DEVELOPMENT OF A GENETIC TRANSFORMATION SYSTEM OF RASPBERRY CULTIVARS FOR GENE FUNCTION ANALYSIS

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

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ABSTRACT

An *Agrobacterium*-mediated transformation system of purple raspberry ‘Amethyst’ was established after a series of experiments that determined the effect of genotype, inoculum density, and co-cultivation time on transformation. In this study, a plant regeneration protocol was established for ‘Joan J’ and ‘Polana’ (the regeneration protocol of ‘Amethyst’ was previously developed). *Agrobacterium*-mediated transformation was conducted for all three cultivars. The minimum killing level of hygromycin B and kanamycin was determined. Inoculum density and co-cultivation time were optimized. Polymerase chain reaction (PCR) verified a successful transformation of ‘Amethyst’ with the frequency of 3.3 ~ 4.4 % when leaves were infected with *Agrobacterium* EHA105 at the cell density of OD_{600} 0.3 and co-cultivated for 3 days in the medium with 25.0 $mg \cdot l^{-1}$ kanamycin. Transgenic lines with the *PtFIT* gene were hydroponically grown under iron sufficiency or deficiency. The real-time quantitative PCR verified the gene expression in response to iron sufficiency and deficiency conditions.

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DEDICATION

I am dedicating this thesis to my wife, Jeehye Kim, for her unconditional support, advice, patience, and love, which always encourage me, to my parents-in-law for their understanding and encouragement, and lastly to my father, Jeong-Su Kim, mother, Bok-Soon Ji, and sister, Hyang-Sil Kim, for their unchanging support, love, sacrifice, advice, and trust in me throughout my life with my infinite respect.

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LIST OF ABBREVIATIONS

AFLP.....Amplified fragment length polymorphism

AS .....Acetosyringone

BA.....6-benzylaminopurin

bHLH.....*basic helix-loop-helix*

CaMV.....Cauliflower mosaic virus

Carb.....Carbenicillin

Cef.....Cefotaxime

CK.....Cytokinin

CRD .....Completely randomized design

C_t value .....Threshold cycle value

DNA.....Deoxyribonucleic acid

EDTA.....Ethylenediaminetetraacetic acid

EtBr.....Ethidium bromide

FAO.....Food and Agriculture Organization

FRO.....*Ferric reduction oxidase*

GA_3 .....Gibberellic acid

GAPDH.....Glyceraldehyde 3-phosphate dehydrogenase

GFP.....Green fluorescent protein

GUS.....β-glucuronidase

hpt .....Hygromycin B phosphotransferase

IBA.....Indolebutyric acid

IRT.....*Iron-regulated transporter*

LB medium .....Luria-Bertani medium

LSD.....Least Significant Difference

MS medium.....Murashige and Skoog medium

NAA.....1-naphthaleneacetic acid

nos.....Nopaline synthase

nptII.....Neomycin phosphotransferase II

PCR.....Polymerase chain reaction

PGR.....Plant growth regulator

pro.....Promoter

PtFIT.....*FER-LIKE Iron Deficiency-Induced Transcription Factor 1* cloned from *Populus tremula*

PVP.....Polyvinylpyrrolidone

RAPD.....Random amplified polymorphic DNA

RNA.....Ribonucleic acid

RT-PCR.....Real-time quantitative PCR

SNP.....Single nucleotide polymorphism

SSR.....Simple sequence repeats

TDZ.....Thidiazuron

ter.....Terminator

Ti-plasmid.....Tumor inducing plasmid

UV light.....Ultraviolet light

Vir genes.....Virulence genes

WPM.....Woody plant medium

CHAPTER I. GENERAL INTRODUCTION

Raspberries are considered a high-value crop throughout history because of their unique flavor, nutritional value in human health, and importance for studies of *Rubus* genus (Buricova et al., 2011; Graham and Jennings, 2009; Jennings, 1988; Pritts, 2016). With the increasing demand of this small fruit in the U.S., conventional breeding approaches have been attempted to improve performance of this small fruits (FAO, 2016; Hall et al., 2009). Although there are few reports of success in breeding, these approaches resulted in a limited genetic diversity and increased susceptibility to a virus infection (Hall et al., 2009; Jennings, 1988). Additionally, the complicated genetic background and perennial nature of raspberry make the conventional breeding difficult for the improvement (Graham and Woodhead, 2009). To cope with problems, researchers have focused on plant biotechnology, which is not only a novel approach but also fundamental for molecular and genetic research (Aldwinckle and Malnoy, 2009; Birch, 1997; Trigiano and Gray, 2011). However, only limited numbers of studies for *Rubus* species are available in the area of plant biotechnology (Aldwinckle and Malnoy, 2009; Hall et al., 2009).

Establishment of an efficient in vitro regeneration is a prerequisite for study of plant biotechnologies in woody plant species since in vitro regeneration has been used for novel trait selection, germplasm preservation, and genetic transformation (Dhar and Joshi, 2005; Huetteman and Preece, 1993; Trigiano and Gray, 2011). Plant regeneration frequency are significantly influenced by explant types, age of explants, supplemented plant growth regulators (PGRs), and genotypes (Birch, 1997; George et al., 2008; Makunga et al., 2005; Trigiano and Gray, 2011). To optimize a plant regeneration protocol, the influence of these factors should be investigated. The previous micropropagation studies of raspberry species are mostly dealt with the effect of PGRs. However, the results showed inefficient in plant regeneration with the non-consistent

regeneration rate (Millan-Mendoza and Graham, 1999). Additionally, limited numbers of cultivars have attempt to develop their own regeneration protocol and there are no studies about an influence of explant ages (Aldwinckle and Malnoy, 2009; Pelto and Clark, 2001).

Agrobacterium-mediated transformation is also important in performance improvement and study of a gene function in raspberry (Birch, 1997; Faria, 1993; Graham et al., 1990; Mathews et al., 1995). The transformation system can be optimized by an evaluation of transformation frequency influenced by in vitro regeneration system, selection systems, inoculum densities and co-cultivation times (Birch, 1997). Yet, only four cultivars of raspberry had been studied to establish *Agrobacterium*-mediated transformation with the instable frequencies (Faria, 1993; Graham et al., 1990; Mathews et al., 1995). Furthermore, there is no precise studies of identification of an influence of these factors, which are important in an optimization of transformation (Aldwinckle and Malnoy, 2009; Birch, 1997).

The objectives of this study were to develop and optimize an efficient transformation system by *Agrobacterium*-mediated transformation with three raspberry 'Amethyst', 'Joan J', and 'Polana'. Prior to transformation, in vitro regeneration methods will be developed for 'Joan J' and 'Polana' by investigating an influence of PGRs, explant types, explant ages on the shoot and root regeneration. Following the establishment of micropropagation protocol and the result of Lenz et al. (2016), *Agrobacterium*-mediated transformation will be optimized for the three raspberry cultivars based on an evaluation of selection systems, inoculum densities, and co-cultivation times. With confirmed transgenic lines by PCR analysis, an expression level of the *PtFIT* gene under iron sufficient and deficient conditions will be characterized to alleviate iron chlorosis within raspberry species. Consequently, an established transformation system could

improve performance of raspberries as a practical tool and extend the understanding of physiological characteristics in *Rubus* genus as a research tool.

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CHAPTER II. LITERATURE REVIEW

Characteristics of Raspberry

The raspberry

Raspberries (*Rubus* spp.) are perennial woody species belonging to the subgenus *Idaeobatus* of the genus *Rubus* in the Rosaceae family (Graham and Jennings, 2009; Hall et al., 2009; Jennings, 1988). Raspberries have two years of lifespan for woody shoots (canes) on a perennial crown and underground root system (Graham and Jennings, 2009). The growth cycle of raspberries includes dormancy breaking, vegetative growth, flower initiation, flowering, and fruiting (Heide and Sønsteby, 2011). After the breaking of dormancy, the crown and root develop primocanes (first-year canes), which become floricanes in the first or second year of growth cycle (Smith et al., 2007). These floricanes produce flowers (0.5 – 1.5 cm in size) with five sepals and petals including one short hypanthium and a gynoecium (Graham and Jennings, 2009). The pollinated flowers develop fruits, which are aggregations of drupelets from ovaries in a same flower, within 30 to 36 days following the three developmental stages: exponential growth, slow growth, and fruit ripening (Gotame, 2014; Graham and Jennings, 2009; Hall et al., 2009; Jennings, 1988).

Raspberries are classified into two groups based on their flowering and fruiting habits: primocanes (annual-fruiting) and floricanes (biennial-fruiting) (Gotame, 2014; Heide and Sønsteby, 2011). Primocanes complete their growth cycle including dormancy release, vegetative growth, flower initiation, and fruiting within one year (Heide and Sønsteby, 2011). Primocanes have two major fruiting periods in early August and late September because of different timing of new canes production (Heide and Sønsteby, 2011). On the other hand, floricanes undergo a two-year growth cycle. In the first year, canes developed from the crown

and root system continue vegetative growth until late summer (Gotame, 2014). In the spring of the second year, the canes release dormancy and elongate their shoots again (Heide and Sønsteby, 2011). Floricanes initiate flowers in early June and produce most of the fruit in late summer on the second year cane (Heide and Sønsteby, 2011). Once the canes completed flowering and fruiting within their life cycle, the shoots of both types of canes finish their lifecycle in the winter (Heide and Sønsteby, 2011). Both floricane and primocane raspberries repeatedly grow new canes from a crown or root suckers. The major differences between the two groups are that primocanes have many canes but shorter height (1 – 1.8 m) than floricanes (2.5 – 3 m) (Jennings, 1988). These distinctions in growth habits between raspberries suggest that two major types can be distinguished based on flowering timing, which caused by differential responses to the day length and temperature for flowering among these cultivars (Gotame, 2014). Based on the fruit color, raspberries can also be classified into red raspberries (*R. idaeus*), black raspberries (*R. occidentalis*), and purple raspberries (hybrid between red and black raspberries), which are explained by the early domestication described in Jennings (1988).

Origin, domestication, and commercialization of raspberries

Raspberries are widely occurred around the temperate regions of North America, Europe, and Asia, especially in the northern hemisphere (Jennings, 1988). Although up to 200 species of the subgenus *Idaeobatus* are characterized, only a few species, mostly European red raspberries (*R. idaeus* L. subsp. *idaeus*), North American red raspberries (*R. idaeus* subsp. *strigosus* Michx) and black raspberries (*R. occidentalis* L.) were domesticated and considered as commercially important (Jennings, 1988; Pritts, 2016). The Ide Mountains of Turkey are considered as the place of origin for the raspberries and East Asia is the center of germplasm diversity (Graham and Jennings, 2009; Jennings, 1988). In the Ancient Greek history, Cato, the Pliny the Elder,

wrote the name of raspberries in Greeks (they called it as “ida” fruit) and collected the fruits from wild about 45 A.D. (Crandall, 1995; Jennings, 1988). However, description of raspberries could not be found from 45 A.D. to the 16th century (Jennings, 1988). After the 16th century, raspberries have been described in literature about the taste, usage, cultivation conditions, and lists of cultivated varieties (Jennings, 1988). These historical records suggest that some wild raspberries were successfully domesticated during the past 400 – 500 years and it has undergone dramatic change of their genetic background due to relatively late domestication than other domesticated crops such as bean and wheat (Jennings, 1988).

After the domestication, raspberries became commercialized with multi-purposes since it was well known as a medicine and an expensive fruit for the upper class (Jennings, 1988). Cultivation of raspberries in Europe was well characterized in the many gardening books and the first sale of raspberry seedling in North America was recorded in 1771 with the description of 20 cultivars (Jennings, 1988; Pritts, 2016). In the Europe and North America, many enthusiastic gardeners made an effort to have fruit quality and productivity with native plants in the 19th and early 20th century, which led to the dramatic increase in harvesting areas in North America (Jennings, 1988; Pritts, 2016). Jennings (1988) and Graham and Woodhead (2009) described the dominant five parent cultivars of red raspberries; ‘Lloyd George’, ‘Pynes Royal’, ‘Preussen’, ‘Cuthbert’, and ‘Newburgh’.

