UNIVERSITY OF TARTU
Faculty of Science and Technology
Institute of Technology

Oleksii Yadlos

Development of visual selection markers for plant transformation

Bachelor's Thesis (12 ECTS)

Curriculum Science and Technology

Supervisor:
Associate professor, Yuh-Shuh Wang

Tartu 2023

Development of visual selection markers for plant transformation

Abstract: The generation of transgenic *Arabidopsis thaliana* plants relies heavily on the choice of selection markers. We investigated three potential visual seed-selection markers: the RUBY reporter, the amilCP chromoprotein, and the endogenous *TTG1* gene. The RUBY reporter, which produces dark-red betalain pigment, successfully led to distinguishable seed color changes in *Arabidopsis* Col-0 and *ttg1* mutant backgrounds. However, the use of the amilCP chromoprotein was limited by its low transformation efficiency and germination rate, likely due to its toxicity in plants. Attempts to use the *TTG1* gene as an endogenous selection marker did not produce observable seed color changes in the first transgenic generation, possibly due to insufficient TTG1 protein expression. Overall, this study demonstrates the potential of the RUBY reporter as an effective visual marker for seed selection and highlights the need to explore endogenous markers to improve the efficiency and convenience of generating transgenic *Arabidopsis* plants.

Keywords: Seed-selection marker; *Arabidopsis thaliana*; TTG1; RUBY; amilCP.

CERCS: B310 Physiology of vascular plants, B225 Plant Genetics.

Visuaalsete markerite arendamine taimede transformatsiooniks

Lühikokkuvõte: Transgeensete hariliku müürlooga (*Arabidopsis thaliana*) taimede loomisel on oluline selektsioonimarkeri valik. Uurisime kolme selektsioonimarkerit eesmärgiga leida sellised, mis võimaldavad transgeenseid seemneid visuaalselt värvi järgi eristada. Töös uuritud markerid olid RUBY reporter, amilCP kromovalk ja endogeenne *TTG1* geen. RUBY reportervalk toodab betalaiini – tumepunast pigmenti, mis tõi kaasa nähtavad muutused transgeensete seemnete värvuses nii metsiktüüpi Col-0 taimedes kui ka *ttg1* mutandiliinis. Kromovalgu amilCP edukat kasutamist markerina takistasid madal transformatsiooni efektiivsus ja seemnete idanevus, mida võis põhjustada amilCP toksiline mõju taimedele. Endogeense *TTG1* geeni kasutamine seemne selektsioonimarkerina ei olnud edukas: esimeses transgeenses põlvkonnas seemnevärvi muutusi ei täheldatud. Võimalik, et seda põhjustas TTG1 valgu madal ekspressioonitase. Uurimus näitab, et RUBY reporterit saab tõhusalt kasutada seemnevärvuse visuaalsel selektsioonil põhineva transgeensete taimede markerina ning et endogeensete geenide markerina kasutamine tõhusaks ja mugavaks transgeensete seemnete selekteerimiseks väärib täiendavat uurimist.

Võtmesõnad: seemne selektsioonimarker; *Arabidopsis thaliana*; TTG1; RUBY; amilCP.

CERCS: B310 Soontaimede füsioloogia, B225 Taimegeneetika

TABLE OF CONTENTS

TERMS, ABBREVIATIONS AND NOTATIONS

FAST – Fluorescent Accumulating Seed Technology

TTG1 – TRANSPARENT TESTA GLABRA 1 transcription regulator

ttg1 – *Arabidopsis* loss-of-function *TTG1* mutant

T-DNA – Transfer DNA

GFP – green fluorescent protein

RFP – red fluorescent protein

R2R3-MYB – R2R3 MYELOBLASTOSIS transcription factor

bHLH – basic HELIX-LOOP-HELIX transcription factor

RB – right border, 25-nucleotide repeat sequence

LB – left border, 25-nucleotide repeat sequence

PCR – polymerase chain reaction

Col-0 – *Arabidopsis thaliana* Columbia-0 ecotype

CRISPR – clustered regularly interspaced short palindromic repeats

Cas9 – CRISPR-associated protein 9

T1 generation – first generation of transgenic/transformed plants

p35S – *CaMV35S* or Cauliflower Mosaic Virus 35S promoter

pOLE1 – *OLEOSIN1* promoter

pTTG1 – *TTG1* promoter

TTG1t – *TTG1* terminator

MS medium – Murashige and Skoog medium

INTRODUCTION

The increasing importance of convenient and reliable selection markers in generating transgenic plants

The generation of transgenic plants has become a vital approach in plant biology, agriculture, and biotechnology, as it enables targeted gene manipulation that drives plant physiology research and also improves valuable traits such as yield, disease resistance, and abiotic stress tolerance. With the increasing demand for sustainable agricultural practices and the need to address global food security challenges, fast generation of various transgenic plants to understand plant biological processes is crucial.

An essential aspect of plant transformation is the identification and selection of transformed individuals from a pool of plants. This process would greatly benefit from using convenient and reliable selection markers to ensure the successful recovery of transgenic plants.

Selection markers are genes that are introduced into plants alongside the gene of interest and provide a distinguishable phenotype that can be easily identified and selected. An ideal selection marker should be highly specific, non-destructive, easy to use, have minimal ecological and health risks, and be compatible with various plant species and techniques.

In recent years, researchers have been exploring alternative selection markers to address the limitations and concerns associated with some of the traditional markers, such as antibiotic resistance genes. These alternatives include fluorescent proteins and pigmentation markers among others. The development of new selection markers and the improvement of existing ones are essential for advancing the field of plant transformation and ensuring the successful generation of transgenic plants with desired characteristics.

In this work, we generated various transgenic plants to test the following potential selection markers: the RUBY reporter, amilCP chromoprotein and the *TTG1* gene as an endogenous selection marker, highlighting their positive and negative aspects.

1 LITERATURE REVIEW

1.1 Antibiotic selection markers

A variety of selection markers are used in plant transformation studies, with antibiotic resistance markers being among the most popular and widely used. Antibiotic selection markers have contributed to numerous successful plant transformations and the development of various genetically modified crops. These markers involve the incorporation of antibiotic resistance genes into the plant genome alongside the gene of interest. The transformed plants can then be selected based on their ability to survive and grow in the presence of the corresponding antibiotic, while non-transformed plants are eliminated.

One of the most commonly used antibiotic selection markers is the neomycin phosphotransferase II (*NPTII*) gene, which confers resistance to kanamycin, neomycin, and G418 (geneticin) (Bevan *et al.*, 1983; Fraley *et al.*, 1983). These antibiotics belong to the aminoglycoside group and bind to the 16S or 18S rRNA of small ribosomal subunits in bacteria and plants, respectively (Eustice and Wilhelm, 1984; Moazed and Noller, 1987). This interaction disrupts the process of protein synthesis, causing misreading of the genetic code and the production of nonfunctional proteins, which leads to cell death (Eustice and Wilhelm, 1984; Moazed and Noller, 1987). Discovered in transposon *Tn5* in *E. Coli* (Beck *et al.*, 1982), *NPT*II, when introduced into plant cells, transfers a phosphate group from ATP to the hydroxyl group of the antibiotic molecule (Berg *et al.*, 1975). Phosphorylation of aminoglycoside antibiotics renders them incapable of binding to the 18S rRNA in the 40S ribosomal subunit. As a result, the antibiotic cannot interfere with protein synthesis, allowing transformed plant seedlings to survive on antibiotic-selective medium (Bevan *et al.*, 1983; Fraley *et al.*, 1983).

Other examples of antibiotic selection markers include the hygromycin B resistance gene (hph), which confers resistance to hygromycin B (Gritz and Davies, 1983), and the bialaphos resistance gene (bar), which provides resistance to the herbicide phosphinothricin (De Block *et al.*, 1987).

Antibiotic selection markers offer several advantages for plant transformation studies. They are easy to use, well-characterized, and compatible with a wide range of host organisms (Rosellini, 2012). Their straightforward application in selecting transformed cells has made them the popular choice in plant transformation research. Moreover, the use of antibiotic resistance markers has contributed to the development of efficient transformation protocols

in numerous plant species, enabling the generation of transgenic plants for both research and commercial purposes (Potrykus, 1991; Rosellini, 2012)

However, these markers are not the most labor- and time-efficient. To obtain homozygous plants with single T-DNA (Transfer DNA) insert (single-insert homozygous transgenic lines) that are important for further research, transgenic plants must be propagated for at least three generations using antibiotic medium plates for selection of transformed seedlings (Figure 1) (Kammar *et al.*, 2023). Handling many lines is necessary to isolate single-insert lines with 3:1 phenotypic segregation (75% of antibiotic resistant and 25% of non-resistant seedlings) in second generation (T2) and homozygous seeds in T3 generation (Figure 1). The selection process requires sterile conditions, meaning that all seeds must be sterilized. Moreover, after transformation, the antibiotic selection marker remains permanently in the plant genome alongside the genes of interest, which may not be the best option for some experiments or the development of commercial products (Rosellini, 2012).

1.2 Fluorescent seed-selection markers

Fluorescent seed-selection markers provide a valuable alternative to traditional antibiotic resistance markers for generating transgenic plants. These markers involve the expression of fluorescent proteins, such as a green fluorescent protein (GFP) or a red fluorescent protein (RFP), under the control of seed-specific promoters (Figure 1). This approach enables early and rapid selection based on the presence of a fluorescent signal in transformed seeds (Figure 1). These markers have been implemented in various plant species, including *Arabidopsis*, oat, maize, rice, and cotton (Kaeppler *et al.*, 2000; Kaeppler *et al.*, 2001; Wenck *et al.*, 2003; Shaked *et al.*, 2005; Stewart, 2005; Nishizawa *et al.*, 2006; Shimada *et al.*, 2010; Wang *et al.*, 2018; Xu *et al.*, 2021).

RFPs are especially useful because they are excited by green-to-red wavelengths, and plants do not exhibit significant background fluorescence in the red spectrum when excited at these wavelengths. Additionally, some variants such as AsRed, DsRed, DsRed2 and TagRFP produce red coloration under visible light, allowing selection without a fluorescence device (Figure 2) (Wenck *et al.*, 2003; Nishizawa *et al.*, 2006; Xu *et al.*, 2021).

TagRFP (Merzlyak *et al.*, 2007) is characterized by its high brightness and extended fluorescence lifetime. This protein exhibits distinct chromoprotein properties, causing seeds transformed with it to display a prominent red color under visible light (Figure 2). Unlike other red fluorescent proteins that form tetramers or dimers, TagRFP is monomeric, making

it an ideal candidate for use as a fusion protein. These advantages facilitated the development of an efficient seed-selection system called Fluorescence-Accumulating Seed Technology (FAST) (Shimada *et al.*, 2010). FAST is based on a fusion of the *Arabidopsis* OLEOSIN1 with a fluorescent protein driven by the *OLEOSIN1* promoter. This promoter enables seed-specific expression of the fusion protein, and the OLEOSIN1 protein directs TagRFP (for FAST-R), or GFP (for FAST-G), to the oil body membrane in seeds. The accumulation of TagRFP or GFP allows easy identification of transformed seeds immediately after harvesting, either under visible light (Figure 2) or using a fluorescent stereomicroscope (Figures 1 and 2). Figure 1 shows an example of FAST-G utilization. In this thesis, FAST-R is employed as an alternative marker to conventional antibiotic-based selection methods for generating transgenic plants.

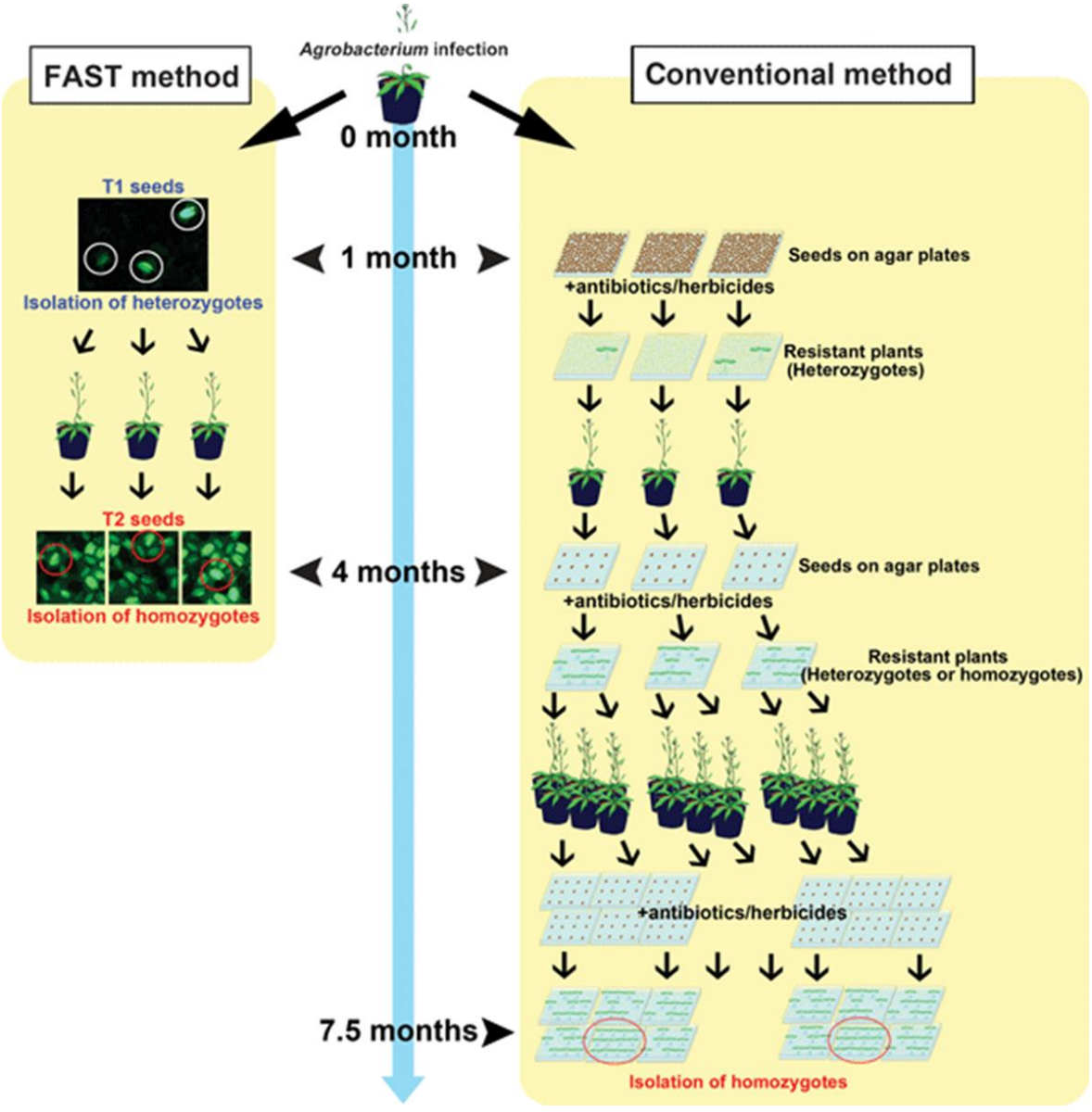

Figure 1. Comparison of the single-insert homozygous line identification process using FAST and conventional antibiotic selection methods. The FAST-G marker is a fluorescent seed-selection marker that uses the seed-specific *OLEOSIN1* promoter to drive the expression of the OLEOSIN1-GFP fusion (Shimada *et al.,* 2010). Transformed T1 seeds can be easily distinguished under a fluorescent stereomicroscope and planted directly into soil. A single-insert line (3:1 ratio for fluorescent seeds versus non-fluorescent seeds) is determined during the T2 generation segregation assessment under a stereomicroscope and homozygous seeds, possessing the strongest fluorescence intensity from population, are easily selected. In contrast, the conventional antibiotic selection method requires T1 seed sterilization and plating on selective medium, followed by the selection of transformed seedlings (Kammar *et al.*, 2023). T2 seeds are harvested from multiple lines, also sterilized, and plated to observe line segregation and line with 3:1 ratio for resistant to non-resistant seedlings is selected. Single-insert resistant plants are transferred to soil, and T3 seeds are harvested. Seeds from several lines must be sterilized and plated onto medium once more to identify homozygous lines where all seeds germinate on the medium. The FAST-G marker significantly reduces the time required for the selection from the conventional 7.5 months to approximately 4 months. Source: Shimada *et al.* (2010).

