTCCGAGCGGAGACTCTAGTTAAGCGTCAATTTGTT
TACACCACA**A**TATAAGGGGTTGTTGAAGAACATCTGCAATAAAATCTCCT
TCTAAACTCTCACTTATTTCGTACTGATAAACAAAAAGAGCATCTTAAAT
CTCTTGATCGGTTTTCGAAG**ATG**TATCACCAAAATCTGATTTCATCGACC
M Y H Q N L I S S T

Figure 11. In the *grt-D* mutant, the T-DNA insertion site is located in the *MYB115* promoter region.

The product of TAIL-PCR was sequenced to find the T-DNA insertion site. T-DNA is located 111 bp upstream of the initiation codon of the *MYB115* (At5g40360). The underlined sequences are the 5x *UAS* repeat sequence. A in the underlined is an Arabidopsis flanking sequence with T-DNA inserted.

6. *MYB115* was overexpressed in *grt-D*

To determine whether the *MYB115* is overexpressed in the *grt-D*, RT-PCR was performed with *MYB115*-specific primers using cDNA synthesized from total RNA isolated from the roots of *grt-D*.

The RT-PCR results showed that the transcripts for *MYB115* was abundantly observed in *grt-D* as compared with those in WT (Figure 12). Thus, it could be concluded that the *grt-D* phenotype is indeed by the ectopic expression of *MYB115*, in roots where no endogenous *MYB115* transcript is expressed.

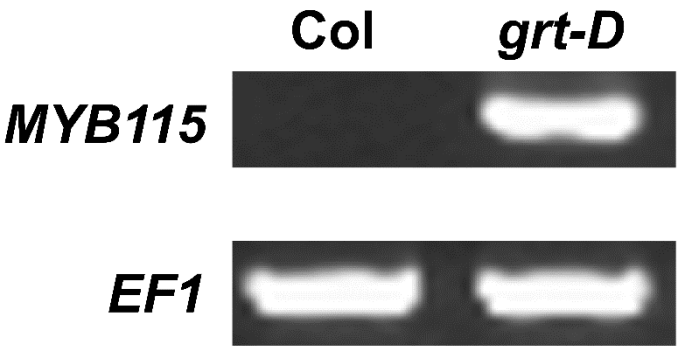


Figure 12. *MYB115* is highly expressed in the *grt-D*.

The seedlings at 5 dag were used for the extraction of the total RNA. The cDNA was synthesized from total RNA and then PCR amplified with the specific primers for *MYB115* and *EF1* for 25 cycles.

7. The overexpression of *MYB115* and *MYB118* under the regulation of *Q2610* recapitulate the *grt-D* phenotypes

To further confirm that the gene inducing *grt-D* phenotype is *MYB115*, it was tested whether the ectopic expression of *MYB115* recapitulate the *grt-D* phenotypes. Firstly, *MYB115* was cloned into pCB302 binary vector possessing 5x *UAS* promoter and nos terminator and then introduced into plants to prepare *UASp:MYB115* transgenic plants. Subsequently, the phenotype was studied in the F_1 generation by the genetic crosses with *Q2610*. At 4 dag, the *Q2610>>MYB115* lines developed the conspicuous green-colored roots as compared with the WT-like sibling (Figure 13). Therefore, *Q2610>>MYB115* exhibited the green-root phenotype similar to that of *grt-D*. These results suggest that the *grt-D* phenotype was induced by overexpression of the *MYB115*. As *MYB118*, the closest homologue of *MYB115*, in Arabidopsis genome, is 36.4% identical to *MYB115* in amino acid sequence, it was examined whether the ectopic expression of MYB118 under the control of *Q2610* also induces *grt-D*-like phenotypes. *Q2610>>MYB118* also reproduced the green-root phenotype, which indicates that both *MYB115* and *MYB118* induce the biogenesis of chloroplasts in the underground parts (Figure 13).