These earlier attempts on the improvement of raspberries were considered very successful, but it brought a degeneration on virus infection because of the narrowed genetic background (Hall et al., 2009; Jennings, 1988). In addition, the softness of raspberry fruit became an obstacle to be delivered to a distant market or processed for multi-purposes (Jennings, 1988). These problems led breeders to develop hybrids between European and American

cultivars to overcome the limited genetic diversity with an improvement of raspberries (Jennings, 1988; Pritts, 2016). Recently, 22 countries in North America, East Asia, and Europe have run 45 raspberry breeding programs in order to develop new cultivars with the improved key traits such as fruit quality, yield, fruit hardness, color, and disease resistance (Graham and Jennings, 2009; Hall et al., 2009). These programs have improved the performance of raspberries through conventional breeding and molecular genetic approaches with the identification of their molecular characteristics (Georgiva et al., 2008; Graham and Woodhead, 2009; Hall et al., 2009; Hyun et al., 2014; Longhi et al., 2014).

Raspberry production in the world has continuously increased for the past 15 years as a result of increased demand (FAO, 2016). Raspberries are often consumed in fresh, frozen, jam, juice, and pastry in the market (Crandall, 1995). The popularity of raspberry has increased because of positive effects on lowering risks of many chronic diseases such as cancer and heart disease (Buricova et al., 2011; Hall et al., 2009). Raspberries contain a high level of polyphenolic compounds (antioxidants) within their fruit and leaves and the ratio of antioxidants determines the color of fruit (Buricova et al., 2011; Jennings, 1988). These polyphenols, including anthocyanins and flavonols, have positive effects on human diet and health since they work as the medicinally active phytochemicals after consumption (Hyun et al., 2014). Raspberries are primarily produced in Russia, Europe and North America (particularly in Washington and Oregon) and the harvesting areas have been extended with the increased production of raspberries (FAO, 2016; Pritts, 2016). Especially, the production in North America has increased to 148,621 tons in 2016, which are doubled from 66,517 tons in 2002 (FAO, 2016). Although the yield and harvesting areas of the North America ware ranked the third largest in the world, the

report indicates that the production of raspberries will be a promising agricultural industry in the United States with the high demand from consumers.

Molecular and genetic characteristics of raspberries

Successfully domesticated raspberries (red and black raspberries) are diploid (2n = 2x =14) with a very small sized genome (275 Mbp), which makes it attractive for molecular genetics research as a model species of Rosaceae family (Graham and Woodhead, 2009). For understanding of the genetic characteristics of raspberries, researchers have developed molecular markers such as RAPD, AFLP, SSR, EST-SSR, and SNP markers to diagnosis a function of gene on morphological variations of raspberries with high rate of transferability between raspberry accessions (Graham et al., 2004; Graham and Woodhead, 2009; Longhi et al., 2014; VanBuren et al., 2016). The phylogenetic studies with the developed markers revealed that the genetic diversity within the commercialized species was very homozygous and a narrow genetic base because of the domestication with the five dominant cultivars (Graham and Woodhead, 2009; Longhi et al., 2014). The first genetic linkage map of red raspberries was published in early 2004 by Graham et al. (2004), which was the relatively late compared to other species in Rosaceae family (Longhi et al., 2014). Based on the first linkage map, Ward et al. (2013) developed the high-density linkage map of red raspberries using Genotyping by Sequencing to develop a genomic selection strategy for improvement of raspberries. With the advance of sequencing technology, Hyun et al. (2014) performed the first RNA sequencing of 'Nova' fruit for the characterization of molecular mechanism related to the ripening process and phytochemical synthesis, which characterized the 42,604 unigenes from the *de novo* assembly. Recently, the draft genome of black raspberry (*Rubus occidentalis* L.) was reported with the identification of 28,005 protein-coding genes by VanBuren et al. (2016) and Jibran et al. (2018) to accelerate

marker-assisted breeding programs. Although the genome of black raspberries can be utilized as a reference for studies in many raspberry cultivars, the sequencing is not completed and limited in only a few accessions of black raspberries (Jibran et al., 2018; VanBuren et al., 2016). Therefore, further studies for other raspberry cultivars are required to improve performance and yield of raspberries based on approach of plant biotechnology.

Plant Genetic Transformation

Plant genetic transformation is a process to transfer foreign DNA into a plant genome with the expression of the inserted gene within the explant (Trigiano and Gray, 2011). By introducing a foreign gene, the plant genome undergoes the genetic alterations that cannot be acquired through conventional breeding methods (Birch, 1997; Pitzschke and Hirt, 2010; Trigiano and Gray, 2011). Over the last 30 years since the first transformation was reported, the techniques of plant transformation have been widely used in the most major crop species to improve a plant performance as a practical tool or to analyze a gene function as a novel research tool (Birch, 1997; Trigiano and Gray, 2011). A plant genetic transformation system has the essential requirements: (1) a competent regeneration system of transformed tissues, (2) a transformation system to introduce a foreign gene, (3) construction of a plasmid vector harboring a gene of interest, (4) a workable selection system to screen non-transgenic plants and (5) a trusted confirmation system to identify a transformed plant by the insertion of the gene of interest (Birch, 1997; Griffiths et al., 2008; Lodge et al., 2007; Trigiano and Gray, 2011).

Development of an efficient plant regeneration system

Plant regeneration is a process to develop a whole plant derived from a plant tissue or cell and proliferate it using in vitro culture techniques (George et al., 2008a; Ikeuchi et al., 2016; Trigiano and Gray, 2011). Plant regeneration is based on the totipotency of plant in which a plant cell has a potential to be differentiated into an entire plant (Ikeuchi et al., 2016; Trigiano and

Gray, 2011). In plant biotechnology, plant regeneration is prerequisite of a genetic transformation to develop a whole plant by using the transformed tissue or cell (Birch, 1997). Birch (1997) described that an efficient regeneration is critical for recovery of transgenic plants. The basic steps for adventitious plant regeneration were well characterized by Trigiano and Gray (2011): (1) selection of explants, (2) maintenance of aseptic conditions, (3) proliferation of shoots, (4) root development, and (5) transplant of regenerated plants.

An efficient plant regeneration system can be established by testing different factors affecting the regeneration efficiency, such as the type of explant sources, combination of plant growth regulators (PGRs), genotypes and age of explants (Birch, 1997; George et al., 2008c; Makunga et al., 2005; Trigiano and Gray, 2011). Additionally, Birch (1997) and Trigiano and Gray (2011) reviewed that development of an efficient regeneration system is important to optimize a transformation frequency during *Agrobacterium*-mediated transformation to transfer a gene of interest.

Plant regeneration can be achieved through indirect and direct organogenesis with non-meristematic explants to develop clones from mother explants (Birch, 1997; Trigiano and Gray, 2011). Plant regeneration in indirect organogenesis is followed by initiation of callus from somatic tissues and shoot or root differentiation from a group of callus cells, but explants can develop shoot or root without formation of undifferentiated cell phase (callus) in direct organogenesis (Trigiano and Gray, 2011). A leaflet and a piece of petiole, representative vegetative plant tissues, are typically used as explants for in vitro organogenesis (Birch, 1997; Ikeuchi et al., 2016; Makunga et al., 2005; Trigiano and Gray, 2011). Regeneration via indirect organogenesis is achieved by subculturing into callus inducing media, shoot inducing media, and root inducing media (Trigiano and Gray, 2011). This approach can be beneficial in mass

multiplication of clones from the explant (Savita et al., 2011). However, indirect organogenesis may lead undesirable results because of somaclonal variation in plant tissue culture (Krishna et al., 2016). Micropropagation by direct organogenesis can develop and proliferate a uniform clone by using a small piece of vegetative explants within relatively rapid pace (Karp, 1995). Direct organogenesis does not require additional subcultures for organogenesis since indirect organogenesis is followed by callus inducing and shoot or root inducing procedures (Trigiano and Gray, 2011). Furthermore, Birch (1997) reviewed that direct organogenesis via vegetative tissue is more appropriate for plant genetic transformation since this approach shows reduced somaclonal variations with minimized exposure to in vitro condition during the transformation.

Application of PGRs has shown a dramatic effect on a shoot and/or root formation from explants at a very low concentration (0.001 – 10 µM) (Trigiano and Gray, 2011). An efficient regeneration system can be optimized by testing the type and concentration of PGRs and different genotypes and materials (Makunga et al., 2005). Two important classes of PGRs for regulating growth and morphogenesis are auxin and cytokinin since they play a critical role in development of root and shoot (Birch, 1997; George et al., 2008b; Trigiano and Gray, 2011). Trigiano and Gray (2011) described the roles of auxin in plant regeneration system, which included a root formation and cell elongation. Synthetic and natural auxins commonly used in tissue culture for root formation are 1-naphthaleneacetic acid (NAA) and indolebutyric acid (IBA) (George et al., 2008b). Cytokinins have been used to initiate shoot formation, bud stimulation, and callus inductions (Huetteman and Preece, 1993; Zawadzka and Orlikowska, 2006). In general, 6-benzylaminopurin (BA) and thidiazuron (TDZ) are used to induce an efficient shoot organogenesis from a vegetative explant (George et al., 2008c; Huetteman and Preece, 1993; Ikeuchi et al., 2016; Trigiano and Gray, 2011). Meng et al. (2004) reported the 5.0 µM BA

treatment on ‘Marion’ blackberries had a high rate of shoot regeneration. However, it may be controversial since Trigiano and Gray (2011) reviewed that high levels of combined PGRs (higher than 5 µM) could inhibit adventitious shoot regeneration of woody plants. In addition, Lenz et al. (2016) reported that the highest regeneration rate of purple raspberry ‘Amethyst’ was 93.3 % in the 1.0 µM TDZ treatment and higher TDZ concentrations decreased the number of shoot formation on the explants. High TDZ concentration may induce hyperhydricity on regenerated shoot or translucent stem tissue in plant tissue culture (Huetteman and Preece, 1993; Wang et al., 2003). These reports suggested that an evaluation of various combinations of PGRs is required to develop an efficient regeneration system.

Age of explants has a crucial effect on plant regeneration. The regeneration study of *Jatropha curcas L.* by Mazumdar et al. (2010) revealed that the young explants had higher potential of direct shoot regeneration than old explants. Additionally, the youngest explants (0 day of the cotyledonary leaf) were shown not only the highest regeneration frequency with 87.5 % but also the significant effects in the *Agrobacterium*-mediated transformation with 90 % of GUS gene expression (Mazumdar et al., 2010). In the study of regeneration of *Miscanthus* × *giganteus*, perennial rhizomatous grass, the youngest callus (1 month old) showed significantly higher regeneration frequency (93 %) than older callus explants (Kim et al., 2010). Thus, a testing of explant sources, various combinations of PGRs, and age of explants should be tested to develop an optimized regeneration system.

Genetic transformation systems

An efficient plant regeneration system can develop more putative transgenic plants in a genetic transformation system. An efficient genetic transformation protocol should also include the construction of plasmid vectors, test of antibiotics, and transformation verification.

Agrobacterium-mediated transformation and particle bombardment transformation are commonly used methods for plant transformation (Birch, 1997; Bourras et al., 2015; Trigiano and Gray, 2011). Selection of transformation method is highly dependent on the plant species because of the host range of the *Agrobacterium* strains (Bourras et al., 2015).