The intensity of fluorescence of FAST-R or FAST-G is strongly correlated with zygosity or insert copy number. T2 seeds derived from T1 plants with a single insertion would display a 3:1 ratio for fluorescent seeds versus non-fluorescent seeds. Among fluorescent seeds, T2 heterozygous seeds exhibit weaker fluorescence than homozygous seeds (Shimada *et al.*, 2010). This feature eliminates the need for time-consuming sterilization and plating on antibiotic selection medium. Rapidly selected T1 seeds can be planted directly into soil, and in the T2 generation, homozygous seeds are selected from lines that display single insert segregation (Figure 1). This method significantly reduces the time required for isolating a single-insert homozygous line, from around 7.5 months with conventional antibiotic selection, to just 4 months (Figure 1).

Figure 2. Coloration and fluorescence of seeds harboring the FAST-R seed-selection marker. Seeds of the homozygous transgenic line (T3) are on the left, displaying a clear red coloration in comparison to the wild type on the right. The fluorescence of the transgenic seeds under a stereomicroscope is shown below, non-flourescent wild-type seeds are on the right (not visible). WT – non-transformed *Arabidopsis* wild-type Col-0.

1.3 Chromoprotein amilCP

Similar to fluorescent proteins, chromoproteins isolated from marine organisms were shown to be suitable selection markers in bacteria (Liljeruhm *et al.*, 2018; Tafoya-Ramirez *et al.*, 2018). They strongly absorb visible light, resulting in a distinct and easily detectable color (Alieva *et al.*, 2008). For example, amilCP is a purple-blue chromoprotein isolated from *Acropora millepora* (Alieva *et al.*, 2008). It is non-toxic to *E. coli* and has been used in different bacterial species for monitoring transgenic events (Tafoya-Ramirez *et al.*, 2018). Such proteins could be used for rapid visual selection of transgenic seeds. There is limited literature on the application of chromoproteins in plants, and we decided to test amilCP in *Arabidopsis* for this purpose.

1.4 Pigment based visual seed-selection markers

Naturally occurring plant pigments, such as flavonoids, have garnered significant interest as reporter genes and selection markers in various plant species since the 1990s (Ludwig *et al.*, 1990; Chawla *et al.*, 1999). Flavonoids are a diverse group of secondary metabolites found in plants and include anthocyanins and proanthocyanidins, which are present in the vegetative tissues and seeds of *Arabidopsis*, respectively (Debeaujon *et al.*, 2003; Xu *et al.*, 2015). Flavonoids play a significant role in attracting pollinators and seed dispersers by providing

distinct red, purple, violet, and blue coloration to plant parts (Grotewold, 2006). These metabolites exhibit antioxidant properties and contribute to protection against drought conditions, UV radiation, low temperatures, pathogens, and herbivores (Sarma and Sharma, 1999; Lorenc-Kukuła *et al.*, 2005; Gould and Lister, 2006).

Late biosynthetic genes (LBGs) encode a set of enzymes involved in the biosynthesis of anthocyanins and proanthocyanidins, and their activation leads to the accumulation of these metabolites. Proanthocyanidins provide the *Arabidopsis* seeds with distinct dark-brown color. In most species, the expression of LBGs is controlled by the MBW complex, which consists of two transcription factors: R2R3-MYB (R2R3-MYELOBLASTOSIS homolog) and bHLH (basic HELIX-LOOP-HELIX), and the WD40 transcription regulator TTG1 (TRANSPARENT TESTA GLABRA 1) (Allan *et al.*, 2008; Gonzalez *et al.*, 2008; Petroni and Tonelli, 2011; Xu *et al.*, 2015; Chaves-Silva *et al.*, 2018). Overexpression of MYB or bHLH transcription factors involved in anthocyanin synthesis allowed the selection of transgenic events in maize (Shen and Petolino, 2006), *N. benthamiana* (Kim *et al.*, 2010), apple, strawberry, and potato (Kortstee *et al.*, 2011). In these studies, the tissues of interest became dark-colored due to increased pigment accumulation. However, this approach has proven not to be universal, meaning that a marker suitable for one species was not fully functional in others (Doshi *et al.*, 2007). Additionally, such overexpression markers did not allow for early selection in the callus stage of strawberry and potato and required the plants to be more mature in order to identify the phenotype (Kortstee *et al.*, 2011).

Some studies have also down-regulated the anthocyanin biosynthesis pathway, leading to the disappearance of anthocyanin pigmentation in tomato (Orzaez *et al.*, 2009) and petunia (Chen *et al.*, 2004). Such a drastic loss of pigmentation can also be seen in mutants of the MBW complex specific for the flavonoid synthesis pathway (Figure 3). Mutations in the *Arabidopsis TTG1* gene lead to a significant reduction in anthocyanin biosynthesis in vegetative tissues and loss of proanthocyanidins synthesis resulting in yellow coloration of seeds (Walker *et al.*, 1999). The light-yellow color of seeds in the *ttg1* mutant is noticeably different from the dark-brown wild-type seeds, and such feature could help to conveniently distinguish between transformed and non-transformed seeds. However, since *TTG1* is also involved in many other regulatory pathways (Figure 3), including trichome initiation (Schiefelbein, 2003), root hair formation (Schiefelbein *et al.*, 2014), and seed coat mucilage production (Xu *et al.*, 2015) among others, disabling the *TTG1* gene during transformation for seed selection might not be the suitable approach. Therefore, in this study, we aim to

transform *ttg1* mutants with a functional *TTG1* gene, to restore seed color as an indication of a successful transformation event.

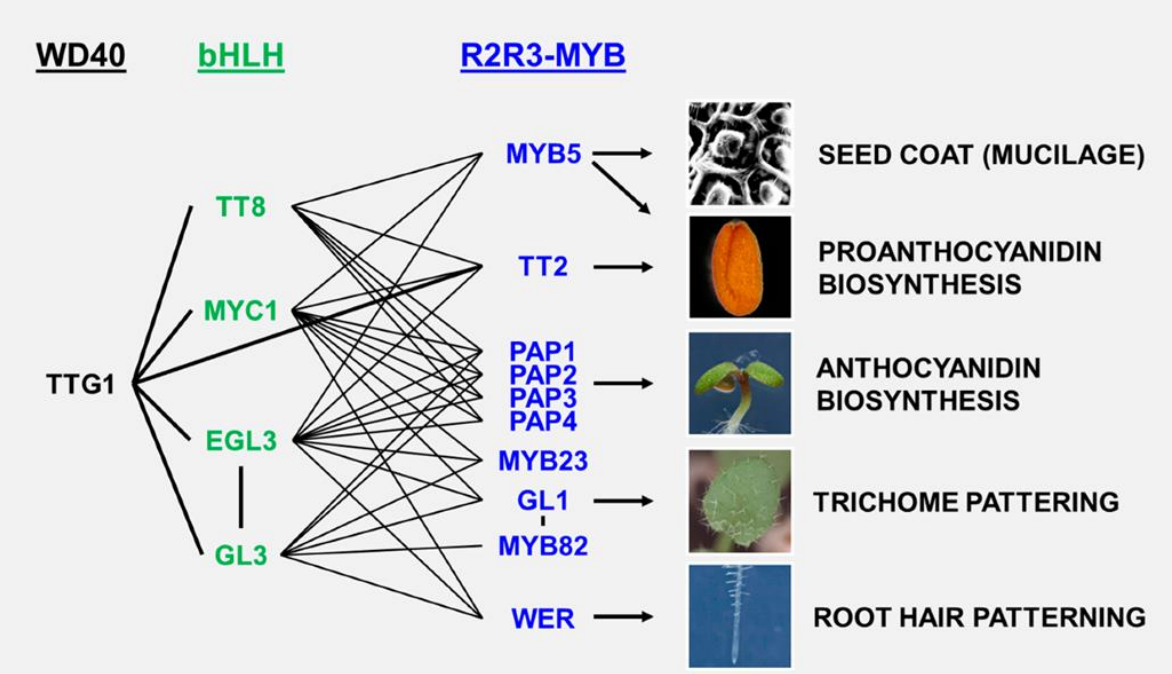

Figure 3. **Functions of the MBW complex in *Arabidopsis*.** The WD40 transcription regulator TTG1, various bHLH and R2R3-MYB transcription factors form a tertiary complex and activate a set of genes involved in seed coat mucilage production, proanthocyanidin and anthocyanin synthesis, as well as trichome and root hair development. Modified from Zhang and Schrader (2017).

Recently, a promising plant pigment-based reporter, RUBY, was developed (He *et al.*, 2020). The RUBY marker is based on the production of betalain pigment in transgenic plants. Betalains are natural pigments found in certain plants, such as beetroot and dragonfruit, and are responsible for their red, violet, or yellow colors. These pigments are derived from the amino acid tyrosine through a multi-step enzymatic process, which is depicted in Figure 4a (Strack *et al.*, 2003; Polturak and Aharoni, 2019; Xu *et al.*, 2020). First, tyrosine is hydroxylated into L-3,4-dihydroxyphenylalanine (L-DOPA) by the enzyme P450 oxygenase CYP76AD1. This enzyme also oxidizes L-DOPA to cyclo-DOPA. Next, L-DOPA is converted into betalamic acid by L-DOPA 4,5-dioxygenase (DODA). In a non-enzymatic reaction, cyclo-DOPA and betalamic acid condense to form betanidin. Lastly, glucosyltransferase (GT) adds a sugar moiety to betanidin, generating the colorful betalain. The RUBY open reading frame contains all three enzymes required for this biochemical pathway (Figure 4b). They are linked together with 2A peptide sequences (Liu *et al.*, 2017; Wang and Chen, 2020), and the stop codons of CYP76AD1 and DODA are removed to ensure the translation of all three coding sequences together (Figure 4b). After translation, the 2A peptides undergo

self-cleavage, releasing the three proteins for correct folding (Figure 4b). This technique enables the use of only one promoter to express all required enzymes. By combining RUBY with the appropriate promoter, seed-specific betalain accumulation can be achieved, facilitating transgene event selection in Arabidopsis seeds.

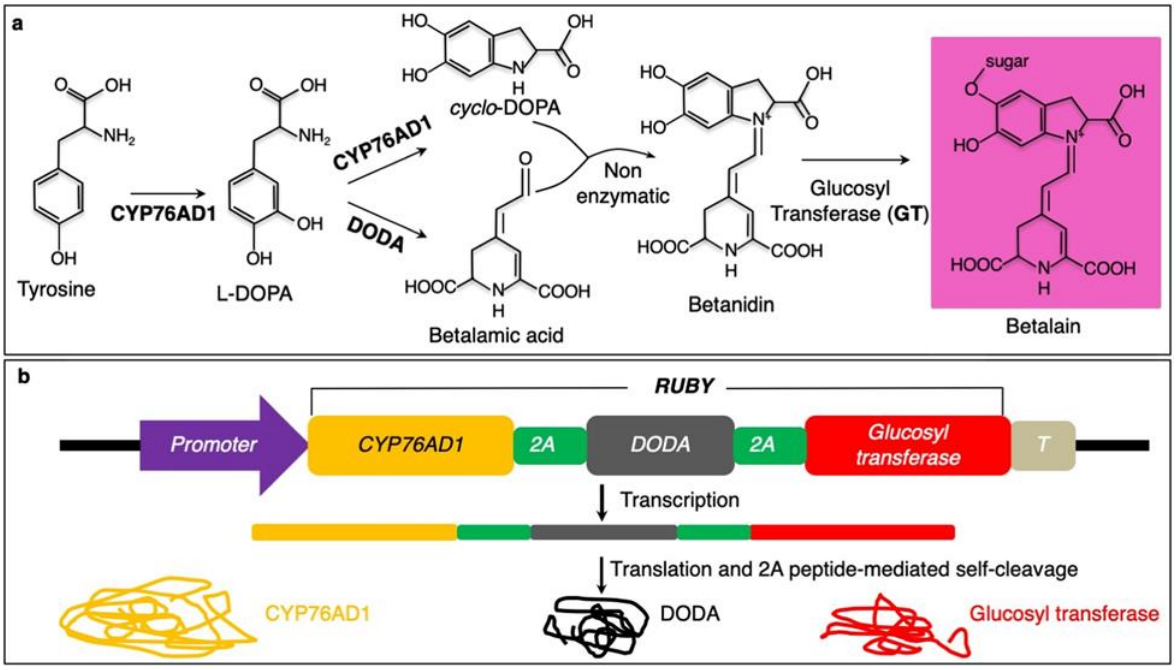

Figure 4. Betalain biosynthetic pathway and the RUBY reporter. a) Biochemical pathway for the conversion of tyrosine to betalain. Tyrosine is hydroxylated to L-3,4-dihydroxyphenylalanine (L-DOPA) and then converted to cyclo-DOPA by the enzyme P450 oxygenase CYP76AD1. L-DOPA is also converted into betalamic acid by L-DOPA 4,5-dioxygenase (DODA). In a non-enzymatic reaction, cyclo-DOPA and betalamic acid react to form betanidin, and a sugar moiety is added by a glucosyltransferase (GT) to form the colorful betalain. **b**) RUBY open reading frame with three enzymes (CYP76AD1, DODA, GT) needed for betalain biosynthesis. Protein coding sequences are separated by 2A peptides, which undergo self-cleavage after translation, releasing individual proteins for further folding. This technique allows for the utilization of only one promoter and terminator for several distinct coding sequences. Source: He *et al.* (2020).

A significant advantage of using the RUBY selection marker is that betalain has a very distinct color compared to the wild-type coloration of *Arabidopsis* seeds, which could make identification quick and straightforward. Furthermore, since the precursor of betalain is the amino acid tyrosine, which is present in every cell, pigment production is ensured in all tissues of interest (He *et al.*, 2020). To assess how distinctive the seed coloration is compared to the dessicated wild-type brown seeds, we transformed *Arabidopsis* with RUBY driven by *CaMV35S* and *OLEOSIN1* promoters for ubiquitous and seed-specific expression, respectively. Two genotypes – Col-0 and the *ttg1* mutant – were used for transformation.

2 THE AIMS OF THE THESIS

The primary objective of this thesis was to develop versatile markers that significantly decrease the amount of time and effort required for transgenic seed selection in *Arabidopsis*.

First, we identified the RUBY reporter as a promising candidate for seed selection due to the distinct betalain coloration produced in fresh seeds of transformed *Arabidopsis*, which can be visually detected without specialized equipment. Our main aim was to investigate whether the resulting betalain pigmentation could be clearly visible in desiccated seeds, as their dark-brown color might potentially mask the RUBY phenotype. We used wild-type *Arabidopsis* Col-0 and plants with a loss-of-function mutation in the gene of the transcription regulator TTG1. The *ttg1* mutant lacks proanthocyanidin production, which is the primary seed pigment, resulting in a yellow seed color instead of dark-brown. The yellow *ttg1* seeds can potentially serve as a better background for detection of transgene-acquired coloration. We transformed RUBY under the control of a constitutive/ubiquitous *CaMV35S* and a seed-specifc *OLEOSIN1* promoters into Col-0 and *ttg1* mutant plants to examine its performance in the first two generations of transgenic plants.

Second, we investigated the use of chromoproteins, small proteins isolated from marine species and commonly employed as reporters in various bacterial species, as seed-selection markers in plants. Specifically, we employed the purple-blue chromoprotein amilCP driven by the *Arabidopsis* ubiquitin 10 and *OLEOSIN1* promoters in Col-0 and the *ttg1* mutant, analyzing two generations of transgenic plants.

Lastly, we attempted to create an endogenous selection marker based on the *TTG1* gene. Ideally, including the *TTG1* gene in the T-DNA for transformation into *ttg1* mutants would restore seed color, indicating a successful transformation event and making this system a convenient visual selection marker. Therefore, we transformed *ttg1* mutants with constructs containing functional *TTG1* and assessed the seeds for brown coloration.

3 EXPERIMENTAL PART

3.1 MATERIALS AND METHODS

3.1.1 Plant material

Arabidopsis thaliana Columbia-0 (Col-0) and the *ttg1* mutant (see below) were used to generate various transgenic plants through *Agrobacterium tumefaciens*-mediated plant transformation (Clough and Bent, 1998).

The *ttg1* mutant was created using a CRISPR/Cas9 technology to target the TTG1 coding sequence. Col-0 plant was transformed with the corresponding construct and the first generation (T1) seeds were selected based on FAST-R fluorescence (Shimada *et al.*, 2010). T2 seeds without red fluorescence (indicating the loss of transgene) but with a clear yellow color phenotype were selected, sown, and sequenced. Homozygous plant line with one-nucleotide deletion in the 5' end of the TTG1 coding sequence was selected and its T3 generation was used for further transformation with the T-DNA (Gelvin, 2017) of interest.

3.1.2 Plant growth conditions

Plants were grown in plastic pots filled with a mixture of peat and perlite (at a 2:1 ratio) inside growth cabinets. The plants were initially kept in a short-day growth cabinet with a daily photoperiod of 10 hours, a day temperature of 23°C, a night temperature of 21°C, and a relative air humidity of 55%. The short-day growth cabinet had a photosynthetic photon flux density of 250 μmol m^{-2} s^{-1}. Once the plants reached a suitable size, they were transferred to a long-day growth room with a photoperiod of 14 hours and the same temperature, humidity, and photon flux conditions.