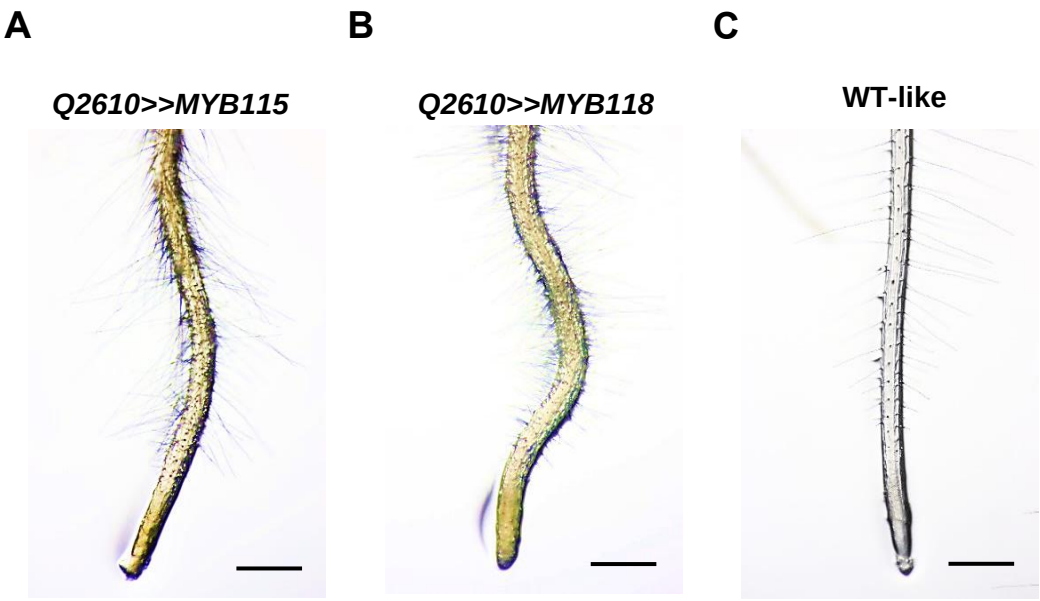


Figure 13. Overexpression of *MYB115* and *MYB118* reproduced green root.
(A) In the *Q2610>>MYB115* line, the development of the ectopic chloroplast, which is the same phenotype as *grt-D*, was observed. (B) *Q2610>>MYB118* line also developed the green root. To obtain the *Q2610>>MYB115* and *Q2610>>MYB118* root phenotypes, *UASp:MYB115* and *UASp:MYB118* were crossed to *Q2610*. Scale bars = 0.5 mm.

8. The petal- and stamen-specific expression of *MYB115* and *MYB118* under the regulation of *AP3* and *PI* promoters induced green-petal phenotypes

To determine whether the ectopic expression of *MYB115* and *MYB118* could induce the development of the green-colored organs in the above-ground parts as well, *MYB115* and *MYB118* were expressed under the control of the putative petal-specific promoters originated from *AP3* and *PI* known to involve in the determination of the stamen and petal identities in Arabidopsis. To induce the petal- and stamen-specifically expression of any target under the control of *UAS* promoter, the 288 bp of *AP3* promoter and 300 bp of *PI* promoter known to be petal- and stamen- specifically active (Hill et al., 1998; Honma and Goto, 2000) were hired to express *GAL4-VP16* transcription factor. The transgenic lines expressing *AP3p:GAL4-VP16* or *PIp:GAL4-VP16* was prepared and then crossed to *UASp:MYB115* or *UASp:MYB118* transgenic lines.

AP3>>MYB115 and *AP3>>MYB118* plants developed green-colored petals and sterile stamens (Figure 14A and 14D). The shape of petal margin of the transgenic lines was similar to wild type (Figure 14A, 14C, 14D and 14F). *PI>>MYB115* and *PI>>MYB118* transgenic lines also exhibited green-colored petals and sterile stamens (Figure 14B and 14E). Unlikely to other transgenic lines, *PI>>MYB118* line developed the undulated (wavy-shaped) -petal margin.

To further confirm that the promoter acting is restricted to stamens and

petals, the length of floral organs of the *AP3>>MYB115*, *AP3>>MYB118*, *PI>>MYB115* and *PI>>MYB118* lines and WT-like sibling were measured. As compared with the WT-like siblings, the petal and stamen lengths of *AP3>>MYB115* and *PI>>MYB115* (Table 4 and Figure 15), *AP3>>MYB118* and *PI>>MYB118* (Table 5 and Figure 16) were reduced whereas the length of carpels and sepals was not altered as compared with that of WT.

Figure 14. Phenotypes of *AP3>>MYB115*, *AP3>>MYB118*, *PI>>MYB115* and *PI>>MYB118* transgenic lines floral organ

(A, B) *AP3>>MYB115* and *AP3>>MYB118* are green in both petals and stamen. (D, E) *PI>>MYB115* and *PI>>MYB118* also petals are greenish and had defects in stamens development. The petals margin of *PI>>MYB118* showed similar to the wavy margin. In addition, pollen was not developed in all transgenic lines stamens. Scale bars = 0.5 mm (A–F left panel), 0.1 mm (A–F right panel).