Agrobacterium-mediated transformation takes the advantage of *Agrobacterium tumefaciens* that causes a tumor on many dicot plant species to deliver genes by a tumor inducing plasmid (Ti-plasmid) (Birch, 1997; Bourras et al., 2015; Păcurar et al., 2011). The Ti-plasmid contains four functional regions: origin of replication, conjugation region, virulence (vir) region, and T-DNA (transfer DNA) region (Birch, 1997; Bourras et al., 2015; Păcurar et al., 2011; Trigiano and Gray, 2011). T-DNA region is integrated into a plant genome after the plant tissues are infected by *Agrobacterium tumefaciens* that is often activated by acetosyringone, a phenolic compound released from a wounded plant (Gelvin, 2003; Păcurar et al., 2011). To facilitate gene delivery by Ti-plasmid, researchers have engineered the plasmid to be suitable for various laboratory purposes such as cloning of selectable marker genes (Gelvin, 2003). Genes related to the tumor formation and opine biosynthesis on the plasmid are removed (disarmed) and marker genes (selectable and/or screenable genes), a gene of interest, and a promoter for driving the expression of the transgenes are inserted into the plasmid (Păcurar et al., 2011; Trigiano and Gray, 2011). Thus, the disarmed *Agrobacteria* cannot cause tumor and any DNA can be inserted to the T-DNA region that will be transferred to the cells in the host plant (Păcurar et al., 2011). To enhance *Agrobacterium*-mediated transformation efficiency, researchers developed a binary vector system in which a Ti helper plasmid only contains *vir* genes and another vector contains the gene of interest and the marker genes (Gelvin, 2003; Păcurar et al., 2011; Trigiano and Gray, 2011). The binary vector system helps transfer a large size of T-DNA and improves

transformation efficiency (Păcurar et al., 2011). Commonly used *Agrobacterium* strains harboring a binary vector system are LBA4404, GV310, MP90, AGL0, EHA101, EHA105, and NT1 (pKPSF2) (Gelvin, 2003).

The process of T-DNA transfer from the plasmid in the *Agrobacterium* to plant cells includes the following steps: (1) a host plant is wounded and releasing natural phenolic compounds such as acetosyringone (AS); (2) *Agrobacterium* contracts with AS and the complex of *vir* genes within Ti-plasmid is activated by an AS signal; (3) *Agrobacterium* is attached to plant cell surface; (4) the T-DNA region in the bacteria is single-stranded and nicked as 25 base pair repeats; (5) single-stranded T-DNAs are transferred into the host plant cells; (6) the T-DNAs are then integrated into the plant genome; and (7) transgenes are expressed in the host genome (Bourras et al., 2015; Gelvin, 2003; Gelvin, 2010; Georgiva et al., 2008; Jackson et al., 2013; Păcurar et al., 2011; Pitzschke and Hirt, 2010).

A protocol of *Agrobacterium*-mediated transformation consists of vector construction, integration of the constructed vector into an *Agrobacterium* strain, proliferation of the strain, preparation of infection solution, infection of explants by co-cultivating the *Agrobacterium* and the explants, selection of transgenic cells, regeneration of the transgenic cells, and confirmation of gene transfer in transgenic plants (Birch, 1997; Trigiano and Gray, 2011). The advantage of *Agrobacterium*-mediated transformation system is minimum disruption on the transgenic plant because of fewer copies of integrated transgenes to the host genome compared to other transformation methods such as gene gun (Birch, 1997). However, *Agrobacterium*-mediated transformation has disadvantages, such as a limited host range, a low regeneration frequency, and random integration of transgenes within the host genome (Birch, 1997; Bourras et al., 2015; Păcurar et al., 2011; Trigiano and Gray, 2011).

Particle bombardment transformation is a DNA-mediated transformation that can address disadvantages of *Agrobacterium*-mediated transformation (Altpeter et al., 2005; Trigiano and Gray, 2011). This approach utilizes a device that directly delivers plasmid DNAs into plant cells. Plasmid DNAs are coated on sub-cellular sized gold or tungsten particles (Rivera et al., 2012). In general, particle bombardment refers to the 'gene-gun' method since the DNAs coated with particles are shot into a plant cell through a device with high pressure (Rivera et al., 2012; Trigiano and Gray, 2011). The transformed plant cells are processed by selection of transgenic cells, regeneration of new plants, and confirmation of transgenic plants (Altpeter et al., 2005; Birch, 1997; Rajasekaran et al., 2000; Rao et al., 2009; Rivera et al., 2012; Trigiano and Gray, 2011). Advantages of particle bombardment transformation are no limitation on plant species, especially for monocot species because these species are not in the host range of *Agrobacterium* (Birch, 1997). However, particle bombardment may cause gene silence as many copies of the transgenes are integrated into the plant genome (Rivera et al., 2012; Trigiano and Gray, 2011). Moreover, specific equipment and supplies such as the gene-gun and gold or tungsten particles are required, which resulted in a high cost to conduct transformation (Altpeter et al., 2005; Trigiano and Gray, 2011).

Construction of a gene transfer vector

Construction of a gene transfer vector is an important process to integrate a foreign gene into plant genome. Plasmid DNA is often used to construct gene transfer vectors. Plasmid DNA is a naturally occurring circular double-stranded DNA founded in various bacteria species with an origin of replication region (Lodge et al., 2007). Advances in science, specifically the discoveries of restriction enzymes and DNA ligases, led to development of recombinant DNA

technologies to manipulate plasmid DNA by deleting or inserting genes within a specific region of the plasmid DNA (Lodge et al., 2007).

Ti-plasmid from *Agrobacterium tumefaciens* is often used to construct gene transfer vectors. The Ti-plasmid is composed of T-DNA, Ti-plasmid conjugation, origin of replication, and virulence region (Bourras et al., 2015). The virulence region contains virulence genes including *VirD2*, *VirF*, and *VirE2*, which form T-complex to transfer T-DNA segment into the host genome as a site-specific endonuclease complex (Bourras et al., 2015; Gelvin, 2003). Genes in a T-DNA region encoded functions such as synthesis of auxin and cytokinin, resulted in not only proliferation of transformed plant tissues but also production of opines for proliferation of the *Agrobacterium* (Bourras et al., 2015; Gelvin, 2010). These characteristics of Ti-plasmid from *A. tumefaciens* allow researchers to modify the plasmid by removing tumor inducing genes and opine biosynthesis to develop a suitable method of *Agrobacterium*-mediated transformation (Gelvin, 2003; Păcurar et al., 2011).

To ensure a gene transfer from the vector into the host genome, T-DNA region has been modified to contain many essential marker genes, including (1) a selectable marker to isolate transgenic cells by conferring a resistance to a specific antibiotic and (2) a screenable marker to identify transgenic cells or tissue by a color change reaction under specific chemicals. In the T-DNA region, the gene of interest and a promoter (s) that drives the expression of the gene of interest are also key components (Lodge et al., 2007; Miki and McHugh, 2004; Trigiano and Gray, 2011). Miki and McHugh (2004) reviewed that the *nptII* and *hpt* genes, which confer kanamycin and hygromycin B resistance, respectively, are commonly used as selectable marker genes in many transformation studies (Joyce et al., 2010; Miki and McHugh, 2004; Sundar and Sakthivel, 2008). Screenable marker genes, such as *GUS* (β-glucuronidase), *Luc* (luciferase),

GFP (green fluorescent protein), and *lacZ* (lactose operon) gene, are utilized to identify transgenic cells based on a color change (Lodge et al., 2007). A promoter, located upstream of a gene of interest, regulates gene expression and transcription procedures (Lodge et al., 2007; Trigiano and Gray, 2011). The most widely used promoter for plant transformation is cauliflower mosaic virus (CaMV) 35S promoter known as a strong constitutive promoter derived from the plant virus, which drives a high level of gene expression in many dicot plants (An et al., 1990; Trigiano and Gray, 2011; Wilkinson et al., 1997).

Integration of the gene transfer vector into *Agrobacterium* strains

Introduction of the cloned Ti-plasmid with essential marker genes into *A. tumefaciens* from *Escherichia coli* has been achieved by electroporation or freeze-thaw procedure (Wise et al., 2006). Majority of cloning have performed in *E. coli* due to their reproducibility, benefits on storage, and availability of various endonuclease (Lodge et al., 2007). To transform a cloned Ti-plasmid into the *Agrobacterium,* transfection of Ti-plasmid into a competent *Agrobacterium* strain was developed in the 1970's with the development of a reproducible transformation system (Holsters et al., 1978). At that time, researchers focused on the freeze-thaw method, which induces cell wall damage of *E. coli* by Ca^{2+} and high temperature and then competent strains uptake the extracted plasmids (Holsters et al., 1978; Lodge et al., 2007). Additionally, high-voltage electroporation was devised to integrate Ti-plasmid into competent cells. The mechanism of this method is that the high voltage pulse leads the interruption on *E. coli* membrane and the DNA within Ti-plasmid moves into the competent cells (Lodge et al., 2007; Mersereau et al., 1990). The competent cells are screened by selectable marker genes to ensure the valid integration of the Ti-plasmid and stored with glycerol stocks at -80 ℃ (Lodge et al., 2007).

Optimization of the conditions for *Agrobacterium*-mediated transformation

Suitable selection system in transformation

Selection of transformed cells with an appropriate integration of transgenes into the plant genome is important (Lodge et al., 2007; Miki and McHugh, 2004; Sundar and Sakthivel, 2008). Selectable marker genes from a Ti-plasmid in *Agrobacterium* strain have been considered a vital feature to identify or isolate the transformed cells by conferring a resistance to a certain level of antibiotics, in which such resistance allows transgenic cells survived in a medium with a specific selective agent (Birch, 1997). Selective agents/antibiotics such as kanamycin and hygromycin B, are generally toxic to plant cells with inhibition of protein synthesis (Sundar and Sakthivel, 2008); therefore, untransformed plant cells cannot be survived in the medium with the antibiotics (Miki and McHugh, 2004). A review from Miki and McHugh (2004) investigated different types of selectable marker genes and their popularity in the use of each marker gene from 450 papers published in the peer-reviewed journals related to plant biotechnology. They found that the *npt*II (Neomycin phosphotransferase II) and *hpt* (Hygromycin B phosphotransferase) selectable marker genes, which conferring kanamycin and hygromycin B resistance, respectively, have been mostly used in development of transgenic plants (Miki and McHugh, 2004).

Toxicity from a high level of a selective agent impedes a regeneration capability of transgenic cells (Birch, 1997; Joyce et al., 2010; Trigiano and Gray, 2011). To optimize a transformation system, the influence of antibiotic level on regeneration capability and survival rate should be precisely determined to establish an antibiotics-killing model (Haddadi et al., 2015; Liu et al., 2004). Optimization of antibiotics determines a minimum level of an antibiotic, which not only allows an efficient regeneration of transformed cells but also completely inhibits the growth of non-transformed cells (Birch, 1997; Trigiano and Gray, 2011). An appropriate level of

a selective agent is highly variable in species or even in cultivars (Haddadi et al., 2015; Sundar and Sakthivel, 2008). Therefore, a test with various antibiotic concentrations is important to ameliorate an efficiency of a transformation system (Birch, 1997; Gelvin, 2003; Miki and McHugh, 2004).

Inoculum density

Inoculum density of *Agrobacterium* is a key factor influencing the efficiency of gene transfer. Plants have defense systems to prevent invasion of plant pathogens, which will reduce infection rate of *A. tumefaciens*, consequently reduces the plant regeneration efficiency (Bourras et al., 2015; Gelvin, 2010; Păcurar et al., 2011; Zhang and Finer, 2016). The activation of plant defense systems to pathogen invasion is related to the inoculum density at a threshold level. Zhang and Finer (2016) reported that *Agrobacterium* infection with low-density increased transformation efficiency without triggering the plant turning on the defensive gene. In addition, Li et al. (2017) reported that an optimized optical density (OD) of *Agrobacterium* at an exponential phase improves ability of *Agrobacterium* to transfer DNA into the plant host genome. Furthermore, a high level of OD (higher than OD_{600} 1.0) may cause overgrowing of *Agrobacterium*, causing explants necrosis, and the gene activity in T-DNA region may be decreased (Jiang et al., 2015; Sanimah et al., 2010).