To ensure successful germination, valuable seeds were sown on 0.5× Murashige and Skoog (MS) 0.6% phytoagar medium (Phygenera) containing cefotaxim (100 mg/L), and only after germination, were they transplanted into peat/perlite-filled pots. For imaging purposes, seeds were sown on 10 mM KNO_3 0.7% agar plates with cefotaxim (100 mg/L). Both MS and KNO_3 plates were kept in a SANYO MLR-351 Versatile Environmental Test Chamber (SANYO Electric Co., Ltd.) with a daily photoperiod of 10 hours, a day temperature of 24°C, a night temperature of 19°C and a photosynthetic photon flux density of 125 μmol m^{-2} s^{-1}. The humidity was not regulated in this incubator, therefore, the plates were sealed with medical tape.

Seeds were sterilized before being sown on KNO_3 agar or MS agar medium plates. The sterilization process included holding the seeds in 95% ethanol for 2-3 minutes, washing them with sterilized deionized water, and then treating them with 2% sodium hypochloride for approximately 3 minutes. After the sodium hypochloride treatment, the seeds were washed three times with sterilized deionized water and kept at 4°C for 1-3 days to undergo stratification before being sown on the plate.

3.1.3 Molecular cloning

The *p35S:RUBY/pHDE* plasmid (He *et al.*, 2020) was kindly provided by Mikael Brosché (University of Helsinki) (Figure 5a). The plasmids *pOLE1:gOLE1-amilCP-V5_OLE1t/HygR_P19_pYSWb1* and *pUBQ10:amilCP-V5_NOSt/P19_pTC217** (Figure 5b-c) were cloned beforehand and used for plant transformation without any modifications.

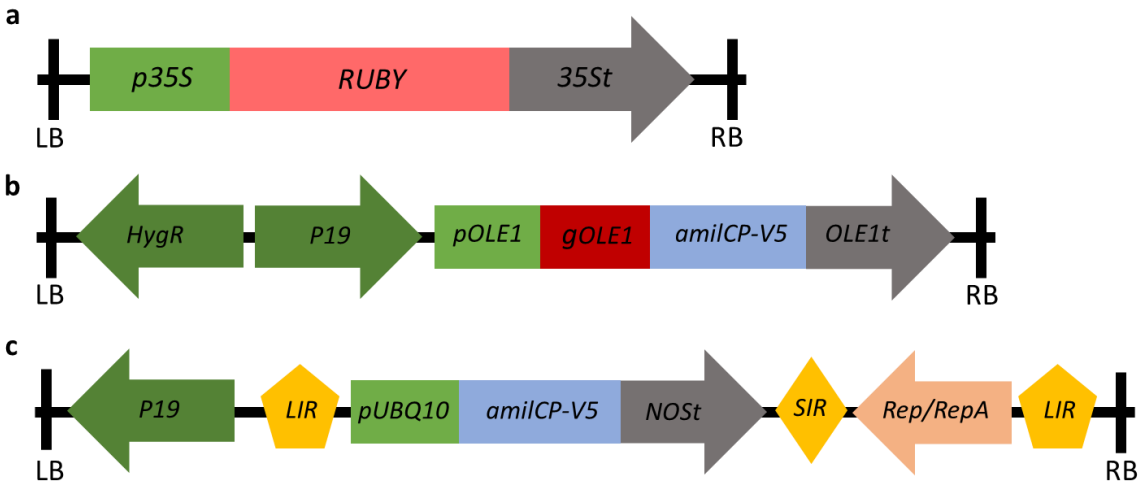

Figure 5. Schematic representation of T-DNA regions of the used constructs. a) *RUBY* selection marker assembled with the *CaMV35S* promoter and terminator in *pHDE* vector (*p35S:RUBY_35St/pHDE*). **b**) Blue chromoprotein amilCP fused with OLEOSIN1 genomic sequence at the N-terminus and a Simian Virus 5 (V5) tag at the C-terminus, and driven by *OLEOSIN1* promoter in *pYSWb1* binary plasmid (*pOLE1:gOLE1-amilCP-V5_OLE1t/HygR_P19_pYSWb1*). **c**) Blue chromoprotein amilCP with a V5 tag driven by the *Arabidopsis* ubiquitin 10 promoter in *pTC217** binary plasmid (*pUBQ10:amilCP-V5_NOSt/P19_pTC217**). LB – Left Border of the T-DNA, RB – Right Border of the T-DNA, *HygR* – Hygromycin B resistance selection marker, *P19* – the tombusviral RNA silencing suppressor, *p35S* – *CaMV35S* promoter, *35St* – *CaMV35S* terminator, *pOLE1* – *OLEOSIN1* promoter, *gOLE1* – genomic OLEOSIN1 coding sequence, *OLE1t* – *OLEOSIN1* terminator, *amilCP-V5* – blue non-fluorescent chromoprotein with V5-tag (McLean *et al.*, 2001), *pUBQ10* – plant ubiquitin 10 promoter, *NOSt* – *A. tumefaciens* nopaline synthase terminator, *LIR* – long intergenic region of the bean yellow dwarf virus (BeYDV), *SIR* – short intergenic region of (BeYDV), *Rep/RepA* – virus replication initiator protein of (BeYDV).

3.1.3.1 Polymerase chain reaction (PCR) settings

All PCR reaction mixtures consisted of a variable amount of DNA template and water (volume details are listed in Table 1). The mixture also contained 0.5 µL of each primer (10 µM, Integrated DNA Technologies), 0.4 µL of 10 mM dNTPs (Thermo Fisher Scientific), 0.2 µL of Phusion PFU DNA Polymerase (Thermo Fisher Scientific), and 4 µL of 5× HF Phusion Buffer (Thermo Fisher Scientific). The PCR program was as follows: an initialization step at 98°C for 30 seconds, followed by 3 cycles of denaturation at 98°C for 10 seconds, primer annealing at 53°C for 15 seconds and extension at 72°C for 30 seconds. For the next 27 cycles the temperature for primer annealing was raised to 58°C while the other steps remained the same. The final extension was carried out at 72°C for 1 minute. The PCR products were either PCR purified or gel purified using the FavorPrep PCR Purification Mini Kit (FAVORGEN Biotech Corporation), following the manufacturer's manual.

3.1.3.2 The Golden Gate cloning

The Golden Gate cloning method (Engler *et al.*, 2008; Engler *et al.*, 2014) was utilized to assemble transcriptional units from DNA parts using Type IIS restriction enzymes. These enzymes create specific, non-palindromic overhangs outside their recognition sequence (Figure 6a). The *Bsa*I recognition sites (GGTCTCN) and specific four-nucleotide linking sequences were introduced to the inserts of interest by PCR (Figure 6b). The linking sequences varied depending on the fragment type: promoter (5' end: GGAG, 3' end: AATG), coding sequence (5' end: AATG, 3' end: GCTT), and terminator (5' end: GCTT, 3' end: CGCT). The vector also contained *Bsa*I recognition sites and four-nucleotide linker sequences (5' end: GGAG, 3' end: CGCT; Figure 6b). After digestion with FastDigest (FD) *Bsa*I restriction enzyme (Thermo Fisher Scientific), 5'-overhangs were formed from the linkers (Figure 6c). The desired inserts were incorporated into the vector through overhang complementarity by T4 DNA ligase (Thermo Fisher Scientific), resulting in a final product without *Bsa*I recognition sites (Figure 6d). Multiple cycles of digestion and ligation in a thermocycler machine, at optimal temperatures of 37°C and 16°C respectively, significantly increased the proportion of correct products. However, some primary plasmids remained in each reaction due to undigested plasmids or self-ligated plasmid fragments.

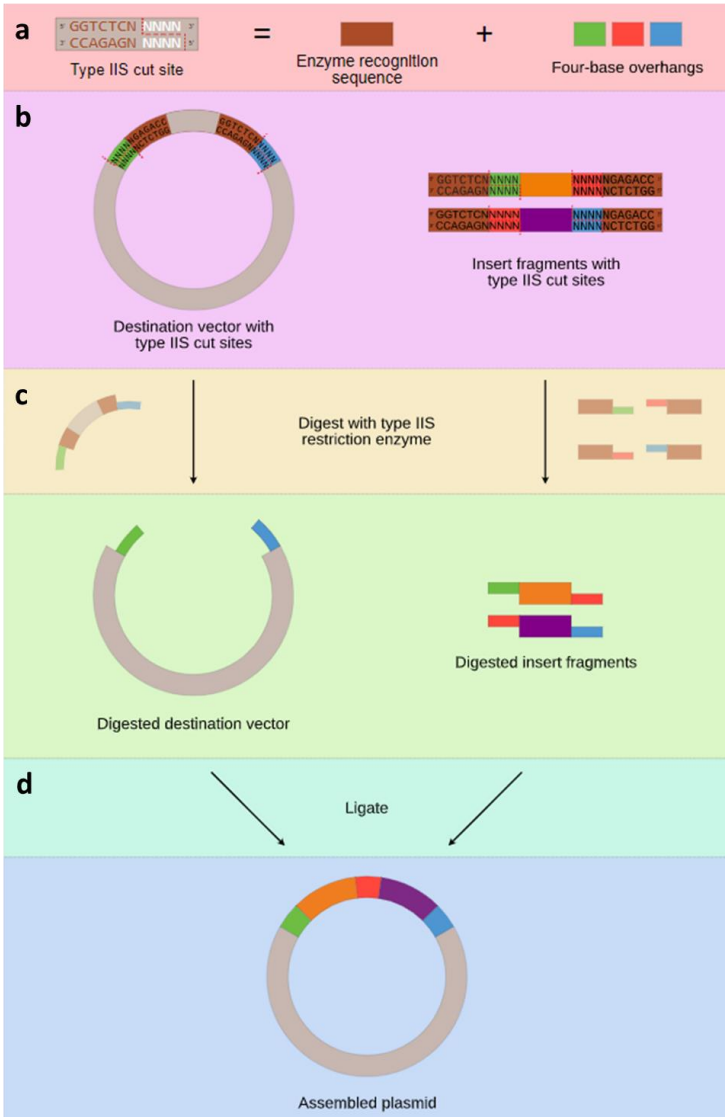

Figure 6. Illustration of Golden Gate assembly. a) Type IIS restriction enzymes can generate unique non-palindromic overhangs beyond their recognition sites. **b, c)** Both the destination vector and inserts of interest, which can include PCR products or plasmid fragments, possess recognition sites and four-base linkers that form four-nucleotide 5'-overhangs after digestion. **d)** These components are then joined by a ligase, based on the complementarity of their overhangs, resulting in the formation of the final plasmid. Modified from: https://en.wikipedia.org/wiki/Golden_Gate_Cloning

The reaction mixture for all samples included 1 µL of 10× Ligase buffer (from Thermo Fisher Scientific), 0.2 µL of T4 DNA ligase, and 0.2 µL of FD *Bsa*I. Water and DNA fragment amounts varied (Table 4). The thermocycler program was 10 cycles of 37°C for 5 minutes and 16°C for 5 minutes, followed by storage at 37°C until transformation to *E. coli*.

3.1.3.3 The Plant MoClo parts

The majority of the fragments utilized in the Golden Gate assembly were previously incorporated into specific Level 0 vectors to create Plant MoClo parts (Engler *et al.*, 2014). The necessary sequences were first PCR amplified, and the *Bsa*I recognition sites and linker sequences were added by primers (Table 1). The amplified products were then purified, digested with FD *Bsa*I restriction enzyme (Table 2) and purified again. After that, the fragments were ligated (Table 3) into pre-digested Level 0 vectors. All Level 0 vectors contained

a spectinomycin resistance selection marker, a pMB1 origin of replication for plasmid propagation in *E. coli*, and a fragment with flanking *Bsa*I recognition sites and linkers that are complementary to the overhandgs on the digested PCR products.

3.1.3.4 Ligation

In the ligation mixture, the amount of DNA fragments and water varied (Table 3). However, all samples contained 1 μL of 10× Ligase buffer and 0.5 μL of T4 DNA ligase. The reactions were then transformed to *E. coli* after being incubated for 1-2 hours at room temperature.

3.1.3.5 *E. coli* transformation

Either Golden Gate or ligation products were transformed into *E. coli* for plasmid amplification. First, 4 μL of the Golden Gate mixture or 10 μL of ligation sample was added to 50-100 μL of thawed competent *E. coli* cells. The cells were incubated on ice for 15 minutes and subjected to heat-shock treatment in a 42°C water bath for 90 seconds. The samples were then cooled down on ice for 2 minutes and placed in a 37°C incubator for 40 minutes after adding 300 μL of liquid LB medium. The cell suspensions were plated on solid LB medium with an appropriate antibiotic based on the selection marker in the vector and incubated at 37°C overnight. A positive colony verified by colony PCR or blue/white selection (for those with an amilCP dropout system) for each construct was selected and cultured in 4 mL of liquid LB medium with the appropriate antibiotic in a shaking incubator at 37°C and 220 rpm overnight. The plasmids were extracted using the FavorPrep Plasmid Extraction Mini Kit (FAVORGEN Biotech Corporation) according to the manufacturer's manual, quantified with a NanoDrop spectrophotometer (Thermo Fisher Scientific) and sent for verification by Sanger sequencing.

3.1.3.6 *Agrobacterium tumefaciens*-mediated plant transformation

The *Agrobacterium tumefaciens* strain GV3101 (Holsters *et al.*, 1980; Koncz and Schell, 1986) was transformed with the desired binary plasmids. Approximately 500 ng of plasmid was added to 20 μL of competent cells, which were then subjected to a heat-shock treatment by incubating them in a water bath at 37°C for 10 minutes. Next, 300 μL of liquid LB medium was added, and the samples were incubated for 1 hour at 28°C. The samples were then plated on solid LB media containing kanamycin (50 mg/L) and gentamicin (50 mg/L) and incubated at 28°C. The addition of gentamicin is necessary for selecting *Agrobacterium* colonies that have the *vir* helper plasmid (Hoekema *et al.*, 1983). After three days, colonies were cultured in 2 mL of LB liquid medium with kanamycin and gentamicin in a shaking

incubator at 28°C and 220 rpm overnight. The culture was then centrifuged, and the pellet was resuspended in 1 mL of a floral dip solution (Clough and Bent, 1998) consisting of 5% sucrose and 0.05% Silwet-L77. This mixture was applied to the flowers using a pipette, followed by two more applications every other day. The mixture was stored at 4°C during the application period. The plants were kept under long-day conditions until seed harvesting.

3.1.3.7 TTG1 coding sequence with native regulatory components

The two variants of *TTG1* gene (Figure 7), with a short and long promoter parts (*short pTTG1:TTG1_TTG1t*, *long pTTG1:TTG1_TTG1t*), were amplified by PCR (Table 1), and then purified. The use of cDNA was not necessary because the protein coding sequence is located in one exon. The purified PCR products were incorporated into the binary vector *amilCP/HygR_P19_pYSWb1* using the Golden Gate cloning method (Figure 7 and Table 4). The subsequent *E. coli* and plant transformation procedures were carried out following the same steps as previously outlined.

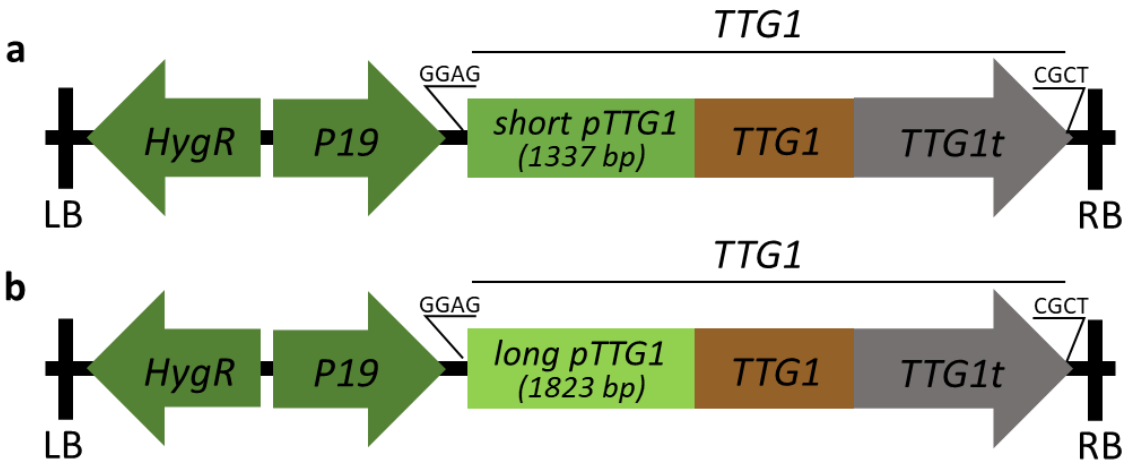

Figure 7. T-DNA regions of constructs with *TTG1* gene. TTG1 coding sequence with either short (**a**) or long (**b**) promoter fragment was assembled in *pYSWb1* binary vector (*short/long pTTG1:TTG1_TTG1t/HygR_P19_pYSWb1*). LB – Left Border of the T-DNA, RB – Right Border of the T-DNA, *HygR* – Hygromycin B resistance selection marker, *P19* – the tombuviral RNA silencing suppressor, *pTTG1* – *TTG1* promoter, *TTG1* – TTG1 coding sequence, *TTG1t* – *TTG1* terminator. The four-nucleotide linkers that were used for Golden Gate assembly are depicted above each construct.