Table 4. Measurement of length in *AP3>>MYB115* and *PI>>MYB115* floral organs

Genotypes	Length of floral organ (mm)			
	petals	stamens	carpels	Sepals
AP3>>MYB115	0.71 ± 0.27	0.66 ± 0.30	2.25 ± 0.27	2.15 ± 0.23
WT-like sibling (*AP3>>MYB115*)	2.27 ± 0.19	2.16 ± 0.32	2.67 ± 0.23	2.21 ± 0.16
PI>>MYB115	1.04 ± 0.18	0.68 ± 0.16	2.38 ± 0.31	2.51 ± 0.27
WT-like sibling (*PI>>MYB115*)	3.05 ± 0.34	2.42 ± 0.29	2.90 ± 0.32	2.36 ± 0.18

'±' is the value of the standard deviation. Each genotypes were collected from at least 20 flowers.

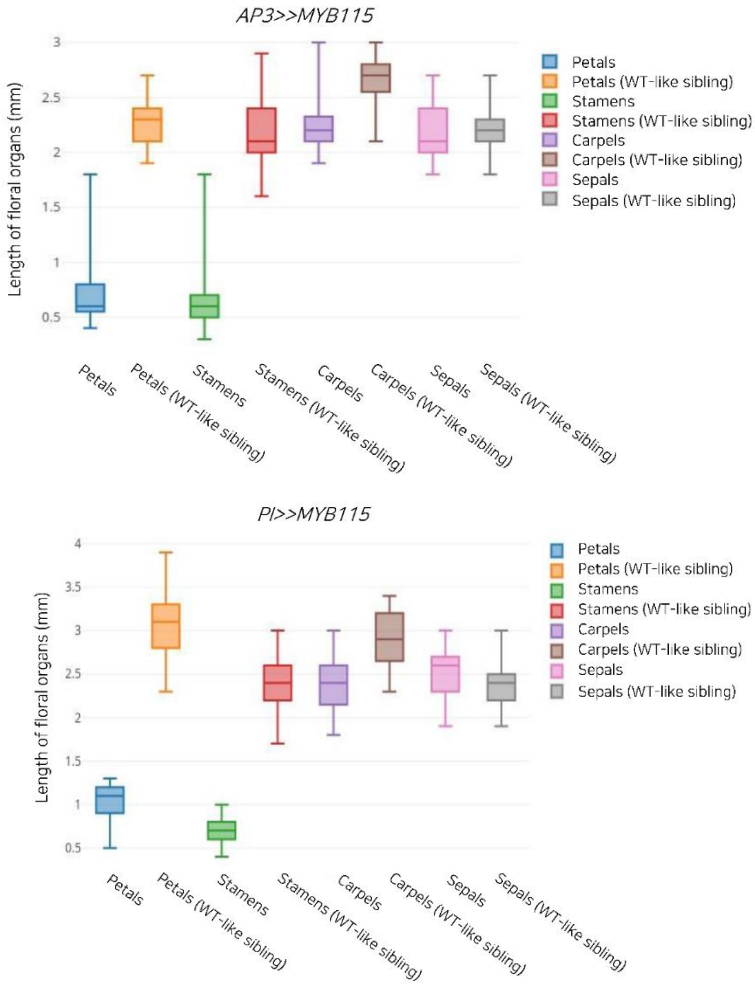


Figure 15. Comparison of floral organ size among *AP3>>MYB115*, *PI>>MYB115* and control.

The lengths of petals, stamens, carpels and sepals of *AP3>>MYB115* and *PI>>MYB115* transgenic lines and WT-like siblings were measured. At least 20 flowers were used to measure length. In *AP3>>MYB115* and *PI>>MYB115*, the length of petals and stamens was shorter than that of control but the lengths of carpels and sepals were almost similar. These box plot charts are based on the measurements in table 4.