Co-cultivation

Co-cultivation time also affects the transformation efficiency (Zhang and Finer, 2016). Co-cultivation times were optimized in the transformation of sunflower and soybean (Li et al., 2017; Santarem et al., 1998; Zhang and Finer, 2016). They found that 15 days for sunflower and 5 days for soybean were the optimized co-cultivation time for *Agrobacterium*-mediated transformation for each species (Li et al., 2017; Zhang and Finer, 2016). Many transformation

studies in raspberry, aspen, and strawberry have adopted 2-3 days co-cultivation time (Dai et al., 2003; Haddadi et al., 2015; Mathews et al., 1995; Zhang and Finer, 2016). Longer co-cultivation time can also trigger the overgrowth of *Agrobacterium* in many dicot species, which can suppress recovery of the transgenic cells (Birch, 1997; Jiang et al., 2015; Sanimah et al., 2010; Zhang and Finer, 2016).

Inoculum density and co-cultivation time in *Agrobacterium*-mediated transformation are affecting *Agrobacterium* growth. Overgrowth of *Agrobacterium* is a major issue during co-cultivation, which must be addressed (Birch, 1997; Zhang and Finer, 2016). In nature, *Agrobacterium* infection of plant tissues occurs at a lower density and a longer co-cultivation time than under the laboratory condition (Bourras et al., 2015). A low density of *Agrobacterium* may be beneficial to control its overgrowth. Zhang and Finer (2016) reported that the use of an inoculum cell density of 6×10^2 bacteria ml^{-1} with a long co-cultivation (15-days) showed the highest transformation frequency (23.7 %) in sunflower transformation without activating the plant defense system and overgrowth of the bacteria. Therefore, both the inoculum density and co-cultivation time should be determined to develop an efficient *Agrobacterium*-mediated transformation system.

Confirmation of transformation

The transgene (s) integrated into the plant genome should be confirmed by detecting the expression of the marker genes and the target gene using various methods (Griffiths et al., 2008; Lodge et al., 2007; Trigiano and Gray, 2011). Selectable marker genes, such as *nptII* and *hpt*, are used to confirm transformation by positive selection of the transgenic cells using selective agents (Birch, 1997). Reporter genes such as *GUS* (β-glucuronidase) and *GFP* (green fluorescent protein) shows color changes in the transformed tissues under a specific condition, for instance,

staining tissues by glucuronide or exposure tissues to ultraviolet (UV) light (Lodge et al., 2007). Gel electrophoresis can separate macromolecule such as DNA, RNA, and protein based on their differences in size (Griffiths et al., 2008). Electric current in a gel electrophoresis apparatus can move molecules, amplified fragments including a gene of interest based on specific primer sets in a PCR, from negative electrode to positive electrode (Lodge et al., 2007). Separated bands on a gel are stained by intercalating agent such as ethidium bromide to be visualized under UV light. Comparison with a standard ladder DNA and positive control (usually plasmid DNA used in the transformation) can confirm transformation of the gene of interest. This is classic and straightforward method to identify an appropriate integration of gene within T-DNA region into a plant genome (Lodge et al., 2007; Trigiano and Gray, 2011).

Southern blot analysis can be used to confirm the integration of a specific DNA into the host genome based on the complementary hybridization of DNA (Griffiths et al., 2008; Lodge et al., 2007). As a result, only hybridized DNA sequence with labeled probes can be visualized on a membrane by autoradiography (Lodge et al., 2007). Real-time quantitative PCR (RT-PCR) is used to quantify the amount of target DNA and the expression level of the target gene in fixed PCR cycles. Required materials for an RT-PCR reaction include SYBR green fluorescent dye, RT-PCR system, a synthesized cDNA fragment, and a specific primer set for a gene of interest (Lodge, et al. 2007). This method utilizes a fluorescent dye such as SYBR Green, which emits fluorescence when a double stranded DNA is formed (Lodge et al., 2007). In a result of RT-PCR, a threshold cycle value (C_t value; level of fluorescent is increased rapidly) is used to quantify an expression level of target DNA from the original sample. Using RT-PCR for a confirmation of transformation is beneficial to analysis a gene function based on an expression level of the foreign gene under a specific condition (Lin-Wang et al., 2010; Nelson et al., 2007).

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CHAPTER III. IN VITRO PLANT REGENERATION OF RED RASPBERRY (*RUBUS IDAEUS*) CULTIVARS 'JOAN J' AND 'POLANA'

Abstract

Plants were regenerated from in vitro tissues of red raspberry cultivars 'Joan J' and 'Polana'. The effects of plant growth regulators (PGRs), explant type, and explant age on in vitro shoot and root regeneration were investigated. In vitro leaf and petiole tissues were inoculated in woody plant medium (WPM) supplemented with 200 $mg \cdot l^{-1}$ polyvinylpyrrolidone (PVP) and various combinations of 6-benzylaminopurine (BA) and/or thidiazuron (TDZ). Results showed that the regeneration rates were 70 % for 'Joan J' and 82.2 % for 'Polana' when the 7-day-old leaf segments were inoculated on the medium with 2.5 µM BA and 1.0 µM TDZ and WPM with 2.5 µM BA and 0.1 µM TDZ, respectively. Regenerated shoots were rooted both in vitro and ex vitro. The in vitro rooting was first conducted in ½ Murashige and Skoog (MS) medium with 5 or 10 µM 1-naphthaleneacetic acid (NAA) and 5 or 10 µM indole-3-butyric acid (IBA) for 'Joan J'. In vitro shoots of 'Polana' were pulsed in ½ MS medium with 10-80 µM NAA or IBA for 7 days, then either kept in the same medium or transferred to auxin-free medium. The result of in vitro rooting showed that 100% and 75% of in vitro shoots of 'Joan J' and 'Polana' developed roots when they were pulsed with 10 µM and 20 µM NAA, respectively. The ex vitro rooting was conducted by dipping the in vitro shoots in a solution of 0, 500 µM IBA or 500 µM NAA for 10 seconds, then inserting to potting mix (Sunshine mix) for a 4-week rooting and acclimatization period. The ex vitro rooting result showed that an average of 80.9% micro-cuttings of the two cultivars were rooted with no significant differences in rooting frequency among three treatments.

Introduction

Raspberries (*Rubus* spp.) have been known as a valuable fruit species and their fruits are available in a variety of forms in grocery products such as jam, juice, and bakery product (Jennings, 1988). Red raspberry (*Rubus idaeus* L.) is one of the most important *Rubus* species due to its enrichment in vitamins and antioxidants (Buricova et al., 2011; Hall et al., 2009). In addition, Graham and Woodhead (2009) reported that red raspberry species (2n = 2x = 14) is a suitable model species of *Rubus* genus to study the molecular genetic characteristics due to a small sized genome (275 Mbp). Food and Agriculture Organization (FAO) reported that the demand of raspberries in North America has continuously increased for the last 20 years (FAO, 2016). Researchers have attempted to improve raspberry yield and quality using conventional breeding; however, drawbacks, such as perennial nature, virus infection, and narrow genetic diversity of raspberry, limit the breeding progress compared to other fruit species (Graham et al., 1997; Graham and Jennings, 2009; Hall et al., 2009; Jennings, 1988). Researchers are looking for new approaches to overcome these limitations in traditional breeding (Graham and Jennings, 2009); however, limited information is available in the field biotechnology for raspberry and other Rubus species (Hall et al., 2009; Pritts, 2016).

Plant biotechnology including genetic transformation can be used to not only develop novel breeding materials, but also help speed up the breeding process. An efficient plant regeneration is a prerequisite for plant genetic transformation. Birch (1997) and Graham et al. (1997) indicated that an efficient regeneration is beneficial to improve the frequency of *Agrobacterium*-mediated transformation with an efficient recovery of transgenic plants. In vitro plant regeneration is based on the cell totipotency in which a living plant cell has potential to be differentiated into an entire plant (Ikeuchi et al., 2016; Trigiano and Gray, 2011).

Plant regeneration is affected by a few factors, such as genotype, explant type and age, and plant growth regulators (PGRs). McNicol and Graham (1990) reported that the five *Rubus* genotypes showed various shoot regeneration rates (15 to 38 %) on the same regeneration medium. McNicol and Graham (1990) and Tsao (1999) reported that leaf discs and stem segments of red raspberries showed different regeneration frequencies. Age of explants has also a crucial role in plant regeneration. A comparison of different explant ages on the regeneration rate of *Saussurea obvallata* and *Eucalyptus camaldulensis* showed a significantly increased frequency in younger explants (10 to 15 days old) (Dhar and Joshi, 2005; Prakash and Gurumurthi, 2009). Similarly, the regeneration of *Miscanthus* × *giganteus* was increased to 93 % by using the youngest callus (1 month old), which was significantly higher than that of the older explants (2 to 12 month old). Auxin and cytokinin play an important role in plant regeneration (Birch, 1997; George et al., 2008c). Plant regeneration of *Rubus* species was achieved in the studies of McNicol and Graham (1990) and Tsao (1999). An application of cytokinin (especially TDZ) has induced the efficient regeneration of raspberry tissues (Graham et al., 1997; Millan-Mendoza and Graham, 1999; Turk et al., 1994). Furthermore, the regeneration study of 'Amethyst' by Dai et al. (2006) and Lenz et al. (2016) showed that the 1.0 µM TDZ treatment developed new shoots with 93.3 % of regeneration rate. Although these factors were characterized in many major fruit crops, a precise effect had not yet studied for many red raspberry cultivars, which are important for the raspberry improvement (Aldwinckle and Malnoy, 2009; Graham and Woodhead, 2009). Therefore, the regeneration studies considering these important factors on red raspberries are necessary.

Two red raspberry cultivars 'Joan J' and 'Polana' used in this study produce relatively large, firm, and glossy red fruits. 'Joan J' is a thornless cultivar and produces fruits over a long

growing season (Jennings, 2008). ‘Polana’ is used to fill the gap during the harvest season between early ripening and late ripening raspberries (Knight, 2004). To our knowledge, no research on plant regeneration has been reported for the two cultivars.

The objective of this study was to develop an efficient plant regeneration system for ‘Joan J’ and ‘Polana’. Such a system will be further used for genetic engineering and gene function analyses for raspberry species; therefore, the present research will be beneficial to breeding and germplasm improvement of raspberry or other *Rubus* species.

Materials and Methods

Plant materials

In vitro plants of the red raspberry ‘Joan J’ and ‘Polana’ were maintained in Murashige and Skoog (MS) medium containing 2.5 µM 6-benzyladenine (BA), 0.5 µM gibberellic acid (GA_3), 3 % sucrose, and 0.525 % agar. The medium pH was adjusted to 5.7-5.8 prior to autoclaving and 40 ml of the medium was poured in the GA7 Magenta boxes (Magenta Corp., Chicago, IL). In vitro cultures were subcultured every 4 weeks. All cultures were placed in tissue culture room with a 16/8 (light/dark) photoperiod at 36 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity and a temperature of 25/18 ℃ (light/dark).

Influence of BA and TDZ on shoot regeneration

Two plant growth regulators, BA at 2.5 and 5.0 µM and TDZ at 0, 0.1, 0.5, and 1.0 µM were used for in vitro shoot regeneration of ‘Joan J’ and ‘Polana’. The experiment consisted of eight treatments (2 BA × 4 TDZ) with three replicates (plates) per treatment. Ten leaf segments (0.5 cm^2) and five petiole pieces (0.5 cm) were inoculated in a petri dish containing 25 ml of Woody Plant Medium (WPM) supplemented with 2 % sucrose, 0.525 % agar, 200 $mg \cdot l^{-1}$ polyvinylpyrrolidone (PVP), and the designated PGRs. The medium pH was adjusted to 5.2 prior

to autoclaving. The petri dishes were firmly sealed with Parafilm M® (Bemis Company, Inc., Neenah, WI, USA). The petri dishes (cultures) were placed in a drawer for dark treatment in a lab (25 ℃). After 4-week dark treatment, cultures were subcultured in the fresh WPM medium with the cytokinin treatments and place in a tissue culture room with 16 light / 8 dark photoperiod for another eight weeks with one subculture at the 4^{th} week. The regeneration performance was evaluated after 12 weeks culture. The number of explants forming shoots, the total number of shoots per petri dish, degree of callusing, and degree of shoot hyperhydricity were recorded. In this experiment, a regenerated shoot was defined as a shoot, which is higher than 0.5 cm ± 0.2 in long, formed a shoot apex and at least two leaves. Degree of callusing was scored as 0, 1, 2, and 3 based on no callus, <1 mm, 1-2 mm, and > 2 mm, in size, respectively. In addition, degree of shoot hyperhydricity was scored as 1, 2, 3, 4, and 5 using the criteria of no, some, moderate, severe, and very severe hyperhydricity, respectively. The experiment was conducted as a Completely Randomized Design (CRD) with split-plot of 2 BA and 4 TDZ levels with three experiments and three replicates (plates) of each treatment.