The binary vector *amilCP/HygR_P19_pYSWb1* (designed by Yuh-Shuh Wang) was chosen as the destination vector. It is called binary because it contains only T-DNA and no virulence region. The T-DNA depends on the virulence helper plasmid in the *Agrobacterium* strain used for transformation (Hoekema *et al.*, 1983). The virulence proteins are expressed from the helper plasmid and recognize two 25-base-pair repeats called Left and Right Border (LB

and RB), which flank the T-DNA region in the binary vector (Yadav *et al.*, 1982). The virulence proteins are required to excise the T-DNA region and mediate its transfer into the plant cell and integration into the host genome (Herreraestrella *et al.*, 1988; Ward and Barnes, 1988; De Vos and Zambryski, 1989; Herreraestrella *et al.*, 1990). The binary vector used was modified from *pHEE2E-TRI* (Wang *et al.*, 2015). The T-DNA of *amilCP/HygR_P19_pYSWb1* contains the tombuviral RNA silencing suppressor *P19* (Garabagi *et al.*, 2012), the blue non-fluorescent chromoprotein from *Acropora millepora* (amilCP) (Alieva *et al.*, 2008) and a hygromycin resistance selection marker. The backbone of the vector (outside the T-DNA part) consists of two origins of replication for propagation in both *E. coli* and *Agrobacterium*, two genes for the *Agrobacterium* system, which encode the replication protein for multiplication of the binary plasmid (pVS1-RepA) and the protein for stable cell segregation (pVS1-staA) (Heeb *et al.*, 2000), and a kanamycin resistance selection marker for selection of transformed bacterial colonies. The *amilCP* part of the vector has flanking regions with a *Bsa*I recognition site and a four-nucleotide linker sequence (GGAG at 5' and CGCT at 3' end). Therefore, the *amilCP* can be substituted by the desired insert via a Golden Gate reaction. Although some undigested plasmids may still be present in the reaction product, the correct plasmid can be easily distinguished after transformation into *E. coli* by selecting white colonies rather than blue ones.

3.1.3.8 TTG1 coding sequence driven by *CaMV35S* promoter

The *CaMV35S* promoter and terminator had previously been cloned into Level 0 vector. The TTG1 coding sequence was amplified using a two-step overlap PCR method to generate synonymous base substitutions that disrupt the protospacer adjacent motif (PAM) of single-guide RNA. This mutation was needed for the *TTG1** knock-in CRISPR/Cas9 experiment, which was not included in this thesis. The 5' and 3' parts of the *TTG1** (asterisk indicates that the fragment was modified) with the overlap region containing the mutations were amplified separately (Table 1). The products were then gel-purified. A second PCR reaction was performed with flanking primers and purified fragments as templates (Table 1). The final product was purified, digested with FD *Bsa*I (Table 2), PCR-purified, and ligated with the Level 0 vector (Table 3). The TTG1* coding sequence, the *CaMV35S* promoter and terminator (*p35S:TTG1*_35St*) were integrated into the binary vector by Golden Gate assembly (Figure 8 and Table 4).

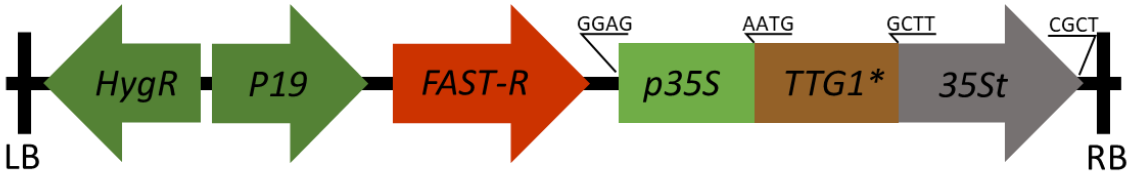

Figure 8. Schematic map of T-DNA with modified TTG1 coding sequence controlled by *CaMV35S* promoter. *TTG1** with *CaMV35S* promoter was integrated into the *pYSWb1* binary plasmid with FAST-R seed-selection marker (*p35S:TTG1*_35St/FAST-R_HygR_P19_pYSWb1*). LB – Left Border of the T-DNA, RB – Right Border of the T-DNA, *HygR* – Hygromycin B resistance selection marker, *P19* – the tombuviral RNA silencing suppressor, *p35S* – *CaMV35S* promoter, *TTG1** – modified TTG1 coding sequence, *35St* – *CaMV35S* terminator. The four-nucleotide linkers that were used for Golden Gate assembly are depicted over construct.

The *amilCP_FAST-R/HygR_P19_pYSWb1* was chosen as the binary vector for this construct. The FAST-R seed-selection marker (Shimada *et al.*, 2010) is integrated into the T-DNA region; otherwise, this vector is identical to the *amilCP/HygR_P19_pYSWb1* described in the previous chapter.

3.1.3.9 Targeting expression of RUBY in seeds by *OLEOSIN1* promoter

The *OLEOSIN1* (*OLE1*) promoter and terminator were chosen for seed-specific expression of the RUBY as research showed that its promoter is most active during seed maturation (Kim *et al.*, 2002).

Figure 9. Schematic representation of the T-DNA region with RUBY assembled with *OLEOSIN1* regulatory elements. The RUBY coding sequence, driven by the *OLEOSIN1* promoter and terminator, was cloned in the *pYSWb1* binary vector (*pOLE1:RUBY_OLE1t/P19_HygR_pYSWb1*). LB – Left Border of the T-DNA, RB – Right Border of the T-DNA, *HygR* – Hygromycin B resistance selection marker, *P19* – the tombuviral RNA silencing suppressor, *pOLE1* – *OLEOSIN1* promoter, *OLE1t* – *OLEOSIN1* terminator. The four-nucleotide linkers that were used for Golden Gate assembly are depicted over construct.

The promoter (*pOLE1*) was cloned into Level 0 plasmid (Tables 1, 2, and 3) and terminator (*OLE1t*) was already available in Level 0 vector. These components were utilized in a Golden Gate assembly to create the *pOLE1:RUBY_OLE1t/HygR_P19_pYSWb1* construct (Figure 9 and Table 4).

Table 1. PCR reaction details.

Sequence	Primer name	Primer sequence	PCR conditions
short/long pTTG1:TTG1_TTG1t/HygR_P19_pYWSb1			
short pTTG1:TTG1_TTG1t	XV-53 forward	GTGGTCTCAGGAGCATCCTTATGTTTAGCGCATAG	*Arabidopsis thaliana* genomic DNA (gDNA) 1 µL, H_2O 13.4 µL
	XV-50 reverse	ATGGTCTCCAGCGCTAATAAGAGCCAATATATATGACT	
long pTTG1:TTG1_TTG1t	XV-43 forward	GTGGTCTCTGGAGTAAACGTGGGTTTGG	gDNA 1 µL, H_2O 13.4 µL
	XV-50 reverse	ATGGTCTCCAGCGCTAATAAGAGCCAATATATATGACT	
p35S:TTG1:35St/pFAST-R_HygR_P19_pYWSb1*			
5' *TTG1** fragment	XV-45 forward	ACGGTCTCAAATGGATAATTCAGCTCCAGATT	gDNA 1 µL, H_2O 13.4 µL
	XV-59 reverse	GATCTCCGGAACTTGGACGACGTAG	
*TTG1** 3' fragment	XV-58 forward	CTACGTCGTCCAAGTTCCGGAGATC	gDNA 1 µL, H_2O 13.4 µL
	XV-46 reverse	GTGGTCTCAAAGCTCAAACTCTAAGGAGCTGCAT	
*TTG1** 5'+3' overlap	XV-45 forward	ACGGTCTCAAATGGATAATTCAGCTCCAGATT	5' *TTG1** 0.1 µL, *TTG1** 3' 0.1 µL, H_2O 14.2 µL
	XV-46 reverse	GTGGTCTCAAAGCTCAAACTCTAAGGAGCTGCAT	
pOLE1:RUBY_OLE1t/HygR_P19_pYWSb1			
RUBY	XVI-68 forward	GTGGTCTCAAATGGATCATGCGACCCTCG	p35S:RUBY/pHDE 0.1 µL, H_2O 14.3 µL
	XVI-69 reverse	GTGGTCTCAAAGCTCACTATCACTGGAGGCTTG	
pOLE1	VI-02 forward	GTGTCGACTCTGTGGTCTCA	pFAST-R/pGEM-T easy 0.1 µL, H_2O 14.3 µL
	XVII-01 reverse	GTGGTCTCACATTTTTTTGTTCTTGTTTACTAGAGAG	

The recognition sequence of *Bsa*I is highlighted in red, while linker sequences or overhangs (after digestion) are highlighted in green.

Table 2. Restriction digestion summary table.

Digested product	DNA sample, H_2O	Reagents
p35S:TTG1:35St/pFAST-R_HygR_P19_pYWSb1*		
TTG1/Bsa*I	purified *TTG1*_XV-45&XV-46* 35.5 µL	FD *Bsa*I 0.5 µL, 10× FD buffer 4 µL
pOLE1:RUBY_OLE1t/P19_HygR_pYSWb1		
*pOLE1/Bsa*I	purified *pOLE1_VI-02&XVII-01* 35.5 µL	FD *Bsa*I 0.5 µL, 10× FD green buffer 4 µL
*RUBY/Bsa*I	purified *RUBY_XVI-68&XVI-69* 35.5 µL	

Table 3. Ligation reaction components and products specification table.

Construct name	DNA fragments, H_2O	Common reagents
TTG1/MoClo_AATG-GCTT*	*TTG1*/Bsa*I 1 µL, *MoClo_AATG-GCTT/Bsa*I 0.5 µL and H_2O 7 µL	T4 DNA ligase 0.5 µL, 10× ligase buffer 1 µL
RUBY/MoClo_AATG-GCTT	*RUBY/Bsa*I 1.5 µL, *MoClo_AATG-GCTT/Bsa*I 0.5 µL, H_2O 6.5 µL	
pOLE1/MoClo_GGAG-AATG	*pOLE1/Bsa*I 1 µL, *MoClo_GGAG-AATG/Bsa*I 0.5 µL, H_2O 7 µL	

Table 4. Constructs assembled with Golden Gate cloning.

Construct name	DNA fragments, H_2O	Common reagents
short pTTG1:TTG1_TTG1t/HygR_P19_pYSWb1	*amilCP/HygR_P19_pYSWb1* (169 ng/µL) 0.5 µL, *short pTTG1:TTG1_TTG1t* 2 µL, H_2O 6.1 µL	T4 DNA ligase 0.2 µL, FD *Bsa*I 0.2 µL, 1 µL of 10× ligase buffer
long pTTG1:TTG1_TTG1t/HygR_P19_pYSWb1	*amilCP/HygR_P19_pYSWb1* (169 ng/µL) 0.5 µL, *long pTTG1:TTG1_TTG1t* 2 µL, H_2O 6.1 µL	
p35S:TTG1:35St/FAST-R_HygR_P19_pYSWb1*	*amilCP_FAST-R/HygR_P19_pYSWb1* (99 ng/µL) 0.5 µL, *p35S/MoClo_GGAG-AATG* (166 ng/µL) 0.7 µL, *TTG1*/MoClo_AATG-GCTT* (374 ng/µL) 0.3 µL, *35St/MoClo_GCTT-CGCT* (251 ng/µL) 0.5 µL and H_2O 6.6 µL	
pOLE1:RUBY_OLE1t/P19_HygR_pYSWb1	*amilCP/HygR_P19_pYSWb1* (169 ng/µL) 0.5 µL, *pOLE1/MoClo_GGAG-AATG* (78 ng/µL) 1 µL, *RUBY/MoClo_AATG-GCTT* (498 ng/µL) 0.25 µL, *OLE1t/MoClo_GCTT-CGCT* (200 ng/µL) 0.5 µL and H_2O 6.35 µL	

3.2 RESULTS

3.2.1 *Arabidopsis* Col-0 and *ttg1* mutant phenotypes

During the development of the CRISPR/Cas9 technology for plant genome editing in our lab, we have chosen the *TRANSPARENT TESTA GLABRA 1* (*TTG1*) as the target gene to create knockouts. The *TTG1* transcription regulator plays a crucial role in numerous molecular pathways and its knockouts have distinct phenotypic characteristics (see the Literature Review and Figure 3), which can be easily visually identified. The pathways regulated by *TTG1* involve trichome development and the production of anthocyanins and proanthocyanidins. In the wild-type *Arabidopsis*, trichomes can be found on the leaves (Figure 10a). The vegetative tissues contain anthocyanin pigments (Figure 10a, c-e), which give the plant its characteristic green and purple coloration in the later stages of the plant's life cycle or when the plant is in stress (Figure 10c). The seeds of *Arabidopsis* primarily contain proanthocyanidins, which impart a distinct dark-brown color in the seeds (Figure 10a).

As a result, the *ttg1* loss-of-function mutants, including the *ttg1* generated by the CRISPR/Cas9 technology used in this study, exhibit deficiencies in all of the aforementioned traits, such as glabrous leaves that are devoid of trichomes (Figure 10b), and lack of anthocyanin and proanthocyanidin pigments. Consequently, the leaf and stem remain light green and later turn light-yellow (Figure 10b, f-h). In the absence of proanthocyanidin accumulation, the seeds appear light-yellow (Figure 10b), as opposed to the dark-brown color in the wild-type seeds (Figure 10a).

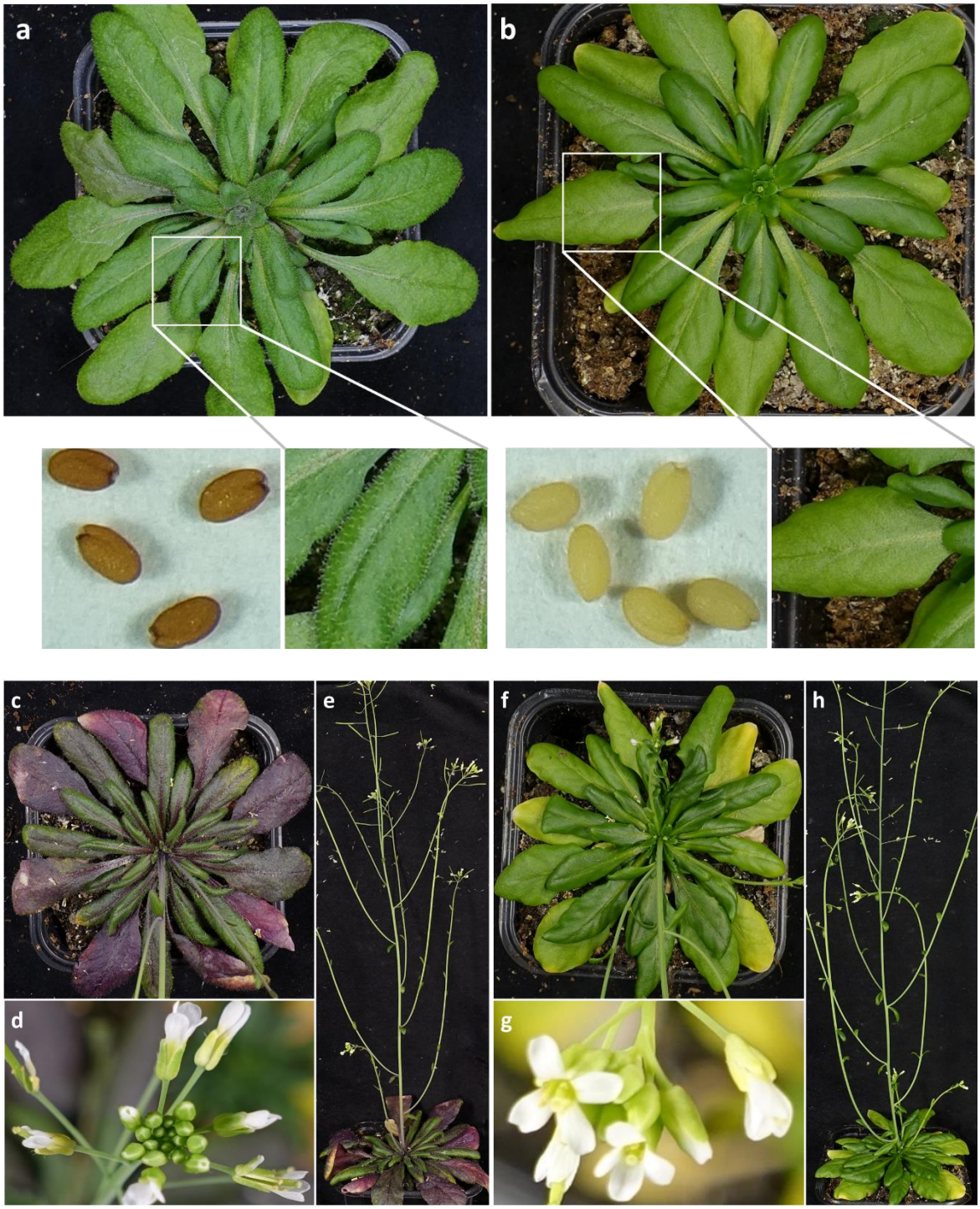

Figure 10. Comparison of *Arabidopsis* Col-0 and *ttg1* mutant plants. a) A 5-week-old Col-0 plant with visible trichomes on leaves and its brown seeds. **b**) A 5-week-old *ttg1* mutant without trichomes and its light-yellow seeds. **c** to **e**) Phenotypes of an 8-week-old mature Col-0 plant. **f** to **g**) Phenotypes of an 8-week-old *ttg1* mutant.