Table 5. Measurement of length in *AP3>>MYB118* and *PI>>MYB118* floral organs

Genotypes	Length of floral organ (mm)			
	petals	stamens	carpels	Sepals
AP3>>MYB118	0.99 ± 0.22	0.77 ± 0.16	2.26 ± 0.28	2.32 ± 0.17
WT-like sibling (*AP3>>MYB118*)	2.90 ± 0.29	2.15 ± 0.24	2.77 ± 0.28	2.30 ± 0.13
PI>>MYB118	1.91 ± 0.39	0.71 ± 0.23	2.46 ± 0.34	2.52 ± 0.26
WT-like sibling (*PI>>MYB118*)	2.73 ± 0.37	2.01 ± 0.38	2.68 ± 0.36	2.27 ± 0.23

'±' is the value of the standard deviation. Each genotypes were collected from at least 20 flowers.

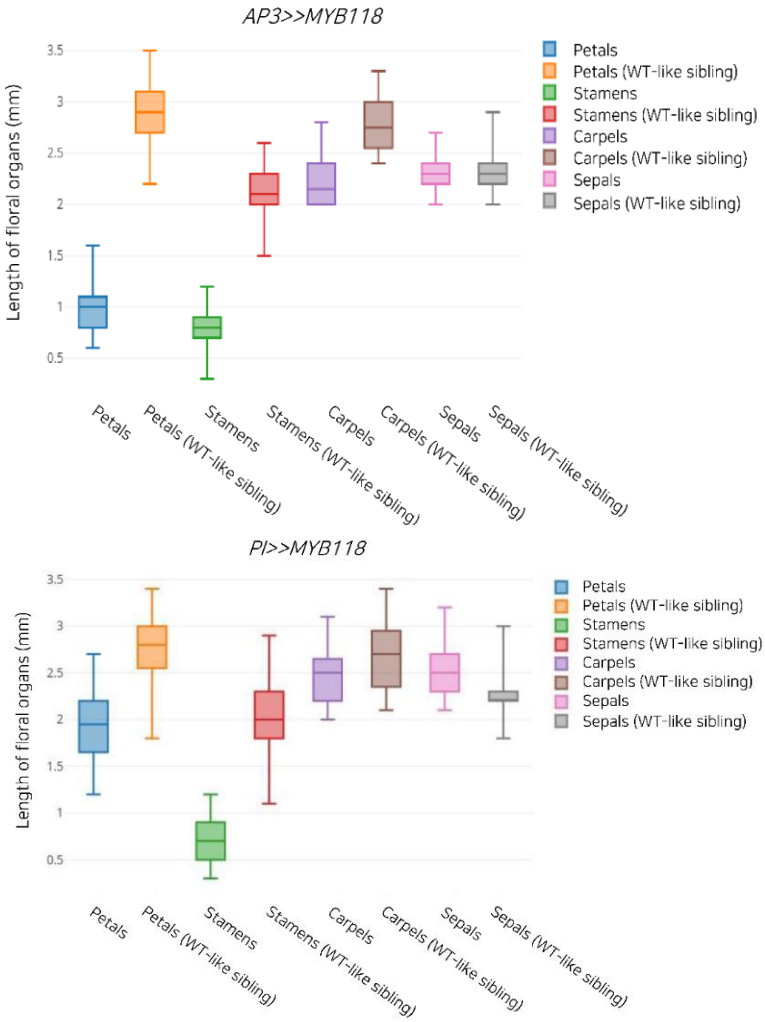


Figure 16. Comparison of floral organ size among *AP3>>MYB118*, *PI>>MYB118* and control.

The lengths of petals, stamens, sepals and carpels of *AP3>>MYB118* and *PI>>MYB118* transgenic lines and WT-like siblings were measured. In *AP3>>MYB118* and *PI>>MYB118*, the length of stamens and petals was shorter than that of control, whereas did not affect the length of sepals and carpels. These box plot charts are based on the measurements in table 5.

9. Analysis of chlorophyll contents in petals organ

According to the results of this study, the petals of the transgenic lines were greenish compared with the WT-like control. Therefore, it was necessary to examine whether the green petals of the transgenic lines are led by the accumulation of chlorophylls. Therefore, chlorophylls were extracted from the petals, and then quantified. The petals of *AP3>>MYB115* and *PI>>MYB115* were collected from floral organs. The concentrations of Chl. a+b in *AP3>>MYB115* petals and *PI>>MYB115* were about 4.78 times and 4.34 times higher than that of WT-like petals, respectively (Table 6 and Figure 17).