Influence of explant age on regeneration frequency

Leaves/petioles at the age of 7-, 14-, and 28-day old were harvested from in vitro cultures. Ten leaf segments (0.5 cm^2) and five petioles (0.5 cm) were inoculated in the optimized medium (2.5 μM BA and 1.0 μM TDZ for 'Joan J'; 2.5 μM BA and 0.1 μM TDZ for 'Polana') of each cultivar. The influence of age and explant types on shoot regeneration was evaluated using the number of regenerated shoots and the degree of callusing. The criteria of a shoot regeneration was same with the cytokinins experiment. The culture conditions were the same as those in the experiment for the PGRs evaluation. This experiment was conducted as a CRD with three age treatments, three experiments, and three replicates.

In vitro rooting

The influence of naphthaleneacetic acid (NAA) and indole-3-butyric acid (IBA) on root initiation was examined. Four regenerated shoots (3 cm long) were inserted the rooting medium in a magenta box (40 ml medium). The rooting medium was ½ MS with 200 $mg \cdot l^{-1}$ PVP, 1.5 % sucrose, and 0.575% agar and various PGR treatments of no PGR, 5 µM NAA, 10 µM NAA, 5 µM IBA, or 10 µM IBA. The pH of medium was adjusted to 5.7 prior to autoclaving. Magenta boxes containing explants were placed in a tissue culture room with the environmental conditions of 16/8 photoperiod and 25 ℃. After 4 weeks, rooting frequency, number of roots per responding shoot, averaged root length, and root thickness were recorded. A regenerated root in this experiment was defined in which a root could penetrate the medium to uptake nutrient and longer than 0.5 cm ±0.2 in length. The experiment was conducted as a CRD with five PGR treatments and three replicates.

For in vitro rooting of 'Polana' cultivar, an additional experiment was conducted due to the low rooting frequency in the previous experiment. Four regenerated shoots (3 cm long) of 'Polana' were pulsed in the rooting medium containing NAA or IBA at 10, 20, 40, or 80 µM for 7 days were then transferred to auxin-free-rooting medium for additional 21 days. One set of cuttings were continuously in the auxin-containing medium for 28 days. The rooting medium was ½ MS with 200 $mg \cdot l^{-1}$ PVP, 1.5 % sucrose, and 0.575% agar (pH as 5.7 prior to autoclaving). Rooting performance was evaluated the aforementioned in vitro rooting. This experiment was performed as a CRD with 16 treatments (4 concentrations × 2 auxins × continuous or pulsing) and three replicates.

Ex vitro rooting and acclimation

In vitro shoots (3 cm long) were transplanted into a 48-hole plug tray containing Sunshine Mix #1 (Fisons Western Corp., Vancouver, Canada) after dipping in a solution of 500 µM NAA, 500 µM IBA, or water. The tray was covered with a plastic lid. After 4 weeks, the lid was gradually removed during a 4-week period. The rooted plants were then transplanted into the 1-litter pots in the greenhouse. Influence of PGRs on rooting was evaluated by calculating the rooting frequency, root number, averaged root length, and root thickness. In addition, the survival rate at 6^{th} weeks in the greenhouse was also recorded. This experiment was conducted as a Randomized Complete Block Design (RCBD) with three treatments and three replicates (blocks). Each block of the treatments had 12 samples to calculate regeneration frequency and survival rate.

Experimental design and statistical analysis

Analysis of variance (ANOVA) and mean separation were conducted using GLM and GLIMMIX (Laplace) methods of SAS software Version 9.4 (SAS Institute Inc, Cary, NC). In the ANOVA of the regeneration results, the GLM procedure was conducted with the calculation of type III sums of squares due to the missing explants in the experiment. The GLIMMIX procedure was used to test a significance of the treatments, especially combination of TDZ and BA. Mean separation was conducted in the GLIMMIX procedure with the calculation of Least Significant Difference at $\alpha \leq 0.05$. The standard errors from the result of SAS software was used to bar graphs for indicating the standard deviation of the sampling distribution. Graphs were developed by SigmaPlot Version 12.5 (Systat Software Inc, San Jose, CA).

Results

Effect of cytokinins on shoot regeneration of 'Joan J' and 'Polana'

Leaf and petiole explants on the regeneration medium developed new shoots during the 12th-week regeneration period (Fig. 3.1A). The new shoots were developed in 2-3 weeks and more than 95 % of initial explants survived during the regeneration period. The explants were subcultured into a fresh WPM medium every four week.

Cytokinin and explant type significantly affected shoot regeneration of 'Joan J' (Table 3.1 and 3.2). Leaf explants showed higher regeneration rates than petiole explants except for the treatment of 2.5 µM BA and no TDZ in which no shoot were regenerated from leaf explants (Fig. 3.2). BA, TDZ, BA × TDZ, and BA × TDZ × experimental replicates had shown a significant effect on shoot regeneration of 'Joan J' (Table 3.1 and 3.2). The overall regeneration was increasing with the increasing TDZ. The highest shoot regeneration frequency was obtained in the medium with 1.0 µM TDZ and 5.0 µM BA (Table 3.3); therefore, the role of TDZ in shoot regeneration might be more significant.

Application of TDZ at various levels significantly influenced the shoot quality/hyperhydricity of regenerated 'Joan J' shoots (Table 3.4). New shoots regenerated in the 1.0 µM TDZ treatment with 2.5 or 5.0 µM BA were severely hyperhydric, while less hyperhydricity were observed in the low TDZ treatments. Although application of BA did not show significant effect on shoot hyperhydricity, the combination of 5.0 µM BA and 1.0 µM TDZ resulted in a higher degree of hyperhydricity than the treatment of 2.5 µM BA and 1.0 µM TDZ. The result also showed that BA did not significantly affect callus development; however, the callusing rate was increasing with an increase of TDZ (Table 3.4). Considering the regeneration

frequency and the quality of regenerated shoots, the treatment of 2.5 µM BA and 1.0 µM TDZ treatment is the most favorable condition for shoot regeneration of 'Joan J'.

Shoot regeneration of 'Polana' was significantly influenced by BA, TDZ, BA × TDZ, and Experiment × TDZ (Table 3.5 and 3.6). For the leaf explants, the highest shoot regeneration frequency (63.3 %) was observed in the treatment of 2.5 µM BA + 0.1 µM TDZ with the significance of the cytokinin treatments; however, other treatments showed that a better shoot regeneration and the shoot number in the 5.0 µM BA medium than in the 2.5 µM BA medium with or without TDZ (Table 3.7). The regeneration frequencies of petioles were higher in the treatment of 2.5 µM BA compared to the treatment of 5.0 µM BA (Table 3.7). In addition, lower TDZ concentration showed higher shoot regeneration frequency in the experiment. It appeared that addition of TDZ did not enhance shoot regeneration but TDZ application significantly decreased shoot regeneration (Table 3.7).

The effect of TDZ on shoot hyperhydricity was significantly increased hyperhydricity degree (Table 3.8). The higher the TDZ concentrations, the severe the shoot hyperhydricity. The BA treatment did not show significance in shoot hyperhydricity. Considering both shoot regeneration frequency (Table 3.8) and the potential hyperhydricity caused by TDZ (Table 3.8). The combination of 2.5 µM BA and 0.1 µM TDZ was used for shoot regeneration of 'Polana' in the transformation study.

Figure 3.1. Plant regeneration of red raspberry 'Joan J'. A: Shoots were regenerated from 7 days old explants. B: In vitro rooting in 1/2 MS with 10 µM NAA. C: Ex vitro rooting after being dipped in 500 µM IBA solution.

Table 3.1. Effects of BA and TDZ on shoot regeneration from leaf explants of 'Joan J'.

Analysis of variance				
Source of Variance	DF	Type III SS	Mean Square	Pr > F
Experiment	2	233.3	116.7	0.5973
Rep (Experiment)	6	941.7	156.9	0.6496
BA	1	355.6	355.6	< 0.0001
BA × TDZ	3	4278.8	1425.9	< 0.0001
Expt × BA	2	311.1	155.6	0.8826
Expt × BA × TDZ	6	7322.2	1220.4	0.0003
TDZ	3	27761.1	9253.7	< 0.0001
Expt × TDZ	6	3555.6	592.6	0.7996
Error	42	9391.7	223.6	
Total	71	54150.0		

Table 3.2. Effects of BA and TDZ on shoot regeneration from petiole explants of 'Joan J'.

Analysis of variance				
Source of Variance	DF	Type III SS	Mean Square	Pr > F
Experiment	2	643.4	321.7	0.1389
Rep (Experiment)	6	673.4	112.2	0.6340
BA	1	144.5	144.5	0.0413
BA × TDZ	3	322.4	107.5	< 0.0001
Expt × BA	2	152.3	76.2	0.6160
Expt × BA × TDZ	6	5048.1	841.4	0.0003
TDZ	3	9917.9	3306.0	0.1567
Expt × TDZ	6	3659.2	609.9	0.0033
Error	42	6527.3		
Total	71	27088.6		

Table 3.3. Regeneration frequency of 'Joan J' affected by various combinations of BA and TDZ.

	Regeneration frequency (%)			
	Leaf		Petiole	
	BA (µM)		BA (µM)	
TDZ (µM)	2.5	5.0	2.5	5.0
0.0	0.0 B d [z]	3.3 A d	6.6 A a	2.2 B a
0.1	40.0 B c	8.8 A c	15.5 A a	7.5 B a
0.5	43.3 B b	47.8 A b	17.8 A a	13.3 B a
1.0	48.9 B a	54.4 A a	33.3 A a	35.6 B a
Significant effect[y]	B^{***}, T^{***}, $B\times T^{***}$		B^{*}, T^{ns}, $B\times T^{***}$	

[z] Mean separation was analyzed by Least Square Means. Capital letter indicates significance of BA treatment and small letter indicates significance of TDZ treatment.
[y] B: BA, T: TDZ.
^{ns}, *, and *** represent nonsignificant or significant at $P \leq 0.05$ and 0.001, respectively.

Table 3.4. Effect of BA and TDZ on regenerated shoot quality of 'Joan J'.