3.2.2 Assessment of the RUBY seed-selection marker

He *et al.* (2020) developed the RUBY reporter composed of three enzymes required for betalain synthesis. Tissues expressing this reporter accumulate betalain pigment, resulting in a dark-red color. When a seed-specific promoter, *At2S3*, was used, the red seeds could be easily distinguished from untransformed green seeds in the developing siliques. However, the dessicated wild-type *Arabidopsis* seeds have a dark-brown color, which could mask the red betalain pigmentation and renders the RUBY reporter less useful in the dry mature seeds.

In order to further assess the potential of the RUBY reporter as a dry seed-selection marker, wild-type Col-0 (dark-brown seeds) and the *ttg1* mutant (bright yellow seeds) were transformed with either the *p35S:RUBY* (Figure 5a) (He *et al.*, 2020) or the *pOLE1:RUBY* (Figure 9) construct. The bright yellow color of the dessicated *ttg1* seeds would be ideal to show any pigmentation changes resulted from the RUBY reporter. The *CaMV35S* promoter (*p35S*) was meant for a strong constitutive and ubiquitous expression, whereas the *OLEOSIN1* promoter (*pOLE1*) was chosen to drive seed-specific expression of the selection marker gene. To reduce transgene silencing, a *P19* gene silencing suppressor was also included in the *pOLE1:RUBY* construct (Figure 9).

3.2.2.1 Seed pigmentation of primary transformants

All four transformants (*p35S:RUBY/Col-0*, *p35S:RUBY/ttg1*, *pOLE1:RUBY/Col-0*, and *pOLE1:RUBY/ttg1*) produced seeds with a distinct dark-red color (Figure 11a). However, the color intensity varied noticeably within the same genotype (Figure 11a), which is normal in the first generation (Gelvin, 2003). To observe the mature plant phenotype, first generation of transgenic (T1) seeds were planted. One-week-old seedlings of *p35S:RUBY* in both Col-0 and *ttg1* backgrounds continued to express the marker gene, while seedlings of *pOLE1:RUBY* lost a significant amount of betalain pigmentation (Figure 11b).

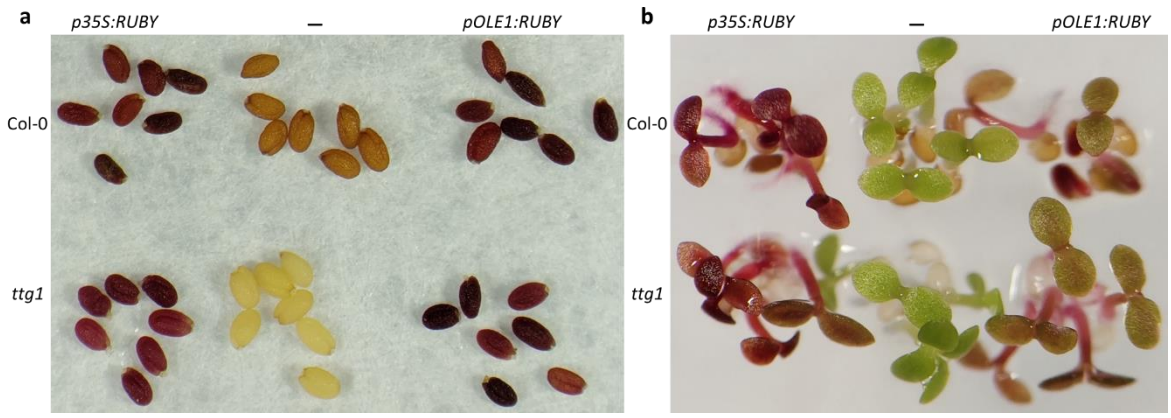

Figure 11. Images of T1 seeds and seedlings transformed with the RUBY selection marker. a) Seeds of Col-0 and *ttg1* plants transformed with *p35S:RUBY* and *pOLE1:RUBY* for ubiquitous and seed-specific expression of RUBY, respectively. **b**) One-week-old seedlings from seeds presented in panel **a**. The middle columns in panels **a** and **b** correspond to untransformed Col-0 and *ttg1* plants.

3.2.2.2 Ubiquitous expression of RUBY in mature plants

Two Col-0 and two *ttg1* plants with *p35S:RUBY* were grown to maturity. Both transformed Col-0 plants exhibited complete dark-red coloration, as expected due to the ubiquitous nature of the *CaMV35S* promoter (Figure 12a-c). Surprisingly, transformed *ttg1* plants displayed no betalain pigmentation, despite being originated from dark-red seeds. Upon examining seven additional *p35S:RUBY/ttg1* plants, only two transformants exhibited complete red pigmentation in mature plants (Figure 12d-f). Another two *p35S:RUBY*/*ttg1* transformants displayed pigmentation only in specific plant parts: one plant exhibited dark coloration exclusively in leaves (Figure 13a-c), while the second plant had pigmentation in petals and pistils (Figure 13d-f). The remaining three plants did not have any visually detectable betalain coloration.

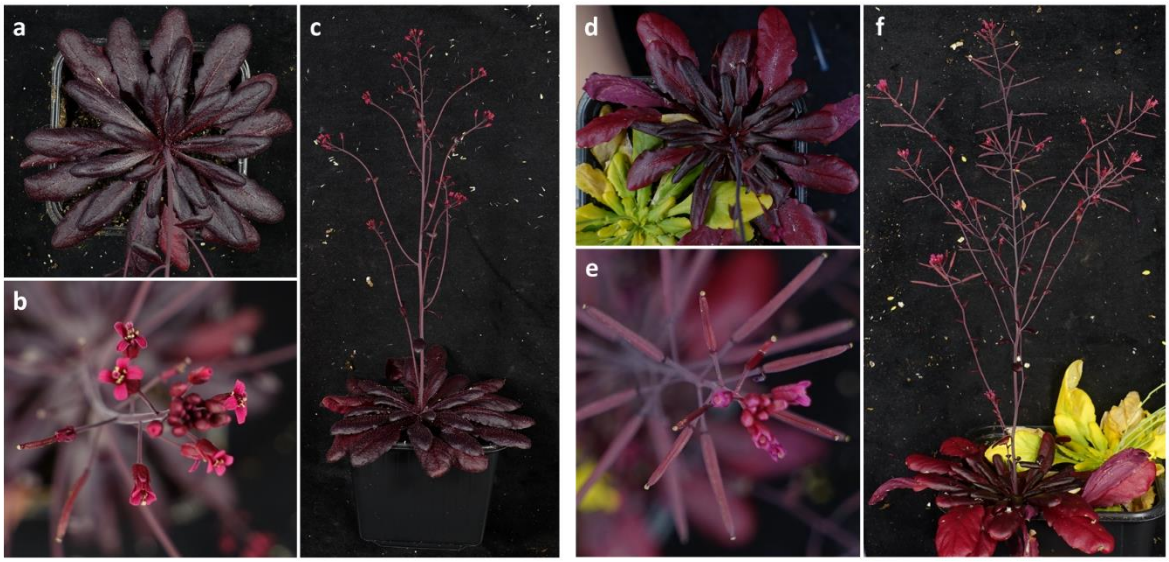

Figure 12. Mature transgenic plants with the *p35S:RUBY* construct. *Arabidopsis* Col-0 (**a-c**) and *ttg1* mutant (**d-f**) were transformed with RUBY selection marker under *CaMV35S* promoter, resulting in the production of betalain in all tissues.

Figure 13. Different expression patterns of T1 *p35S:RUBY/ttg1* plants. The images illustrate two *ttg1* mutants transformed with the RUBY selection marker under the control of the *CaMV35S* promoter. One plant (**a-c**) exhibits distinct pigmentation in leaves (**a**) and another plant (**d-f**) displays betalain coloration in petals and pistils (**e**).

T2 seeds from eight *p35S:RUBY/ttg1* plants out of the nine examined were mostly dark-red, as expected for this generation. Only one *p35S:RUBY/ttg1* plant, which lacked betalain pigmentation in vegetative tissues, produced T2 seeds that were predominantly yellow, with a small fraction of dark-red seeds.

3.2.2.3 Mature plants with RUBY driven by *OLEOSIN1* promoter

All T1 plants with the *pOLE1:RUBY* construct in the Col-0 background exhibited slight betalain pigmentation in their flowers (Figure 14a-c) compared to wild-type Col-0 plants (Figure 10a, c-e) and those expressing *p35S:RUBY* (Figure 12a-c). The purple color in some of the rosette leaves is commonly observed in the wild-type plants in the late reproductive stage or under stress conditions (Figure 10c) and is not related to the *pOLE1:RUBY* expression. On the other hand, no color change was observed in the *ttg1* background transformed with the *pOLE1:RUBY* (Figure 14d-f).

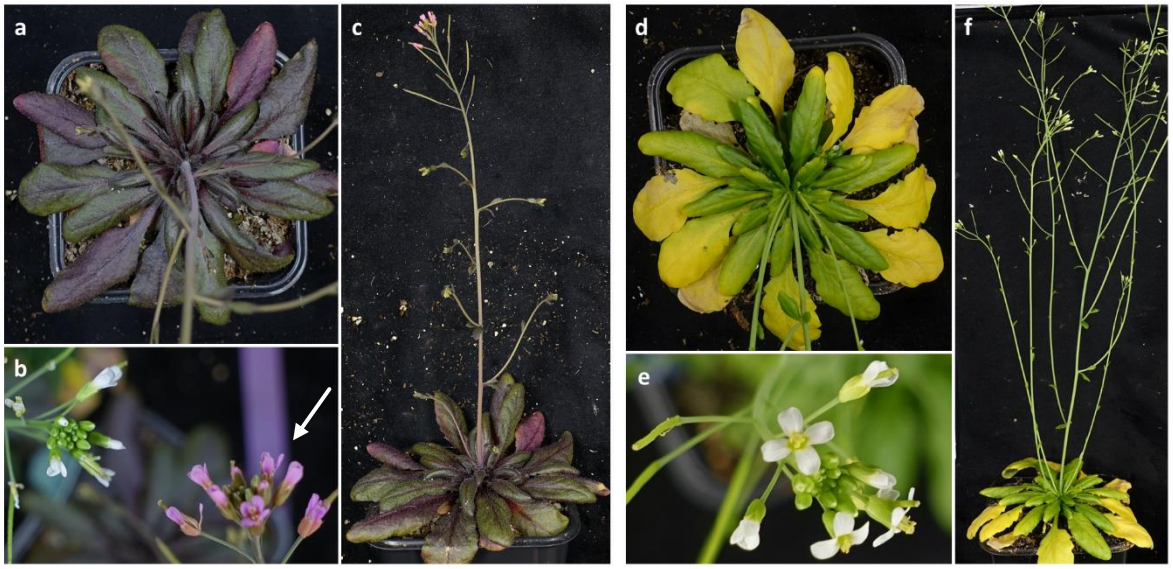

Figure 14. Images of plants with *pOLE:RUBY* insert. Seed-specific expression of RUBY was largely achieved in Col-0 (**a-c**) and the *ttg1* mutant (**d-f**) using the *OLEOSIN1* promoter since no substantial RUBY expression was observed in the mature plant tissues other than seeds, and there was only weak expression in the flowers of the transformed Col-0 (**b**). The arrow in **b** indicates flowers of *pOLE1:RUBY*/Col-0 and the plant on the left is wild type. Five transformants were planted and analyzed for each background.

To test whether the RUBY marker provides distinguishable features for different zygosity in T2 seeds, we examined seeds from plants with the *OLEOSIN1* promoter (three lines of *pOLE1:RUBY*/Col-0 and three lines of *pOLE1:RUBY*/ttg1). Most T2 seeds of the *pOLE1:RUBY* construct in both Col-0 and the *ttg1* background displayed a distinct dark-red coloration (Figure 15). Among the dark-red seeds, only one *ttg1* line exhibited differences in seed coloration (Figure 15c), while transgenic seeds in other lines of both backgrounds appeared almost homogeneous in color (Figure 15a-b).

Figure 15. **Second generation of seeds transformed with RUBY under seed-specific promoter**. **a** & **b**) Transgenic *pOLE1:RUBY* lines in Col-0 (**a**) and *ttg1* (**b**) backgrounds with homogeneous seed coloration. **c**) A T2 *pOLE1:RUBY/ttg1* line with noticeable differences in seed coloration.

3.2.3 Chromoprotein amilCP as a possible seed-selection marker

Various chromoproteins have been employed as efficient reporter genes for bacterial transformation (Liljeruhm *et al.*, 2018). One such example is amilCP, a chromoprotein isolated from *Acropora millepora* (Alieva *et al.*, 2008), which exhibits a distinct purple-blue color. This coloration enables easy selection of amilCP-expressing bacterial colonies (Tafoya-Ramirez *et al.*, 2018). In this study, we aimed to introduce amilCP into *Arabidopsis* to determine whether it could produce a noticeable change in seed color, thereby serving as a convenient seed-selection marker.

Constructs for ubiquitous (*pUBQ10:amilCP*) and seed-specific (*pOLE1:gOLE1-amilCP*) expression of the amilCP were transformed into Col-0 and *ttg1* plants. The amilCP was fused to OLEOSIN1 for specific accumulation in oil body membranes, similar to the FAST seed-selection marker (Shimada *et al.*, 2010). When T1 seeds were examined, no altered seed color was observed in the case of *pUBQ10:amilCP* in both backgrounds. However, a few purple seeds were found in the *pOLE1:gOLE1-amilCP* lines – four in Col-0 and twelve in the *ttg1* backgrounds (Figure 16).

Figure 16. Phenotype of T1 amilCP expressing seeds in Col-0 and *ttg1* backgrounds. Seeds were collected from Col-0 and *ttg1* plants transformed with *pOLE1:gOLE1-amilCP* for seed-specific expression of amilCP.

These purple seeds were stratified and sown on defined (Murashige & Skoog; MS) medium plates to promote better germination and growth. Only three seeds in the *ttg1* background germinated, while all four transformed Col-0 seeds failed to germinate.

The three *pOLE1:gOLE1-amilCP/ttg1* seedlings were transplanted to soil. Mature plants exhibited the same phenotype as the *ttg1* mutant (Figure 17a), and no unusual pigmentation was observed. T2 seeds were collected and examined. All three plants produced seeds with distinct purple color (Figure 17b).