Table 6. Measurement of chlorophyll contents of *AP3>>MYB115* and *PI>>MYB115* petals

Genotypes	Concentrations of Chl. (μg / mL)		
	Chl. a	Chl. b	Chl. a+b
AP3>>MYB115	2.482	0.336	2.818
WT-like sibling (*AP3>>MYB115*)	0.341	0.249	0.590
PI>>MYB115	1.646	0.421	2.066
WT-like sibling (*PI>>MYB115*)	0.344	0.132	0.476

10 mg of *AP3>>MYB115*, *PI>>MYB115* and WT-like sibling petals were collected and chlorophylls were extracted.

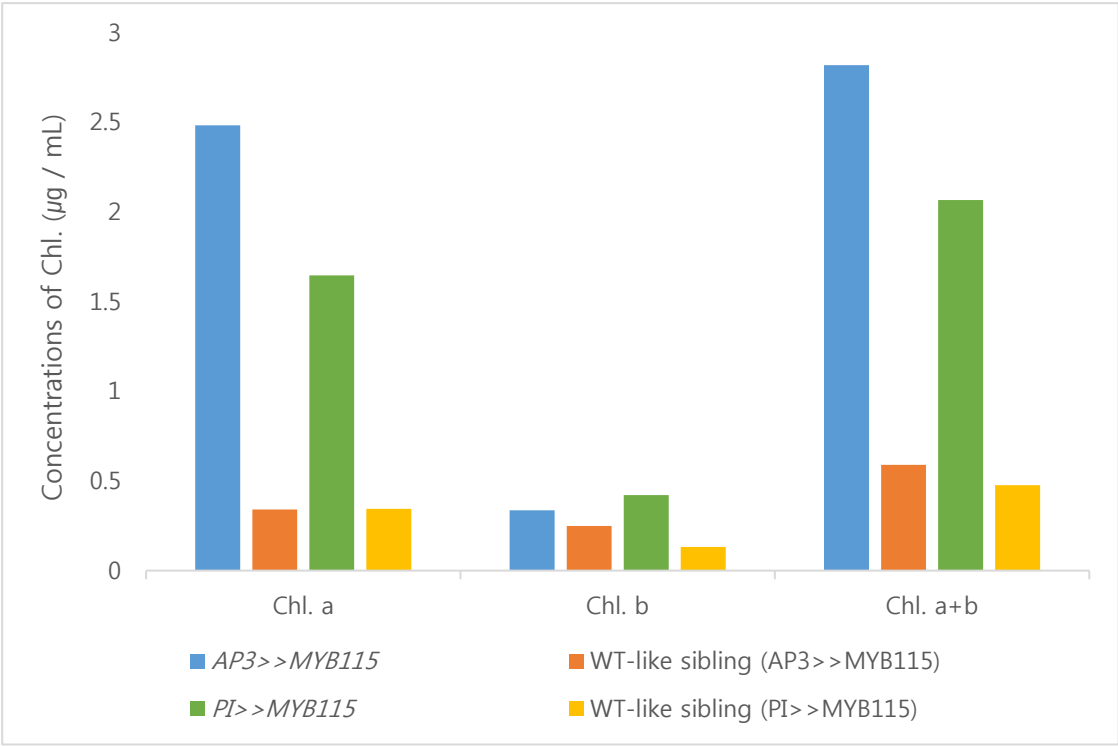


Figure 17. Comparison of Chl. concentrations among *AP3>>MYB115*, *PI>>MYB115* and control petals.

AP3>>MYB115 and *PI>>MYB115* transgenic lines and WT-like sibling petals were extracted in aqueous 80% acetone solution at 4℃ for 24 h. Compared with the WT-like sibling, the contents of chlorophyll a+b were about 4.8 times larger in *AP3>>MYB115* petals and *PI>>MYB115* was 4.3 times larger than that of the control. This chart is based on the measurements in table 6.

Ⅳ. Discussion

Most plant roots are heterotrophic organs that depend on sugars and carbohydrates produced from the leaves (Kobayashi et al., 2012). However, some orchidaceae and mangrove roots turn green when exposed to light (Gill and Tomlinson, 1977; Benzing et al., 1983). Thus, the root appears to have the ability to make functional photosynthesis devices (Kobayashi et al., 2012). As previously reported, the *plant growth activator* (pga) *37* gain-of-function mutant using the estradiol-inducible active tagging system developed chloroplasts in the root (Wang et al., 2009). In addition, some *pga* mutants also developed somatic embryos and shoots in the root (Zuo et al., 2002; Sun et al., 2003).