Treatment (μM)		Regenerated shoot numbers in responsive leaf explants	Regenerated shoot numbers in responsive petiole explants	Callusing in leaf explants[z]	Callusing in petiole explants	Hyperhydricity of shoots from leaves[y]	Hyperhydricity of shoots from petioles
BA	TDZ						
2.5	0.0	0.0 B c[x]	0.7 A b	1.0 A b	1 .0 A b	1.0 A c	1.0 A c
	0.1	2.4 B b	1.2 A ab	2.0 A ab	1.8 A ab	1.3 A bc	1.3 A bc
	0.5	2.0 B a	1.3 A ab	2.1 A a	2.1 A ab	2.1 A ab	1.7 A ab
	1.0	2.0 B a	1.9 A a	3.0 A a	2.9 A a	3.0 A a	3.0 A a
5	0.0	0.4 A c	0.3 A b	1.1 A b	1.0 A b	1.0 A c	1.0 A c
	0.1	1.0 A b	1.1 A ab	1.7 A ab	1.2 A ab	1.2 A bc	1.0 A bc
	0.5	2.1 A a	1.4 A ab	2.1 A a	1.4 A ab	2.7 A ab	2.7 A ab
	1.0	2.0 A a	2.0 A a	2.6 A a	2.1 A a	3.8 A a	3.6 A a
Significant effects[w]		B^{**}, T^{ns}, $B\times T^{ns}$	B^{ns}, T^{ns}, $B\times T^{ns}$	B^{ns}, T^{ns}, $B\times T^{ns}$	B^{ns}, T^{ns}, $B\times T^{ns}$	B^{ns}, T^{*}, $B\times T^{ns}$	B^{ns}, T^{*}, $B\times T^{ns}$

[z] Callusing was scored 0, 1, 2, and 3 based on the callus size of no callus, < 1 mm, 1-2 mm, and > 2 mm, respectively.
[y] Hyperhydricity degree was scored as 1, 2, 3, 4, and 5 based on the criteria of no hyperhydricity, some, moderate, severe, and very severe, respectively.
[x] Mean separation was analyzed by Least Square Means at $\alpha = 0.05$. Capital letter indicates significance of BA treatment (BA) and small letter indicates significance of TDZ treatment (T).
[w] ANOVA based on GLIMMIX (Laplace) procedure was conducted to identify a significant effect among the two important factors. ^{ns}, *, and ** represent nonsignificant or significant at $P \leq 0.05$ or 0.01, respectively.

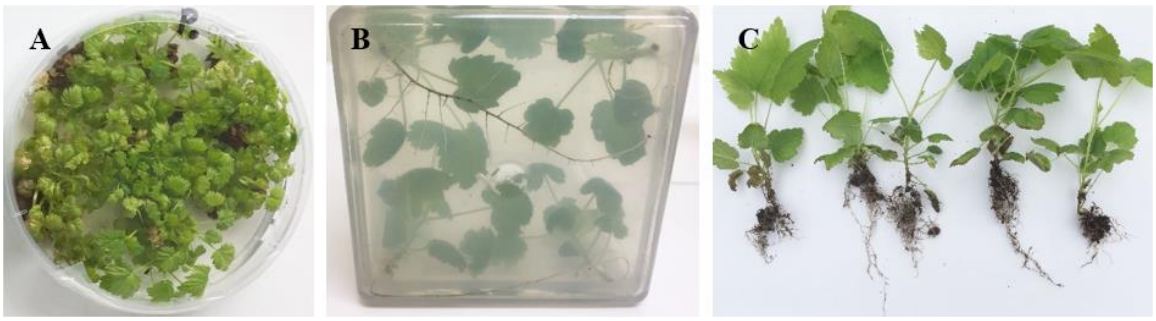

Figure 3.2. Plant regeneration of red raspberry 'Polana'. A: Shoots were regenerated shoots from 7 days old explants. B: In vitro rooting in 1/2 MS medium with the treatment of 7-day 20 µM NAA pulsing and 3-week PGR-free medium. C: Ex vitro rooting after being dipped in 500 µM NAA solution.

Table 3.5. Effects of BA and TDZ on shoot regeneration from leaf explants of 'Polana'.

Analysis of variance				
Source of Variance	DF	Type III SS	Mean Square	Pr > F
Experiment	2	933.3	466.7	0.2042
Rep (Experiment)	6	1791.6	298.61	0.4038
BA	1	22.2	22.2	< 0.0001
TDZ	3	3411.1	1137.0	< 0.0001
BA × TDZ	3	2766.8	892.6	< 0.0001
Expt × BA	2	711.1	355.6	0.2948
Expt × TDZ	6	5855.6	975.9	0.0073
Expt × BA × TDZ	6	3322.2	553.7	0.0936
Error	42	11875.0	282.7	
Total	71	30600.0		

Table 3.6. Effects of BA and TDZ on shoot regeneration from petiole explants of 'Polana'.

Analysis of variance				
Source of Variance	DF	Type III SS	Mean Square	Pr > F
Experiment	2	933.3	466.7	0.2042
Rep (Experiment)	6	1791.7	298.6	0.4038
BA	1	22.2	22.2	< 0.0001
TDZ	3	3411.1	1137.0	< 0.0001
BA × TDZ	3	2677.8	892.6	< 0.0001
Expt × BA	2	711.1	355.5	0.2948
Expt × TDZ	6	5855.5	975.9	0.0073
Expt × BA × TDZ	6	3322.2	553.7	0.0936
Error	42	11875.0		
Total	71	30600.0		

Table 3.7. Regeneration frequency of 'Joan J' affected by various combinations of BA and TDZ.

	Regeneration frequency (%)			
	Leaf		Petiole	
	BA (µM)		BA (µM)	
TDZ (µM)	2.5	5.0	2.5	5.0
0.0	34.4 B c[z]	48.8 A c	33.3 A a	28.8 B a
0.1	63.3 B a	45.5 A a	28.8 A b	24.4 B b
0.5	38.8 B b	44.4 A b	26.6 A d	6.6 B d
1.0	38.8 B d	32.2 A d	26.6 A c	17.7 B c
Significant effect[y]	B***, T***, B×T***		B***, T***, B×T***	

[z] Mean separation was analyzed by Least Square Means at $\alpha = 0.05$. Capital letter indicates significance of BA treatment and small letter indicates significance of TDZ treatment.
[y] B: BA, T: TDZ.
*** represents significant at $P \leq 0.001$.

Table 3.8. Effects of BA and TDZ on regenerated shoot quality of 'Polana'.

Treatment (μM)		Regenerated shoot numbers in responsive leaf explants	Regenerated shoot numbers in responsive petiole explants	Callusing in leaf explants[z]	Callusing in petiole explants	Hyperhydricity of shoots from leaves[y]	Hyperhydricity of shoots from petioles
BA	TDZ						
2.5	0.0	3.0 A a[x]	2.0 B b	3.0 A a	2.4 A a	1.0 A b	1.0 A b
	0.1	2.9 A ab	2.6 B a	3.0 A a	2.7 A a	2.7 A a	2.9 A a
	0.5	2.5 A ab	1.0 B d	2.9 A a	2.7 A a	4.4 A a	4.3 A a
	1.0	1.8 A b	1.4 B c	2.8 A a	2.3 A a	4.2 A a	4.1 A a
5	0.0	2.9 A a	3.0 A b	2.9 A a	1.9 A a	1.3 A b	1.4 A b
	0.1	3.3 A ab	3.1 A a	2.8 A a	2.1 A a	2.9 A a	3.2 A a
	0.5	2.9 A ab	1.3 A d	2.8 A a	2.7 A a	4.1 A a	4.3 A a
	1.0	1.6 A b	2.2 A c	2.6 A a	2.2 A a	4.2 A a	4.9 A a
Significant effects[w]		B^{ns}, T^{ns}, $B\times T^{ns}$	B^{***}, T^{***}, $B\times T^{***}$	B^{ns}, T^{ns}, $B\times T^{ns}$	B^{ns}, T^{ns}, $B\times T^{ns}$	B^{ns}, T^{**}, $B\times T^{ns}$	B^{ns}, T^{**}, $B\times T^{ns}$

[z] Callusing was scored 0, 1, 2, and 3 based on the callus size of no callus, < 1 mm, 1-2 mm, and > 2 mm, respectively.
[y] Hyperhydricity degree was scored as 1, 2, 3, 4, and 5 based on the criteria of no hyperhydricity, some, moderate, severe, and very severe, respectively.
[x] Mean separation was analyzed by Least Square Means at $\alpha = 0.05$. Capital letter indicates significance of BA treatment (BA) and small letter indicates significance of TDZ treatment (T).
[w] ANOVA based on GLIMMIX (Laplace) procedure was conducted to identify a significant effect among the two important factors. ^{ns}, **, and *** represent nonsignificant or significant at $P \leq 0.01$ or 0.001, respectively.

Effect of explant ages on shoot regeneration

The leaves and petioles of 7, 14, or 28 days old in vitro shoots were tested to optimize the regeneration efficiency of ‘Joan J’ and ‘Polana’. The media used for the experiment were WPM 2.5 µM BA + 1.0 µM TDZ for ‘Joan J’ and WPM 2.5 µM BA + 0.1 µM for ‘Polana’. The regeneration frequency significantly increased as decreasing explant age. Seven-day-old leaf explants of ‘Joan J’ had 70 % of regeneration frequency, which is significantly 1.7 times higher than the regeneration frequency of 14-days old explants (Fig. 3.3). In ‘Polana’ regeneration, the regeneration frequency was significantly increased in the 7-day old explants as 82.2 % (Fig. 3.4). In addition, the leaf explants of ‘Polana’ showed higher shoot regeneration frequency than the petiole explants. Therefore, use of 7-days-old explants can efficiently increase shoot regeneration of ‘Joan J’ and ‘Polana’.

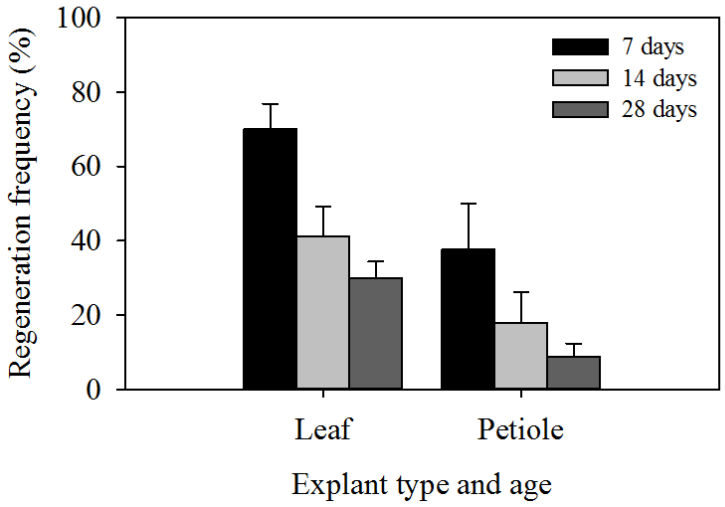

Figure 3.3. Regeneration frequency of 'Joan J' influenced by the age of explants. The bars on the graphs represent standard error.

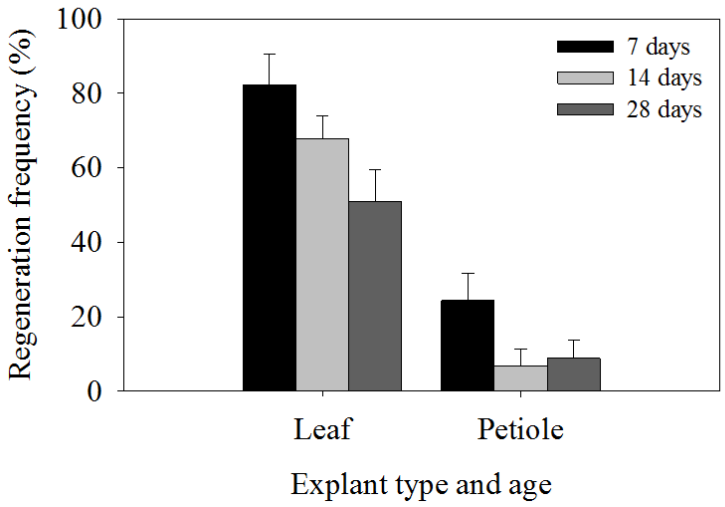

Figure 3.4. Regeneration frequency of 'Polana' influenced by the age of explants. The bars on the graphs represent standard error.

In vitro rooting influenced by auxin treatments

Auxin treatment significantly increased rooting frequency of ‘Joan J’. In vitro rooting of ‘Joan J’ with 10 µM NAA showed 100 % of rooting frequency with 2.2 roots per explant and 1.05 cm in length (Fig 3.1B and Fig 3.5). Root initiation from ‘Joan J’ shoots was observed at 2-3 weeks after the transplant. In this experiment, NAA at 10 µM showed a significantly higher rooting rate than the NAA at 5 µM, while no significant difference in rooting was observed between 5 and 10 µM IBA (Fig. 3.5). Although the auxin treatments showed significant effect in the number of regenerated roots, the auxin treated treatment did not show significant difference in the mean separation by LSD (Table 3.9). In addition, the root length was not significantly affected by the auxin treatments (Table 3.9).