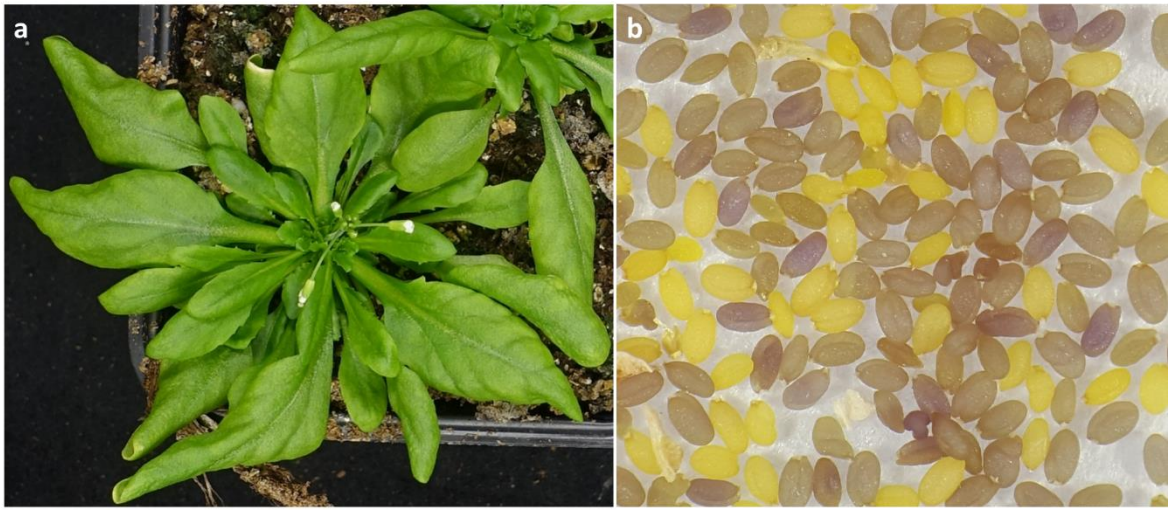

Figure 17. T1 *ttg1* plant with seed-specific expression of amilCP and its T2 seeds. a) Phenotype of mature *ttg1* mutant transformed with *pOLE1:gOLE1-amilCP*. **b**) Different seed colorations observed in the T2 generation collected from the plant shown in panel **a**.

3.2.4 Complementation of the *ttg1* mutant

The purpose of the following experiments was to investigate whether introduction of the *TTG1* gene could restore the natural brown seed color in the *ttg1* mutant. If successful, this technique could facilitate development of transgenic plants without requiring an exogeneous selection marker. This would involve transforming the *TTG1* gene alongside the gene of interest into the *ttg1* mutant and selecting the brown seeds. In this manner, transformed plants would exhibit a phenotype identical to wild type except for phenotypes caused by the gene of interest.

3.2.4.1 Transformation of *TTG1* gene with native regulatory components

First, we aimed to complement the *ttg1* mutant with the TTG1 coding sequence controlled by native regulatory sequences. Constructs with either shorter (1337 bp) or longer (1823 bp) *TTG1* promoter and the TTG1 coding sequence (*short pTTG1:TTG1, long pTTG1:TTG1*) were transformed into *ttg1* plants.

After obtaining and examining the T1 seeds, only a few small brown seeds were found (Figure 18a-b). The brown seeds were grown into mature plants, but they had glabrous leaves (Figure 18a-b) and lacked pigmentation during later stages of plant growth, similar to the *ttg1* plants (Figure 10f-h). Additionally, small light-brown seeds were discovered in unrelated T1 generation of *pUBQ10:amilCP/ttg1* (Figure 18c). Mature plants from these seeds also displayed *ttg1* phenotypes (Figure 18c). T2 seeds from plants derived from these brown

T1 seeds (Figure 18a-c) were gathered and all of them turned out to be yellow (not shown) as the *ttg1* seeds (Figure 10b).

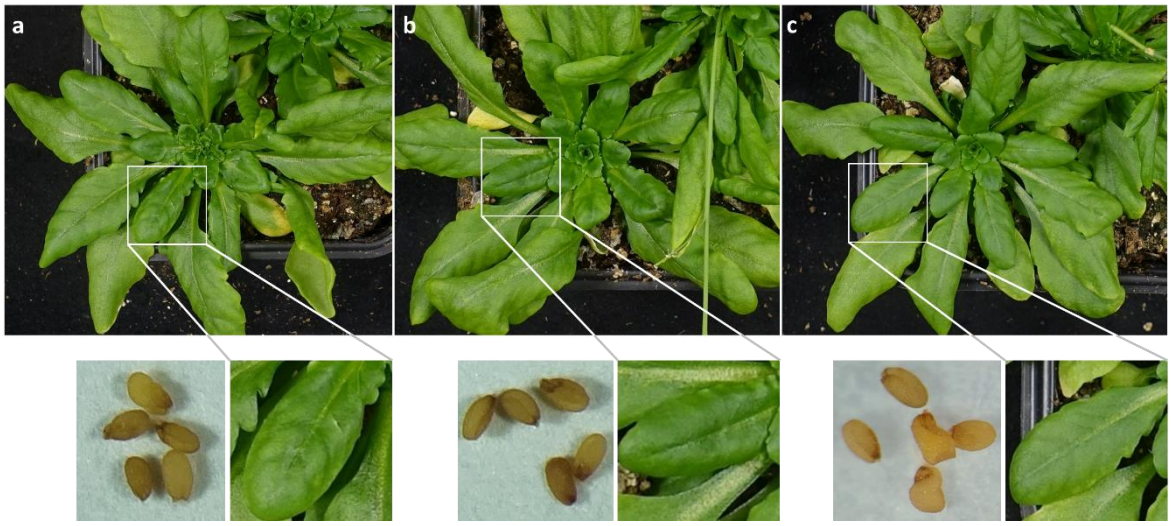

Figure 18. T1 seeds with altered coloration discovered in the pool of *short pTTG1:TTG*/ttg1 (**a**), *long pTTG1:TTG1/ttg1* (**b**), *pUBQ10:amilCP/ttg1* (**c**) lines, and their respective mature plants.

3.2.4.2 Overexpression of *TTG1* with *CaMV35S* promoter

Since transformation of the gene with two native promoter variants showed negative results, we reasoned that these promoters might not be fully functional and/or expression levels were not sufficient for phenotype complementation. We decided to overexpress *TTG1* using the *CaMV35S* promoter (*p35S:TTG1**). In addition, the FAST-R fluorescent marker was included for visual differentiation of transgenic seeds.

The *ttg1* plants were transformed with the constructs and seeds were collected and examined for their phenotype. Seeds showing red fluorescence did not exhibit any brown coloration and appeared red due to FAST-R expression (Figure 19a). Several red seeds were sown to observe the phenotype of mature plants. Surprisingly, all plants developed trichomes (Figure 19a), and after eight weeks, they exhibited pigmentation similar to the wild type (Figure 10c-e). A few seeds displayed a brown and small phenotype (Figure 19b-c), resembling T1 seeds observed in the *short/long pTTG1:TTG1/ttg1* (Figure 18a-b) and *pUBQ10:amilCP/ttg1* (Figure 18c) lines. Some of these brown seeds also showed FAST-R fluorescence (Figure 19b). While brown seeds with fluorescence developed into plants with trichomes (Figure 19b) and pigmentation in later stages similar to the wild type (Figure 10c-e), brown seeds without fluorescence developed into plants with glabrous leaves (Figure 19c) and also lacked wild-type-like pigmentation, resembling the *ttg1* mutant (Figure 10f-h).

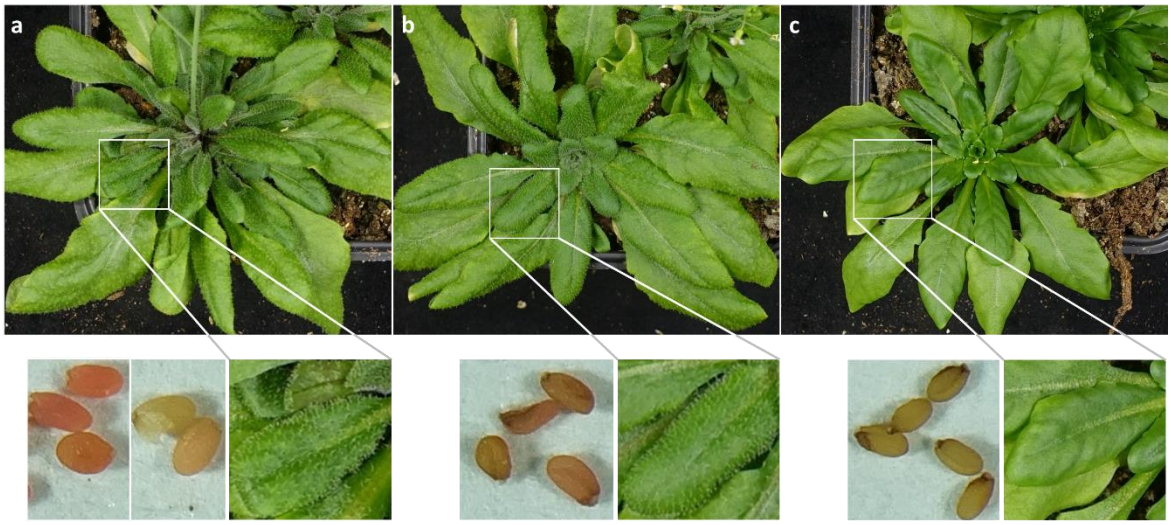

Figure 19. First generation of the *ttg1* seeds transformed with *p35S:TTG1 construct and resulting mature plants. a**) Yellow transformed seeds exhibiting different levels of FAST-R expression, as indicated by the intensity of red coloration. **b**) Brown seeds transformed with the *p35S:TTG1** construct exhibiting FAST-R fluorescence. **c**) Brown seeds from a *p35S:TTG1** transformed plant without any fluorescence.

3.3 DISCUSSION

3.3.1 The RUBY reporter can be used as a promising visual selection marker in dry seeds

Seeds expressing the RUBY marker exhibited a distinct dark-red color in both Col-0 and *ttg1* backgrounds (Figure 11 and 15), allowing for easy visual selection of transgenic dessicated seeds.

However, for the isolation of a homozygous line, it is preferable that the seed-selection marker provides a different phenotype for heterozygous and homozygous seeds in the T2 generation, such as the FAST marker (Shimada *et al.*, 2010), which displays stronger fluorescence intensity in homozygous seeds compared to heterozygous seeds. This feature enables quicker identification of homozygous seeds without the need to propagate numerous lines for a third generation to observe phenotype segregation. Unfortunately, the difference in pigment concentration between heterozygous and homozygous seeds was not distinct enough for convenient visual selection in the case of the p*OLE1:RUBY*-expressing seeds (Figure 15a-b). Additionally, the differences in seed coloration observed in Figure 15c, could be influenced by other variables, such as partial mRNA silencing, and may not correlate with zygosity. To draw a definitive conclusion, further analysis of additional lines in T3

generations is required. Experiments with other seed-specific promoters exhibiting different expression activities may also be promising for efficient seed-selection marker development.

Furthermore, the RUBY marker holds potential for visualizing expression in plant tissues as reported by He *et al.* (2020) and in this study (Figure 12). However, it is unclear why most plants with *p35S:RUBY* in the *ttg1* background were unable to produce betalain in vegetative tissues, either partially (Figure 13) or entirely. One possible explanation is that *TTG1* is involved in the regulation of tyrosine synthesis, which is required for betalain production. However, changes in tyrosine content or its precursors in *ttg1* or other flavonoid biosynthesis mutants have not been reported. Another explanation could be enhanced transgene silencing in the *ttg1* background. The RUBY marker, driven by the *CaMV35S* promoter, exhibits ubiquitous and strong expression, making it susceptible to posttranscriptional gene silencing. This type of silencing typically activates during plant development and resets after meiosis; therefore, the transgene was not silenced in seeds (Schubert *et al.*, 2004). Moreover, the *P19* silencing suppressor was not included in the *p35S:RUBY* construct (He *et al.* (2020); Figure 5), increasing the likelihood of silencing. Although only two T1 plants were analyzed in the Col-0 background (Figure 12a-c) as compared to nine plants in the *ttg1* background, we tend to believe that *TTG1*-dependent regulation may be linked to betalain synthesis, or lack of *TTG1* may trigger or enhance the silencing mechanism. The interesting observation that *pOLE1:RUBY/ttg1* transgenic plants did not show any accumulation of betalain in flowers, compared to the Col-0 background (Figure 14), where all flowers had noticeable pigmentation, may support this hypothesis.

3.3.2 The chromoprotein amilCP cannot serve as an efficient plant reporter due to its potential toxicity in plants

One potential limitation of RUBY is its large size. The open reading frame spans 4kb, excluding regulatory sequences, which may result in decreased transformation efficiency for larger vectors and T-DNAs. This issue can potentially be addressed by using chromoprotein markers, which have considerably shorter protein sequences.

The purple-blue amilCP has a coding sequence of 663 bp. If expressed well, this chromoprotein would produce easily distinguishable color in seeds. However, the amilCP appeared to be toxic to plants based on our results. As mentioned in the Materials and Methods section, we utilized amilCP, along with an antibiotic resistance marker, in *E. coli* as a dropout for blue & white screening in the cloning procedures. While antibiotic resistance of the colony indicates the presence of the plasmid inside the bacteria, the absence of purple-

blue color of the colony suggests that the chromoprotein gene has been exchanged for the gene of interest during the cloning process. The purple-blue amilCP-expressing colonies were consistently smaller compared to white colonies (data not shown), suggesting that amilCP is also toxic to bacteria in our setup, contradictory to the reported non-toxicity for *E. coli* by Tafoya-Ramirez *et al.* (2018). This toxicity could explain the absence of transgenic seeds in the *pUBQ10:amilCP* lines, as well as low transformation efficiency and germination rate in the *pOLE1:gOLE1-amilCP* lines. However, to exclude other possibilities of low transformation efficiency, several repeats of transformation should be performed, and the germination rates of T1 and T2 seeds should be analyzed for more conclusive assessment.

The clear non-homogeneous population of T2 seeds may be a potential advantage of this marker as a quantitative indicator based on the zygosity. In this study, seed coloration could be divided into roughly two groups (Figure 17b), supporting the possibility of differentiation between homozygous and heterozygous seeds. Further investigation of toxicity and the correlation of phenotype with zygosity in the T2 generation is needed to verify this issue.

3.3.3 *TTG1* as a potential endogenous selection marker to restore *ttg1* mutant phenotypes

One of the objectives of this study was to restore the dark-brown wild type seed color in the yellow *ttg1* seeds. This color transition would serve as an efficient visual indicator for successful transformation and, at the same time, other mutant traits such as glabrous leaves and abscence of anthocyanins would be also restored. However, we were unable to achieve this goal completely, as transformed T1 *ttg1* seeds with *p35:TTG1** retained their yellow color with red coloration from the FAST-R marker (Figure 19a). Although some brown seeds were observed in the T1 generation (Figure 19b-c), their coloration appears to be unrelated to the transgene, as some brown seeds did not exhibit FAST-R fluorescence. Furthermore, plants derived from these non-transgenic brown seeds lacked trichome development and anthocyanin synthesis (Figure 19c), similar to the *ttg1* mutant. Additionally, some seeds of the unrelated *pUBQ10:amilCP/ttg1* T1 also displayed brown coloration (Figure 18c). The most plausible explanation for these observations is natural variation in seed color, which is not linked to the *TTG1*-dependent pathway.

On the other hand, T1 plants derived from fluorescent yellow and brown seeds displayed wild-type phenotypes with trichomes and anthocyanin pigmentation (Figure 19a-b). Such partial complementation observed in transformants with *p35S:TTG1** presents an intriguing

result. The absence of brown seed color but presence of trichomes (Figure 19a) and anthocyanins in mature plants indicate that the introduced TTG1* coding sequence was functional and could be expressed, but not sufficient to restore the wild-type seed color. This might explain why seed pools from plants transformed with *TTG1* under the native promoters also lacked true brown seeds; some of them might have had the transgene, but it was not sufficient for seed color restoration.

Initially, it was difficult to explain these results. Studies reported the restoration of brown color by complementation of *TTG1* gene using not only *Arabidopsis* version of the gene but also homologues from other species (Walker *et al.*, 1999; Carey *et al.*, 2004; Humphries *et al.*, 2005; Wang *et al.*, 2016; Wang *et al.*, 2021). Nevertheless, it remains unclear whether this phenotype was present in the first generation, as most research was focused on stable T2 and T3 generations or the generation was not stated explicitly. Few studies specifically reported trichome development in T1 generation (Carey *et al.*, 2004; Humphries *et al.*, 2005).

Upon re-examining the *pTTG1:TTG1* constructs, it was discovered that the terminator sequence (*TTG1t*) had been improperly cloned. The genomic *TTG1* comprises two exons (Figure 20). The complete coding sequence of the protein and the first nucleotide of the 3' UTR are located within the first exon, while the second exon encodes the remainder of the 3' UTR. Insufficient attention was paid to this specific gene structure, leading to mistaking the intron following the first exon for the 3' UTR and the terminator (Figure 20).