In this study, a mutant *grt-D*, developing chloroplasts and somatic embryo-like tissues in the root, was screened through the *GAL4-UAS* active tagging system (Figure 4). The activation tag inserted in the *grt-D* genome was present in the promoter region of *MYB115* (Figure 11) and the *MYB115* was confirmed to be highly expressed (Figure 12). Furthermore, *Q2610>>MYB115* recapitulate *grt-D* phenotype (Figure 13). *MYB118/PGA37*, the closest homologue to *MYB115* in Arabidopsis was reported to be overexpressed for the green-root development in the *pga37* mutant (Zhang et al., 2009). As the *grt-D* phenotype is similar to the estradiol-treated *pga37* phenotype and the overexpression of both *MYB115* and *MYB118* driven by *Q2610* led to the equivalent green-root phenotypes, it can be concluded that *MYB118* share the function of *MYB115* in Arabidopsis Although they share only 36.4% amino acid sequence identity (Zhang et al., 2009).

As reported previously, a *myb115 myb118* double mutant did not exhibit apparent developmental defect (Wang et al., 2009). Recently, the roles of *MYB115* and *MYB118* have been elucidated for the suppression of endosperm maturation and the production of ω−7 monounsaturated fatty acids in the maturing endosperm by activating the two⊿−9 palmitoyl−ACP desaturase genes (Barthole et al., 2014; Troncoso−Ponce et al., 2016). It has also been reported that the seed−specific overexpression of *MYB115* increased the ω−7 monoenoic fatty acid contents. It was also reported that *MYB115* and *MYB118* are involved in the control of benzoyloxy glucosinolate pathway (Zhang et al., 2015). However, with these known functions of *MYB115* and *MYB118* it is difficult to clearly explain how the green root phenotypes is induced by the ectopic expression of *MYB115* and *MYB118*. Therefore, it is needed to unravel the unidentified roles of the *MYB*.

In this study, the cells residing in the cortex and epidermis of the *grt−D* root were increased compared with those in the wild−type root (Figure 5). As reported, cell proliferation activity in the cambium of plant roots has an effect on the radial growth and secondary metabolite yield of roots (Jang et al., 2015). For example, it has been reported that sweetpotato MADS−box protein cDNA in sweet potato root promotes the proliferation of metaxylem and cambium cells (Noh et al., 2010). The radial growth, which is dependent on cytokinin, has been shown to increase the yield of root crops (Jang et al., 2015). Therefore, the growth of plant−derived biomass is a major target characteristics in plant science. Furthermore, some *pga* mutants developing green calli or shoot produced a large amount of cytokinin by the overexpression of *AtIPT8* (Sun et al., 2003). Thus it is important to determine

whether the roots of *grt-D*, *Q2610>>MYB115*, and *Q2610>>MYB118* overproduce cytokinins as well.

In the previous studies, attempts were made to produce green-petal flowers by reducing chlorophyll degradation processes. When the *SGR* encoding a Mg-dechaelatase function was blocked, chlorophylls in petals of chrysanthemums and carnations were preserved (Ohmiya et al., 2017). In this study, in order *MYB115* and *MYB118* were specifically expressed in petals and stamen by using *AP3* and *PI* promoters. Consequently, the transgenic lines developed green-colored petals and sterile stamens (Figure 14). Previously reported, *MYB118* inhibited the maturation of endosperm in the seeds (Barthole et al., 2014). Therefore, it is possible that the *MYB118* overexpression might suppress the maturation of petals and stamens thereby inhibit the chlorophyll decomposition in petals.

It was reported that genes for the chloroplast development are inactive in petals (Ohmiya et al., 2017). Therefore, *MYB115* and *MYB118* are expected to activate genes for for the chloroplast development. In the petal margin where chloroplasts are not developed. It would be intriguing to find out the target activated by *MYB115* and *MYB118* for the development of chloroplasts.

In 2005, roses with green petals were breeded and commercialized at the Gyeonggi provincial agricultural research institute, and genetically modified (GM) blue roses of purple petal phenotype based on the delphinidine synthesis route were developed (Katsumoto et al., 2007). The production of flowers developing novel colored petals by using molecular techniques provides a useful tool to pigment researches and greatly improve the commercial value of the flowers (Ohmiya, 2018). However, outdoor cultivation of living modified

organism (LMO) plants is environmentally unsafe, so it is difficult to commercialize. On the other hand, *AP3>>MYB115*, *PI>>MYB115*, *AP3>>MYB118* and *PI>>MYB118* transgenic plants developed in this study are rather safe in the environmental point of view as they fail to produce pollen.

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