In vitro rooting of ‘Polana’ appeared to be more difficult than ‘Joan J’ as no roots were developed when the same auxin treatments were used (data not shown). To induce root development of ‘Polana’, in vitro shoots were pulsed in the high concentrations (10, 20, 40, or 80 µM) of NAA or IBA for 7 days were attempted. The 7-day-pulse treatment enhanced the rooting

frequencies (Fig. 3.6). After auxin pulsing treatment, cuttings were continuously staying in the NAA containing medium could not develop any root, while cuttings were transferred to the NAA-free medium produce roots in all four treatments in which cuttings pulsed with 10 and 20 µM NAA showed a higher rooting frequency than other two treatments (40 and 80 µM NAA). Pulsed with IBA showed different results in which cuttings pulsed with IBA and continuously remained in the same medium produce roots except the treatment of 80 µM IBA (no roots developed). Cuttings pulsed with IBA for 7 days were then transferred to IBA-free medium developed roots in all four treatments. The effect of auxin pulsing showed significance in the root number and length, which were corresponded with the significance of the rooting frequency (Table 3.10). However, the result of mean separation showed that the averaged root number and length from the treatments were not significantly different with same letters. Considering these results, the 7-day-pulsing of 20 µM NAA was used for in vitro rooting of 'Polana' in the further transformation study.

Table 3.9. Effects of auxin treatments on in vitro root quality of 'Joan J'.

Treatment (µM)[z]	Root number	Root length (cm)
Control	0.67 b[y]	0.79 a
5 NAA	2.19 a	0.67 a
10 NAA	2.22 a	1.05 a
5 IBA	2.07 a	0.99 a
10 IBA	2.26 a	0.69 a
LSD	0.9[**]	0.5[ns]

[z] Performance was evaluated using root number and length, which are the mean of experimental repeats.
[y] Mean separation within *columns* and *factors* was determined by Least Significant Difference ($\alpha \leq 0.05$). Means associated with different letters represent significant differences.
[ns] and [**] represent nonsignificant or significant at $P \leq 0.01$, respectively.

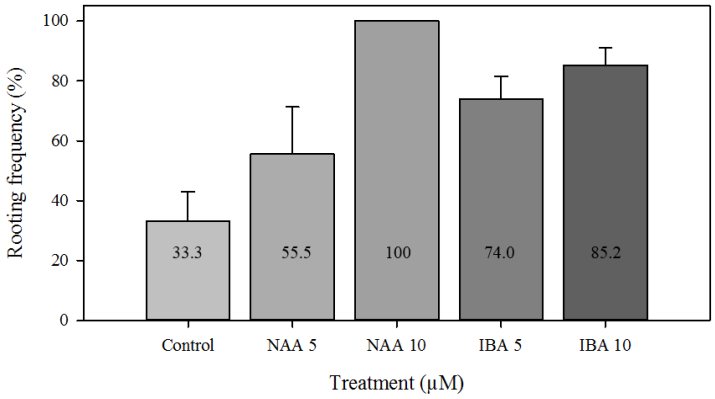

Figure 3.5. In vitro rooting of 'Joan J' influenced by auxin type and concentration. Bars on the graph represent standard errors within the treatment. NAA 10 treatment did not have bar since all experimental repeats developed roots.

Table 3.10. Effects of auxin treatments on in vitro root quality of ‘Polana’.

Treatment (µM)[z]	Root number	Root length (cm)
NAA 10	0.0 c[y]	0.0 b
NAA 20	0.0 c	0.0 b
NAA 40	0.0 c	0.0 b
NAA 80	0.0 c	0.0 b
7 days NAA 10 to 0	1.8 a	1.4 a
7 days NAA 20 to 0	1.8 a	1.3 a
7 days NAA 40 to 0	0.4 bc	0.1 b
7 days NAA 80 to 0	0.5 bc	0.2 b
IBA 10	1.7 a	1.4 a
IBA 20	1.3 ab	0.4 b
IBA 40	0.2 bc	0.1 b
IBA 80	0.0 c	0.0 b
7 days IBA 10 to 0	0.6 bc	0.5 b
7 days IBA 20 to 0	1.7 a	1.5 a
7 days IBA 40 to 0	0.5 bc	0.2 b
7 days IBA 80 to 0	1.8 a	1.7 a
LSD	1.0***	0.8***

[z] Cuttings were remained in the same medium or transferred to auxin-free medium after 7-day-pulsed with different auxins at different concentrations.
[y] Mean separation within *columns* and *factors* was determined by Least Significant Difference (α = 0.05). Means associated with different letters represent significant differences.
*** represents significant at $P \leq 0.001$.

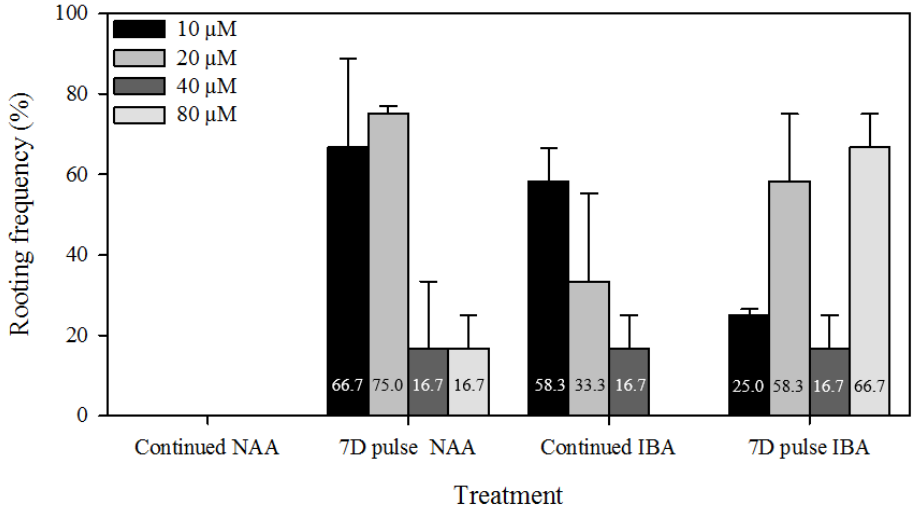

Figure 3.6. In vitro rooting of 'Polana' influenced by continuous and 7-day-pulse auxin treatments in 1/2 MS medium. Bars on the graph represent standard errors within the treatment.

Ex vitro rooting and acclimation

‘Joan J’ and ‘Polana’ successfully developed in the rooting medium (Fig. 3.1C; Fig. 3.2C). ‘Joan J’ showed 91.6 % of rooting frequency in the 500 µM IBA treatment and ‘Polana’ showed 94.4 % in the 500 µM BAA treatment (Fig. 3.7; Fig. 3.8). The water treatment (control) also showed a high rooting frequency of 80%, which were higher than that of in vitro control treatment (33.3 %). ‘Joan J’ in the 500 µM NAA treatment showed 47.2 % rooting frequency, which was the lowest in all ex vitro rooting treatments. The rooting frequency of ‘Joan J’ and ‘Polana’ was corresponding to the results of rooting performance. Although no significant differences in the ex vitro rooting rate of ‘Joan J’ and ‘Polana’ between the control (water) and 500 µM auxin treatments, considering the overall rooting performance (root number, length, and survival rate), the treatments of 500 µM IBA and 500 µM NAA were used for ex vitro rooting of ‘Joan J’ and ‘Polana’, respectively in further transformation experiment.

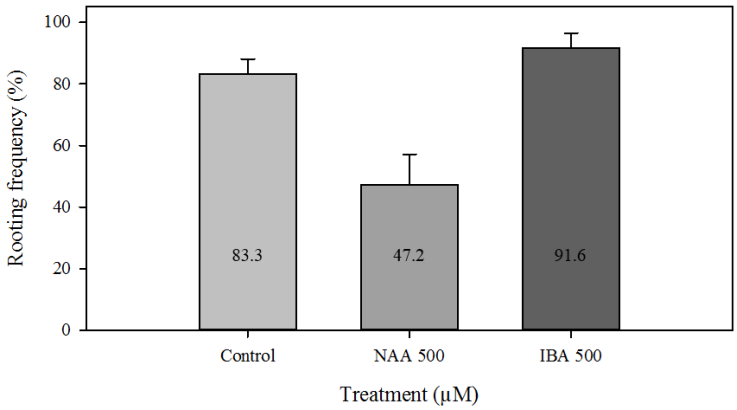

Figure 3.7. Ex vitro rooting of 'Joan J' influenced by auxin treatment. The bars on the graph represent standard errors among the experimental repeats.

Table 3.11. Effect of auxin treatments on ex vitro rooting performance of 'Joan J'.

Treatment (µM)[z]	Root number	Survival rate in the greenhouse (%)	Root length (cm)
Water	4.3 ab[y]	74.9 a	2.9 a
500 NAA	2.7 b	38.8 b	2.7 a
500 IBA	6.1 a	91.6 a	5.2 a
LSD	2.3[*]	30.9[*]	2.5[ns]

[z] Performance was evaluated using root number and length, which are the mean of experimental repeats.
[y] Mean separation within *columns* and *factors* was determined by Least Significant Difference (α = 0.05). Means associated with different letters represent significant differences.
[ns] and [*] represent nonsignificant or significant at $P \leq 0.05$, respectively.

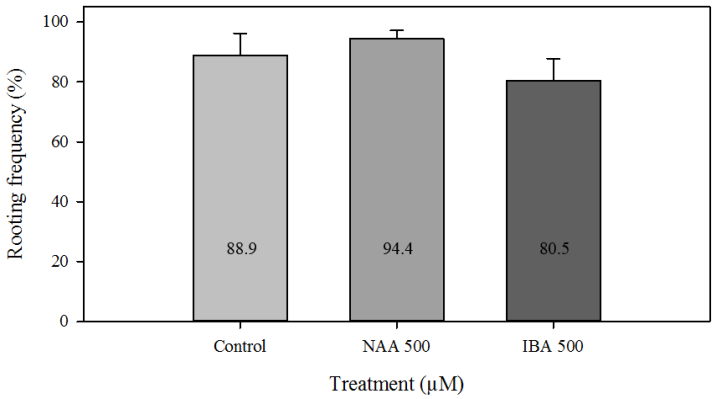

Figure 3.8. Ex vitro rooting of 'Polana' influenced by auxin treatment. The bars on graph represent standard errors among the experimental repeats.

Table 3.12. Effects of auxin treatments on ex vitro rooting performance of ‘Polana’.

Treatment (µM)[z]	Root number	Survival rate in the greenhouse (%)	Root length (cm)
Water	4.4 a[y]	88.8 ab	2.2 b
500 NAA	6.2 a	94.4 a	4.2 a
500 IBA	5.0 a	75.0 b	3.3 a
LSD	2.6[ns]	18.4[ns]	1.0[**]

[z] Performance was evaluated using root number and length, which are the mean of experimental repeats.
[y] Mean separation within *columns* and *factors* was determined by Least Significant Difference ($\alpha = 0.05$). Means associated with different letters represent significant differences.
[ns] and [**] represent nonsignificant or significant at $P \leq 0.01$, respectively.

Discussion

The objective of this study was to develop an efficient plant regeneration system for two red raspberry cultivars ‘Joan J’ and ‘Polana’. Previous studies of shoot regeneration of red raspberries showed inefficient with non-consistent results (Millan-Mendoza and Graham, 1999). Information on plant regeneration of red raspberry was limited to small number of cultivars (Pelto and Clark, 2001). Since no studies of plant regeneration of ‘Polana’ and ‘Joan J’, we conducted this research to test the influence of cytokinin, explant age, and explant type on shoot regeneration in order to develop an efficient plant regeneration protocol for two cultivars.