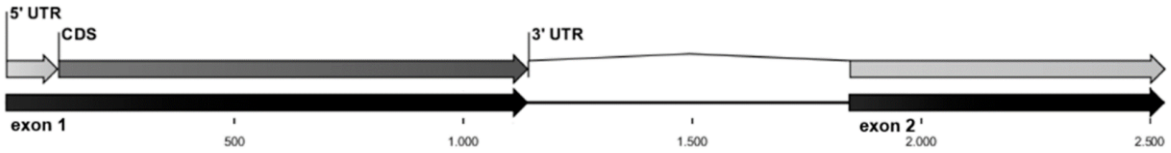

Figure 20. Genomic DNA sequence of *Arabidopsis TTG1*. The coding sequence of the protein resides entirely in the first exon, while the 3' UTR is primarily located in the 2nd exon, with one nucleotide in the first exon. Modified from: Zhang and Schrader (2017).

Without a proper 3' UTR and terminator, the efficiency of expression may be extremely low, if present at all. The transcribed mRNA can be excessively long, resulting in instability, and a partial intron sequence can lead to incorrect splicing or disrupted regulation (Friede *et al.*, 2017; Zhang and Schrader, 2017). Furthermore, lack of the 3' UTR also significantly reduces the stability of the mRNA (Mayr, 2019). Consequently, the absence of the complete intron, 3' UTR, and terminator sequences in our *pTTG1:TTG1* constructs could reduce the amount of functional TTG1 protein in the cells, preventing the restoration of seed color.

This hypothesis is supported by the *ttg1-10* mutant, which features a one-nucleotide substitution in the 5' UTR of the *TTG1* gene that generates a start codon, creating an additional upstream reading frame with nonsense codons (Larkin *et al.*, 1999). This nonsense reading frame may substantially reduce mRNA stability via nonsense-mediated mRNA decay. As a result of this mutation, the *TTG1* mRNA transcript level in *ttg1-10* is considerably lower than that in the wild type, implying a reduced TTG1 protein concentration level. The *ttg1-10* mutant produced yellow seeds, but its trichomes and anthocyanin synthesis were not significantly affected.

The *p35S:TTG1** with the *CaMV35S* terminator also may have provided low efficiency of expression during seed development, unable to restore seed coloration. However, it may have been sufficient for the recovery of anthocyanin synthesis and trichomes on leaves, as observed in the T1 plants (Figure 19a-b). It is known that the activity of *p35S* can vary among tissues, and also can be affected by developmental stages and growth conditions (Amack and Antunes, 2020). In particular, the activity has been reported to be lower in siliques and the inflorescence stems of *Arabidopsis* (Kiselev *et al.*, 2021) and in the embryos of cotton (Sunilkumar *et al.*, 2002).

Several expression assay studies further support these assumptions. It has been demonstrated that bHLH and R2R3-MYB transcription factors (Figure 3) can form dimers and activate expression of enzymes required for anthocyanin synthesis independently of TTG1 (Baudry *et al.*, 2004; Zimmermann *et al.*, 2004; Appelhagen *et al.*, 2010; Appelhagen *et al.*, 2011; Xu *et al.*, 2014; Zhang and Schrader, 2017). However, dimers of bHLH and TT2 (R2R3-MYB) (Figure 3), which can activate enzymes for proanthocyanidin synthesis responsible for the seed color, were largely unable to induce expression of several important target genes without TTG1. The addition of TTG1 to the bHLH-TT2 dimers significantly increased target gene expression, and hence the synthesis of anthocyanin in the older plants. These findings, along with the *ttg1-10* mutant, suggest that bHLH-TT2 may be less stable than other bHLH-R2R3-MYB dimers without the TTG1 (Tian and Wang, 2020). Therefore, with low levels of TTG1, the bHLH-TT2 dimers may be incapable to activate genes responsible for brown seed pigmentation. On the other hand, other bHLH-R2R3 MYB dimers involved in anthocyanin synthesis and trichome development can still interact and induce sufficient expression of target genes for visible phenotype (Payne *et al.*, 2000).

Overall, the potential of using *TTG1* as an endogenous seed-selection marker may still be valid provided that a full genomic *TTG1* clone is used for complementation. Moreover, it

may be promising to consider genes specifically involved in proanthocyanidin synthesis, such as the TT2 R2R3-MYB transcription factor (Figure 3). Loss-of-function mutants of *TT2* gene exhibit only altered seed color (Nesi *et al.*, 2001) without affecting any other traits, which would be ideal for our selection system.

3.3.4 Conclusions

In conclusion, the RUBY marker has demonstrated its immense value as a tool for selecting transgenic *Arabidopsis* seeds. The dark-brown coloration of desiccated Col-0 seeds poses no significant interference with the detection of betalain pigmentation. Our study, along with He et al. (2020), highlights the convenience of utilizing RUBY as a reporter gene for tissue-specific expression. However, the issue of RUBY silencing in vegetative tissues of *Arabidopsis*, especially in the *ttg1* mutant background, may be a substantial challenge. To ensure proper visualization of tissue-specific expression, it is crucial to carefully select a promoter that is both specific and not overly active to minimize the likelihood of silencing. Future research should focus on optimizing seed-specific promoters for RUBY expression, which may enable the differentiation of T2 seed genotypes based on betalain coloration intensity for convinient selection of homozygous lines.

Additionally, our study demonstrates that the 663 bp purple-blue chromoprotein *amilCP* gene can produce distinct seed color. Nevertheless, the primary limitation of using chromoprotein amilCP is its toxicity. Identifying and screening alternative chromoproteins with non-toxic properties for plants could lead to the development of efficient and compact visual seed-selection markers for future research.

Our attempt to complement the *TTG1* gene for use in a seed-selection system was unsuccessful, as *ttg1* mutants transformed with three constructs containing the TTG1 coding sequence retained their yellow seed color in the first generation. The probable deficiency in TTG1 protein levels prevented the restoration of the seed phenotype, but was sufficient for anthocyanin synthesis and trichome formation. Although our efforts to develop an endogenous marker from this gene were hindered in part due to a cloning error, our results underscore the significance of the 3' UTR and terminator sequences in proper *TTG1* gene expression.

SUMMARY

The generation of transgenic *Arabidopsis* plants is grealy dependent on the seed-selection marker being utilized. In this bachelor's thesis, we investigated different potential visual selection markers for their usability and convenience in genetic experiments.

The RUBY reporter comprises three enzymes that convert tyrosine into colorful betalain, changing the color of the targeted tissue to dark-red. The RUBY marker, driven by a ubiquitous *CaMV35S* (*p35S*) and a seed-specific *OLEOSIN1* promoter, was transformed into *Arabidopsis* Col-0 and *ttg1* plants to assess the diffrence between seed coloration of transformed and non-transformed seeds in the background of the dark-brown Col-0 and light-yellow *ttg1* seeds. Results in this study demonstrated that RUBY provided distinct color for transgenic seeds of both backgrounds, and the dark-brown color of desiccated Col-0 seeds did not interfere with betalain detection.

Chromoproteins are efficient reporter genes in bacterial transformations, providing a distinct color when expressed in bacteria. This study explored the potential use of small purple-blue chromoprotein amilCP as a seed-selection marker in plants. The amilCP, driven by the *Arabidopsis* ubiquitin 10 and *OLEOSIN1* promoters, was transformed and analyzed in two generations of transgenic plants. Although purple seeds distinct from non-transformed seeds were produced, the transformation efficiency and germination rate were low, indicating amilCP toxicity. Therefore, amilCP utilization as a selection marker in plants is limited. Identifying alternative, non-toxic chromoproteins could yield efficient and compact visual seed-selection markers for future research.

Lastly, an attempt was made to create an endogenous selection marker based on the TTG1 transcription regulator. The *ttg1* mutants lack proanthocyanidin production, resulting in yellow seeds instead of dark-brown. Including the *TTG1* gene in the T-DNA for transformation into *ttg1* plants could potentially restore seed color, indicating successful transformation and providing a convenient visual selection marker. However, transforming *ttg1* mutants with *TTG1*-containing constructs yielded no change in seed color in the first generation and insufficient TTG1 protein levels likely prevented seed phenotype restoration.

REFERENCES

Alieva N. O.; Konzen K. A.; Field S. F.; Meleshkevitch E. A.; Hunt M. E.; Beltran-Ramirez V.; Miller D. J.; Wiedenmann J.; Salih A.; Matz M. V. Diversity and Evolution of Coral Fluorescent Proteins. *Plos One*, 2008, vol. 3, no. 7.

Allan A. C.; Hellens R. P.; Laing W. A. MYB transcription factors that colour our fruit. *Trends in plant science*, 2008, vol. 13, no. 3, pp. 99-102.

Amack S. C.; Antunes M. S. CaMV35S promoter – A plant biology and biotechnology workhorse in the era of synthetic biology. *Current Plant Biology*, 2020, vol. 24, no.

Appelhagen I.; Huep G.; Lu G.-H.; Strompen G.; Weisshaar B.; Sagasser M. Weird fingers: functional analysis of WIP domain proteins. *FEBS letters*, 2010, vol. 584, no. 14, pp. 3116-3122.

Appelhagen I.; Jahns O.; Bartelniewoehner L.; Sagasser M.; Weisshaar B.; Stracke R. Leucoanthocyanidin dioxygenase in Arabidopsis thaliana: characterization of mutant alleles and regulation by MYB–BHLH–TTG1 transcription factor complexes. *Gene*, 2011, vol. 484, no. 1-2, pp. 61-68.

Baudry A.; Heim M. A.; Dubreucq B.; Caboche M.; Weisshaar B.; Lepiniec L. TT2, TT8, and TTG1 synergistically specify the expression of BANYULS and proanthocyanidin biosynthesis in Arabidopsis thaliana. *The Plant Journal*, 2004, vol. 39, no. 3, pp. 366-380.

Beck E.; Ludwig G.; Auerswald E. A.; Reiss B.; Schaller H. Nucleotide sequence and exact localization of the neomycin phosphotransferase gene from transposon Tn5. *Gene*, 1982, vol. 19, no. 3, pp. 327-336.

Berg D. E.; Davies J.; Allet B.; Rochaix J. D. Transposition of R factor genes to bacteriophage lambda. *Proc Natl Acad Sci U S A*, 1975, vol. 72, no. 9, pp. 3628-3632.

Bevan M. W.; Flavell R. B.; Chilton M.-D. A chimaeric antibiotic resistance gene as a selectable marker for plant cell transformation. *Nature*, 1983, vol. 304, no. 5922, pp. 184-187.

Carey C. C.; Strahle J. T.; Selinger D. A.; Chandler V. L. Mutations in the pale aleurone color1 regulatory gene of the Zea mays anthocyanin pathway have distinct phenotypes relative to the functionally similar TRANSPARENT TESTA GLABRA1 gene in Arabidopsis thaliana. *The Plant Cell*, 2004, vol. 16, no. 2, pp. 450-464.

Chaves-Silva S.; Dos Santos A. L.; Chalfun-Júnior A.; Zhao J.; Peres L. E.; Benedito V. A. Understanding the genetic regulation of anthocyanin biosynthesis in plants–tools for breeding purple varieties of fruits and vegetables. *Phytochemistry*, 2018, vol. 153, no. pp. 11-27.

Chawla H. S.; Cass L. A.; Simmonds J. A. Developmental and environmental regulation of anthocyanin pigmentation in wheat tissues transformed with anthocyanin regulatory genes. *In Vitro Cellular & Developmental Biology-Plant*, 1999, vol. 35, no. 5, pp. 403-408.

Chen J.-C.; Jiang C.-Z.; Gookin T.; Hunter D.; Clark D.; Reid M. Chalcone synthase as a reporter in virus-induced gene silencing studies of flower senescence. *Plant molecular biology*, 2004, vol. 55, no. pp. 521-530.

Clough S. J.; Bent A. F. Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. *Plant Journal*, 1998, vol. 16, no. 6, pp. 735-743.

De Block M. D.; Botterman J.; Vandewiele M.; Dockx J.; Thoen C.; Gossele V.; Movva N. R.; Thompson C.; Montagu M. V.; Leemans J. Engineering herbicide resistance in plants by expression of a detoxifying enzyme. *Embo Journal*, 1987, vol. 6, no. 9, pp. 2513-2518.

De Vos G.; Zambryski P. Expression of Agrobacterium nopaline-specific VirD1, VirD2, and VirC1 proteins and their requirement for T-strand production in E. coli. *Mol Plant Microbe Interact*, 1989, vol. 2, no. 2, pp. 43-52.

Debeaujon I.; Nesi N.; Perez P.; Devic M.; Grandjean O.; Caboche M.; Lepiniec L. Proanthocyanidin-accumulating cells in Arabidopsis testa: regulation of differentiation and role in seed development. *Plant Cell*, 2003, vol. 15, no. 11, pp. 2514-2531.

Doshi K.; Eudes F.; Laroche A.; Gaudet D. Anthocyanin expression in marker free transgenic wheat and triticale embryos. *In Vitro Cellular & Developmental Biology-Plant*, 2007, vol. 43, no. pp. 429-435.

Engler C.; Kandzia R.; Marillonnet S. A One Pot, One Step, Precision Cloning Method with High Throughput Capability. *Plos One*, 2008, vol. 3, no. 11.

Engler C.; Youles M.; Gruetzner R.; Ehnert T. M.; Werner S.; Jones J. D. G.; Patron N. J.; Marillonnet S. A Golden Gate Modular Cloning Toolbox for Plants. *Acs Synthetic Biology*, 2014, vol. 3, no. 11, pp. 839-843.

Eustice D. C.; Wilhelm J. M. Mechanisms of action of aminoglycoside antibiotics in eucaryotic protein synthesis. *Antimicrob Agents Chemother*, 1984, vol. 26, no. 1, pp. 53-60.

Fraley R. T.; Rogers S. G.; Horsch R. B.; Sanders P. R.; Flick J. S.; Adams S. P.; Bittner M. L.; Brand L. A.; Fink C. L.; Fry J. S.; Galluppi G. R.; Goldberg S. B.; Hoffmann N. L.; Woo S. C. Expression of bacterial genes in plant cells. *Proc Natl Acad Sci U S A*, 1983, vol. 80, no. 15, pp. 4803-4807.

Friede A.; Zhang B.; Herberth S.; Pesch M.; Schrader A.; Hülskamp M. The second intron is essential for the transcriptional control of the Arabidopsis thaliana GLABRA3 gene in leaves. *Frontiers in Plant Science*, 2017, vol. 8, no. pp. 1382.

Garabagi F.; Gilbert E.; Loos A.; McLean M. D.; Hall J. C. Utility of the P19 suppressor of gene-silencing protein for production of therapeutic antibodies in Nicotiana expression hosts. *Plant Biotechnology Journal*, 2012, vol. 10, no. 9, pp. 1118-1128.

Gelvin S. B. Agrobacterium-mediated plant transformation: the biology behind the "gene-jockeying" tool. *Microbiology and molecular biology reviews*, 2003, vol. 67, no. 1, pp. 16-37.

Gelvin S. B. Integration of Agrobacterium T-DNA into the Plant Genome. *Annual Review of Genetics, Vol 51*, 2017, vol. 51, no. pp. 195-217.

Gonzalez A.; Zhao M.; Leavitt J. M.; Lloyd A. M. Regulation of the anthocyanin biosynthetic pathway by the TTG1/bHLH/Myb transcriptional complex in Arabidopsis seedlings. *The Plant Journal*, 2008, vol. 53, no. 5, pp. 814-827.

Gould K. S.; Lister C. Flavonoid functions in plants. *Flavonoids: Chemistry, biochemistry and applications*, 2006, no. pp. 397-441.

Gritz L.; Davies J. Plasmid-encoded hygromycin B resistance: the sequence of hygromycin B phosphotransferase gene and its expression in Escherichia coli and Saccharomyces cerevisiae. *Gene*, 1983, vol. 25, no. 2-3, pp. 179-188.

Grotewold E. The genetics and biochemistry of floral pigments. *Annu Rev Plant Biol*, 2006, vol. 57, no. pp. 761-780.

He Y. B.; Zhang T.; Sun H.; Zhan H. D.; Zhao Y. D. A reporter for noninvasively monitoring gene expression and plant transformation. *Horticulture Research*, 2020, vol. 7, no. 1.

Heeb S.; Itoh Y.; Nishijyo T.; Schnider U.; Keel C.; Wade J.; Walsh U.; O'Gara F.; Haas D. Small, stable shuttle vectors based on the minimal pVS1 replicon for use in gram-negative, plant-associated bacteria. *Molecular Plant-Microbe Interactions*, 2000, vol. 13, no. 2, pp. 232-237.

Herreraestrella A.; Chen Z. M.; Vanmontagu M.; Wang K. Vird Proteins of Agrobacterium Tumefaciens Are Required for the Formation of a Covalent DNA Protein Complex at the 5' Terminus of T-Strand Molecules. *Embo Journal*, 1988, vol. 7, no. 13, pp. 4055-4062.

Herreraestrella A.; Vanmontagu M.; Wang K. A Bacterial Peptide Acting as a Plant Nuclear Targeting Signal - the Amino-Terminal Portion of Agrobacterium Vird2 Protein Directs a Beta-Galactosidase Fusion Protein into Tobacco Nuclei. *Proceedings of the National Academy of Sciences of the United States of America*, 1990, vol. 87, no. 24, pp. 9534-9537.

Hoekema A.; Hirsch P. R.; Hooykaas P. J. J.; Schilperoort R. A. A Binary Plant Vector Strategy Based on Separation of Vir-Region and T-Region of the Agrobacterium-Tumefaciens Ti-Plasmid. *Nature*, 1983, vol. 303, no. 5913, pp. 179-180.

Holsters M.; Silva B.; Vanvliet F.; Genetello C.; Deblock M.; Dhaese P.; Depicker A.; Inze D.; Engler G.; Villarroel R.; Vanmontagu M.; Schell J. The Functional-Organization of the Nopaline a Tumefaciens Plasmid Ptic58. *Plasmid*, 1980, vol. 3, no. 2, pp. 212-230.

Humphries J. A.; Walker A. R.; Timmis J. N.; Orford S. J. Two WD-repeat genes from cotton are functional homologues of the Arabidopsis thaliana TRANSPARENT TESTA GLABRA1 (TTG1) gene. *Plant molecular biology*, 2005, vol. 57, no. pp. 67-81.

Kaeppler H. F.; Carlson A. R.; Menon G. K. Routine utilization of green fluorescent protein as a visual selectable marker for cereal transformation. *In Vitro Cellular & Developmental Biology - Plant*, 2001, vol. 37, no. 2, pp. 120-126.

Kaeppler H. F.; Menon G. K.; Skadsen R. W.; Nuutila A. M.; Carlson A. R. Transgenic oat plants via visual selection of cells expressing green fluorescent protein. *Plant Cell Rep*, 2000, vol. 19, no. 7, pp. 661-666.

Kammar S. S.; Bhat A. R.; Hegde S.; Rajulu C.; Mohan T. C. Assessment of homozygosity in transgenic plants using selectable markers. *STAR Protoc*, 2023, vol. 4, no. 1, pp. 102031.

Kim C. Y.; Ahn Y. O.; Kim S. H.; Kim Y. H.; Lee H. S.; Catanach A. S.; Jacobs J. M.; Conner A. J.; Kwak S. S. The sweet potato IbMYB1 gene as a potential visible marker for sweet potato intragenic vector system. *Physiologia Plantarum*, 2010, vol. 139, no. 3, pp. 229-240.

Kim H. U.; Hsieh K.; Ratnayake C.; Huang A. H. C. A novel group of oleosins is present inside the pollen of Arabidopsis. *Journal of Biological Chemistry*, 2002, vol. 277, no. 25, pp. 22677-22684.

Kiselev K. V.; Aleynova O. A.; Ogneva Z. V.; Suprun A. R.; Dubrovina A. S. 35S promoter-driven transgenes are variably expressed in different organs of Arabidopsis thaliana and in response to abiotic stress. *Molecular Biology Reports*, 2021, vol. 48, no. 3, pp. 2235-2241.

Koncz C.; Schell J. The Promoter of Tl-DNA Gene 5 Controls the Tissue-Specific Expression of Chimeric Genes Carried by a Novel Type of Agrobacterium Binary Vector. *Molecular & General Genetics*, 1986, vol. 204, no. 3, pp. 383-396.

Kortstee A.; Khan S.; Helderman C.; Trindade L.; Wu Y.; Visser R.; Brendolise C.; Allan A.; Schouten H.; Jacobsen E. Anthocyanin production as a potential visual selection marker during plant transformation. *Transgenic research*, 2011, vol. 20, no. pp. 1253-1264.

Larkin J. C.; Walker J. D.; Bolognesi-Winfield A. C.; Gray J. C.; Walker A. R. Allele-specific interactions between ttg and gl1 during trichome development in Arabidopsis thaliana. *Genetics*, 1999, vol. 151, no. 4, pp. 1591-1604.

Liljeruhm J.; Funk S. K.; Tietscher S.; Edlund A. D.; Jamal S.; Wistrand-Yuen P.; Dyrhage K.; Gynna A.; Ivermark K.; Lovgren J.; Tornblom V.; Virtanen A.; Lundin E. R.; Wistrand-Yuen E.; Forster A. C. Engineering a palette of eukaryotic chromoproteins for bacterial synthetic biology. *J Biol Eng*, 2018, vol. 12, no. pp. 8.

Liu Z.; Chen O.; Wall J. B. J.; Zheng M.; Zhou Y.; Wang L.; Vaseghi H. R.; Qian L.; Liu J. Systematic comparison of 2A peptides for cloning multi-genes in a polycistronic vector. *Sci Rep*, 2017, vol. 7, no. 1, pp. 2193.

Lorenc-Kukuła K.; Jafra S.; Oszmiański J.; Szopa J. Ectopic Expression of Anthocyanin 5-O-Glucosyltransferase in Potato Tuber Causes Increased Resistance to Bacteria. *Journal of Agricultural and Food Chemistry*, 2005, vol. 53, no. 2, pp. 272-281.

Ludwig S. R.; Bowen B.; Beach L.; Wessler S. R. A regulatory gene as a novel visible marker for maize transformation. *Science*, 1990, vol. 247, no. 4941, pp. 449-450.

Mayr C. What are 3′ UTRs doing? *Cold Spring Harbor perspectives in biology*, 2019, vol. 11, no. 10, pp. a034728.

McLean P. J.; Kawamata H.; Hyman B. T. alpha-synuclein-enhanced green fluorescent protein fusion proteins form proteasome sensitive inclusions in primary neurons. *Neuroscience*, 2001, vol. 104, no. 3, pp. 901-912.

Merzlyak E. M.; Goedhart J.; Shcherbo D.; Bulina M. E.; Shcheglov A. S.; Fradkov A. F.; Gaintzeva A.; Lukyanov K. A.; Lukyanov S.; Gadella T. W.; Chudakov D. M. Bright monomeric red fluorescent protein with an extended fluorescence lifetime. *Nat Methods*, 2007, vol. 4, no. 7, pp. 555-557.

Moazed D.; Noller H. F. Interaction of antibiotics with functional sites in 16S ribosomal RNA. *Nature*, 1987, vol. 327, no. 6121, pp. 389-394.

Nesi N.; Jond C.; Debeaujon I.; Caboche M.; Lepiniec L. The Arabidopsis TT2 gene encodes an R2R3 MYB domain protein that acts as a key determinant for proanthocyanidin accumulation in developing seed. *Plant Cell*, 2001, vol. 13, no. 9, pp. 2099-2114.

Nishizawa K.; Kita Y.; Kitayama M.; Ishimoto M. A red fluorescent protein, DsRed2, as a visual reporter for transient expression and stable transformation in soybean. *Plant Cell Reports*, 2006, vol. 25, no. 12, pp. 1355-1361.

Orzaez D.; Medina A.; Torre S.; Fernández-Moreno J. P.; Rambla J. L.; Fernández-del-Carmen A.; Butelli E.; Martin C.; Granell A. A visual reporter system for virus-induced gene silencing in tomato fruit based on anthocyanin accumulation. *Plant physiology*, 2009, vol. 150, no. 3, pp. 1122-1134.

Payne C. T.; Zhang F.; Lloyd A. M. GL3 encodes a bHLH protein that regulates trichome development in Arabidopsis through interaction with GL1 and TTG1. *Genetics*, 2000, vol. 156, no. 3, pp. 1349-1362.

Petroni K.; Tonelli C. Recent advances on the regulation of anthocyanin synthesis in reproductive organs. *Plant science*, 2011, vol. 181, no. 3, pp. 219-229.

Polturak G.; Aharoni A. Advances and future directions in betalain metabolic engineering. *New Phytol*, 2019, vol. 224, no. 4, pp. 1472-1478.

Potrykus I. Gene Transfer to Plants: Assessment of Published Approaches and Results. *Annual Review of Plant Physiology and Plant Molecular Biology*, 1991, vol. 42, no. 1, pp. 205-225.

Rosellini D. Selectable Markers and Reporter Genes: A Well Furnished Toolbox for Plant Science and Genetic Engineering. *Critical Reviews in Plant Sciences*, 2012, vol. 31, no. 5, pp. 401-453.

Sarma A. D.; Sharma R. Anthocyanin-DNA copigmentation complex: mutual protection against oxidative damage. *Phytochemistry*, 1999, vol. 52, no. 7, pp. 1313-1318.

Schiefelbein J. Cell-fate specification in the epidermis: a common patterning mechanism in the root and shoot. *Current opinion in plant biology*, 2003, vol. 6, no. 1, pp. 74-78.

Schiefelbein J.; Huang L.; Zheng X. Regulation of epidermal cell fate in Arabidopsis roots: the importance of multiple feedback loops. *Frontiers in Plant Science*, 2014, vol. 5, no. pp. 47.

Schubert D.; Lechtenberg B.; Forsbach A.; Gils M.; Bahadur S.; Schmidt R. Silencing in Arabidopsis T-DNA transformants: the predominant role of a gene-specific RNA sensing mechanism versus position effects. *Plant Cell*, 2004, vol. 16, no. 10, pp. 2561-2572.

Shaked H.; Melamed-Bessudo C.; Levy A. A. High-frequency gene targeting in Arabidopsis plants expressing the yeast RAD54 gene. *Proc Natl Acad Sci U S A*, 2005, vol. 102, no. 34, pp. 12265-12269.

Shen L. Y.; Petolino J. F. Pigmented maize seed via tissue-specific expression of anthocyanin pathway gene transcription factors. *Molecular Breeding*, 2006, vol. 18, no. pp. 57-67.

Shimada T. L.; Shimada T.; Hara-Nishimura I. A rapid and non-destructive screenable marker, FAST, for identifying transformed seeds of Arabidopsis thaliana. *Plant Journal*, 2010, vol. 61, no. 3, pp. 519-528.

Stewart C. N., Jr. Monitoring the presence and expression of transgenes in living plants. *Trends Plant Sci*, 2005, vol. 10, no. 8, pp. 390-396.

Strack D.; Vogt T.; Schliemann W. Recent advances in betalain research. *Phytochemistry*, 2003, vol. 62, no. 3, pp. 247-269.

Sunilkumar G.; Mohr L.; Lopata-Finch E.; Emani C.; Rathore K. S. Developmental and tissue-specific expression of CaMV 35S promoter in cotton as revealed by GFP. *Plant molecular biology*, 2002, vol. 50, no. 3, pp. 463-479.

Tafoya-Ramirez M. D.; Padilla-Vaca F.; Ramirez-Saldana A. P.; Mora-Garduno J. D.; Rangel-Serrano A.; Vargas-Maya N. I.; Herrera-Gutierrez L. J.; Franco B. Replacing Standard Reporters from Molecular Cloning Plasmids with Chromoproteins for Positive Clone Selection. *Molecules*, 2018, vol. 23, no. 6.

Tian H.; Wang S. TRANSPARENT TESTA GLABRA1, a key regulator in plants with multiple roles and multiple function mechanisms. *International Journal of Molecular Sciences*, 2020, vol. 21, no. 14, pp. 4881.

Walker A. R.; Davison P. A.; Bolognesi-Winfield A. C.; James C. M.; Srinivasan N.; Blundell T. L.; Esch J. J.; Marks M. D.; Gray J. C. The TRANSPARENT TESTA GLABRA1 locus, which regulates trichome differentiation and anthocyanin biosynthesis in

Arabidopsis, encodes a WD40 repeat protein. *The Plant Cell*, 1999, vol. 11, no. 7, pp. 1337-1349.

Wang J.; Chen H. A novel CRISPR/Cas9 system for efficiently generating Cas9-free multiplex mutants in Arabidopsis. *aBIOTECH*, 2020, vol. 1, no. 1, pp. 6-14.

Wang P.; Zhang J.; Sun L.; Ma Y.; Xu J.; Liang S.; Deng J.; Tan J.; Zhang Q.; Tu L.; Daniell H.; Jin S.; Zhang X. High efficient multisites genome editing in allotetraploid cotton (Gossypium hirsutum) using CRISPR/Cas9 system. *Plant Biotechnology Journal*, 2018, vol. 16, no. 1, pp. 137-150.

Wang W.; Zhang Q.; Guo D. An artemisia WD40-repeat gene regulates multiple cellular functions in Arabidopsis. *Journal of Biosciences and Medicines*, 2016, vol. 4, no. 05, pp. 30.

Wang Y.; Tian H.; Wang W.; Wang X.; Zheng K.; Hussain S.; Lin R.; Wang T.; Wang S. The carboxyl-terminus of TRANSPARENT TESTA GLABRA1 is critical for its functions in Arabidopsis. *International Journal of Molecular Sciences*, 2021, vol. 22, no. 18, pp. 10039.

Wang Z. P.; Xing H. L.; Dong L.; Zhang H. Y.; Han C. Y.; Wang X. C.; Chen Q. J. Egg cell-specific promoter-controlled CRISPR/Cas9 efficiently generates homozygous mutants for multiple target genes in Arabidopsis in a single generation. *Genome Biology*, 2015, vol. 16, no.

Ward E. R.; Barnes W. M. Vird2 Protein of Agrobacterium-Tumefaciens Very Tightly Linked to the 5' End of T-Strand DNA. *Science*, 1988, vol. 242, no. 4880, pp. 927-930.

Wenck A.; Pugieux C.; Turner M.; Dunn M.; Stacy C.; Tiozzo A.; Dunder E.; van Grinsven E.; Khan R.; Sigareva M.; Wang W. C.; Reed J.; Drayton P.; Oliver D.; Trafford H.; Legris G.; Rushton H.; Tayab S.; Launis K.; Chang Y. F.; Chen D. F.; Melchers L. Reef-coral proteins as visual, non-destructive reporters for plant transformation. *Plant Cell Rep*, 2003, vol. 22, no. 4, pp. 244-251.

Xu J.; Yin Y.; Jian L.; Han B.; Chen Z.; Yan J.; Liu X. Seeing is believing: a visualization toolbox to enhance selection efficiency in maize genome editing. *Plant Biotechnology Journal*, 2021, vol. 19, no. 5, pp. 872-874.

Xu J. J.; Fang X.; Li C. Y.; Yang L.; Chen X. Y. General and specialized tyrosine metabolism pathways in plants. *aBIOTECH*, 2020, vol. 1, no. 2, pp. 97-105.

Xu W.; Dubos C.; Lepiniec L. Transcriptional control of flavonoid biosynthesis by MYB-bHLH-WDR complexes. *Trends Plant Sci*, 2015, vol. 20, no. 3, pp. 176-185.

Xu W.; Lepiniec L.; Dubos C. New insights toward the transcriptional engineering of proanthocyanidin biosynthesis. *Plant signaling & behavior*, 2014, vol. 9, no. 6, pp. e28736.

Yadav N. S.; Vanderleyden J.; Bennett D. R.; Barnes W. M.; Chilton M. D. Short Direct Repeats Flank the T-DNA on a Nopaline Ti Plasmid. *Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences*, 1982, vol. 79, no. 20, pp. 6322-6326.

Zhang B.; Schrader A. TRANSPARENT TESTA GLABRA 1-Dependent Regulation of Flavonoid Biosynthesis. *Plants (Basel)*, 2017, vol. 6, no. 4.

Zimmermann I. M.; Heim M. A.; Weisshaar B.; Uhrig J. F. Comprehensive identification of Arabidopsis thaliana MYB transcription factors interacting with R/B-like BHLH proteins. *The Plant Journal*, 2004, vol. 40, no. 1, pp. 22-34.

NON-EXCLUSIVE LICENCE TO REPRODUCE THESIS AND MAKE THESIS PUBLIC

I, Oleksii Yadlos

1. herewith grant the University of Tartu a free permit (non-exclusive licence) to

reproduce, for the purpose of preservation, including for adding to the DSpace digital archives until the expiry of the term of copyright,

“Development of visual selection markers for plant transformation”

supervised by Associate Professor Yuh-Shuh Wang.

2. I grant the University of Tartu a permit to make the work specified in p. 1 available to the public via the web environment of the University of Tartu, including via the DSpace digital archives, under the Creative Commons licence CC BY NC ND 3.0, which allows, by giving appropriate credit to the author, to reproduce, distribute the work and communicate it to the public, and prohibits the creation of derivative works and any commercial use of the work until the expiry of the term of copyright.

3. I am aware of the fact that the author retains the rights specified in p. 1 and 2.

4. I certify that granting the non-exclusive licence does not infringe other persons’ intellectual property rights or rights arising from the personal data protection legislation.

Oleksii Yadlos

24/05/2023