Direct organogenesis is always pursed for plant regeneration since shoot regeneration by indirect organogenesis via callus stage has more chances to cause somaclonal variations (Pelto and Clark, 2001). We used leaf and petiole as initial explants to regenerate shoots. Our results showed that in vitro shoots were regenerated with a uniform morphology (Fig. 3.1; Fig. 3.2), which agrees with Wu et al. (2009) that *Rubus* species could successfully regenerate shoots with the uniformity and vigorous growth.

Many regeneration studies in *Rubus* species have focused on the ratio of cytokinin to auxin (Pelto and Clark, 2001). The effect of combined cytokinin, such as TDZ, BA, or zeatin,

and auxin, such as IBA or NAA, on shoot regeneration was studied (Mezzetti et al., 1997; Pelto and Clark, 2001; Tsao, 1999); however, the results showed no reliable shoot regeneration (McNicol and Graham, 1990; Pelto and Clark, 2001; Tsao, 1999).

Research demonstrated that low concentrations (0.0 - 1.0 µM) of TDZ combined with cytokinins such as BA, kinetin or zeatin can efficiently stimulate shoot formation in woody plant tissue culture (Trigiano and Gray, 2011). The regeneration experiment by Graham et al. (1997) showed that application of TDZ induced shoot regeneration of *Rubus idaeus* with a wide range of regeneration frequency (6-90 %). In addition, Turk et al. (1994) and Millan-Mendoza and Graham (1999) reported that application of TDZ induced direct organogenesis of *Rubus* species. Furthermore, Dai et al. (2006) showed that the purple raspberry 'Amethyst' was more sensitively responded to TDZ than BA during in vitro regeneration in which the regeneration rate was achieved by 93.3 % in WPM with 1.0 µM TDZ, indicating TDZ is a good cytokinin source for plant regeneration of *Rubus* species.

In our experiment, the regeneration frequency of 'Joan J' significantly increased with the increasing concentration of TDZ and BA, whereas, the regeneration frequency of 'Polana' showed a decreasing tendency with a high TDZ and low BA level (Table 3.3; Table 3.7). Moreover, the highest TDZ treatment (1.0 µM) significantly stimulated shoot hyperhydricity. A similar result was derived from the study by Tsao (1999) in which a high concentration of TDZ (5.0 and 10.0 µM) decreased the number of regenerated shoots and stimulated shoot hyperhydricity. Although the highest concentration of TDZ in our experiment (1.0 µM) was lower than that in the study of Tsao (1999), the exposure to combined TDZ and BA (5.0 µM) might be the cause of the glassy appearance of shoots, which were also reported by Huetteman and Preece (1993) and Hazarika (2006). Responses of raspberry cultivars to TDZ and BA varied

in different genotypes (Mokotedi et al., 2000; Pelto and Clark, 2001; Wu et al., 2009). Such different responses were observed in different genotypes in *Rubus idaeus* including 'Ruby', 'Autumn Bliss', 'Heritage', and 'Latham' cultivars (Mezzetti et al., 1997; Millan-Mendoza and Graham, 1999; Tsao, 1999; Turk et al., 1994; Zawadzka and Orlikowska, 2006a). Thus, determination of the effect of cytokinin type and level should be done for each of individual cultivars/genotypes, particularly for certain cytokinins including TDZ.

Age of explants is an important factor influencing plant regeneration (Gilissen et al., 1996; Prakash and Gurumurthi, 2009; Tsao, 1999). The result of the age experiment showed that the 7-day-old explants produced the highest shoot regeneration frequencies for 'Joan J' and 'Polana' cultivars. Dhar and Joshi (2005) reported that the shoot differentiation was more efficiently initiated from the 10- and 15-day-old seedling of *Saussurea obvallata* than the 20-day-old seedling. Prakash and Gurumurthi (2009) showed the higher callus regeneration frequency with 10-day-old explants than use of 15-day old explants of *Eucalyptus camaldulensis*. The regeneration rate in the transgenic plants of *Jatropha curcas* L. was significantly higher in the youngest explants than other tissues (Mazumdar et al., 2010). This efficient regeneration of younger explants is because of metabolic activities in young explants, which provide great potential to be differentiated into a desirable morphology under suitable PGRs (Famiani et al., 1994; Mazumdar et al., 2010). In addition, Mazumdar et al. (2010) reported that variant concentrations of endogenous hormones based on explant ages resulted in the improved frequency in shoot regeneration of young explants.

Different plant tissues show different responses to PGRs in micropropagation (Pelto and Clark, 2001). In plant regeneration of *Rubus* species, various plant tissues such as immature leaves, petiole segments, axillary buds, and cotyledons, were tested (McNicol and Graham, 1990;

Pelto and Clark, 2001; Tsao, 1999). In our experiment, leaf explants showed a significantly higher shoot regeneration frequency than petioles in the same PGR condition (Fig. 3.2; Fig. 3.4; Fig. 3.5; Fig. 3.6). These results are corresponded with the results of McNicol and Graham (1990) and Georgieva et al. (2004). McNicol and Graham (1990) reported that the average numbers of regenerated shoots from leaf and petiole explants were 25.4 and 5.4 shoots, respectively. Georgieva et al. (2004) also reported leaf explants had a higher shoot regeneration rate (40 %) than petioles (18 %) in Bulgarian raspberry cultivars. Mezzetti et al. (1997) reported that the shoot formation rate in leaf explants (37 %) was higher than petiole explants (20%) in the 'Hull Thornless' cultivar. Previous and our studies reported that leaf tissues should be better explants than petioles for plant regeneration of *Rubus* species (Debnath, 2014; Graham et al., 1997; Lenz et al., 2016; Turk et al., 1994; Zawadzka and Orlikowska, 2006b).

Rooting of in vitro shoots can be achieved by either in vitro or ex vitro with or without auxin treatment (George et al., 2008b; Makunga et al., 2005). To optimize in vitro rooting for 'Joan J' and 'Polana', we tested concentrations and types of auxin based on the experimental result from Lenz et al. (2016). In our experiment, 'Joan J' at 10 µM NAA showed 100 % of rooting frequency (Fig. 3.5). 'Polana' did not show any root formation in the same experiment as 'Joan J'. Therefore, we treated in vitro shoots/cuttings in various auxins for seven days (pulsed), and then either left the cuttings in the medium or transferred to the auxin-free medium. 'Polana' showed an improved rooting frequency (75.0 %) in the auxin-free medium after the 7-day-pulsing of 20 µM NAA, while no root developed if cutting left in the NAA-containing medium (Fig. 3.6). A similar result was seen in the research of Woo and Wetzstein (2008) in which 5-day-pulsed in 100 or 150 µM IBA significantly increased rooting percentage (90 %) of *Elliottia racemosa*. The efficiency of auxin pulsing is explained by the study of Klerk et al. (1995) that an

application of auxin on cuttings initiates the first cell division at 24 to 96 hours from the auxin treatment and supplemented auxin treatment after 96 hours inhibits a root differentiation by an outgrowth of root primordia in apple micro cuttings.

The effect of being pulsed of in vitro cuttings with high concentration of auxin on rooting was verified in ex vitro rooting of 'Joan J' and 'Polana'. Similar results were also reported from other species (George et al., 2008b; Madhulatha et al., 2004; Trigiano and Gray, 2011; Wu et al., 2009). We gradually acclimated 'Joan J' and 'Polana' during 4 weeks with a high plant survival rate of 91.6 and 94.4 % in the greenhouse, respectively. The higher rooting frequency of ex vitro rooting than in vitro rooting indicates that rooting via ex vitro more efficiently induced root formation. George et al. (2008a) reviewed that in vitro plants are less effective to develop root system themselves because of inhibited photosynthesis from a long exposure to sucrose and in low light condition. However, once in vitro cuttings are transplanted into the soil and being adapted to ex vitro condition, these plants recovery their own ability to synthesis carbon complexes and then efficiently develop a rooting system to uptake nutrients from the soil. These inherited characteristics of plants resulted in the different frequency of root formation between ex vitro and in vitro.

In conclusion, plant regeneration protocol of two raspberry cultivars 'Joan J' and 'Polana' was developed. Based on the results, plant regeneration of 'Joan J' can be achieved using WPM supplemented with 2.5 µM BA + 1.0 µM TDZ for shoot regeneration and ½ MS with 10 µM NAA for in vitro rooting. The plant regeneration of 'Polana' can be achieved using WPM with 2.5 µM BA + 0.1 µM TDZ for shoot regeneration and ½ MS with 7-day-pulsing of 20 µM NAA for in vitro rooting. Ex vitro rooting with the treatment of 500 µM IBA for 'Joan J' and 500 µM NAA for 'Polana' can be also used.

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CHAPTER IV. *AGROBACTERIUM*-MEDIATED TRANSFORMATION OF RASPBERRY SPECIES (*RUBUS* SPP.)

Abstract

An *Agrobacterium*-mediated transformation was developed for a purple raspberry (*R. occidentalis* × *R. idaeus*) cultivar 'Amethyst'. *Agrobacterium*-mediated transformation method was used to transfer the *FER-LIKE Iron Deficiency-Induced Transcription Factor 1* gene cloned from *Populus tremula* (*PtFIT*) into the raspberry cultivar. The transformation was verified by the expression of the gene. Concentrations of selective agents (kanamycin or hygromycin B), inoculum densities of *Agrobacterium* suspension, co-cultivation times, and co-cultivation method were optimized to improve transformation frequency. 'Joan J' and 'Polana' could not regenerate transgenic lines, but 'Amethyst' produced the 12 transgenic lines that were confirmed to be transgenic lines by polymerase chain reaction (PCR) analysis with the specific primers. 'Amethyst' showed 3.3 ~ 4.4 percentages of transformation frequency under 25 $mg \cdot l^{-1}$ kanamycin selection, 3-day co-cultivation, and the inoculum density of OD_{600} 0.3. To study an expression level of the *PtFIT* gene of the transgenic lines under iron deficiency or sufficiency, clones of each transgenic line were grown in a hydroponic culture system. The result of real-time quantitative PCR further verified the expression of transgene (*PtFIT*) in the transgenic raspberry lines, but such expression was not corresponding to the iron status (sufficiency or deficiency).

Introduction

Plant genetic transformation of crop species has been widely used to improve plant performance and to analyze gene functions (Birch, 1997; Pitzschke and Hirt, 2010; Trigiano and Gray, 2011). *Agrobacterium*-mediated transformation usually includes the following steps: (1) preparation of gene vector and *A. tumefaciens* strain, (2) infection of explants with the bacteria

suspension, (3) co-cultivation, (4) selection of transgenic cells, (5) regeneration of the transgenic cells, and (6) verification of gene transfer (Birch, 1997; Griffiths et al., 2008; Lodge et al., 2007; Trigiano and Gray, 2011). Although general steps for *Agrobacterium*-mediated transformation are similar across many species, transformation rate is varied in species or even in cultivars (Birch, 1997). This implies that a precise optimization for each step is necessary to maximize the transformation frequency for a specific species or cultivar. Accordingly, researchers have studied the influence of selection system, inoculum densities, and co-cultivation times to develop an optimized transformation method (Jiang et al., 2015; Kim et al., 2016; Kokko and Kärenlampi, 1988; Li et al., 2017; Mathews et al., 1995; Zhang and Finer, 2016).

Selection system in *Agrobacterium*-mediated transformation is developed based on that transgenic cells with certain genes are resistant to corresponding antibiotics such as kanamycin and hygromycin B (Miki and McHugh, 2004). Expression of a selectable marker gene in the host genome confers such resistance to such a selectable agent that transgenic cells are able to survive, but non-transgenic cells are killed (Sundar and Sakthivel, 2008); therefore, the level of the agent must be determined. Individual plant genotypes have their own resistance threshold to a selectable agent for survival and regeneration. For a specific genotype and a specific gene vector, type and level of a selectable agent should be tested to determine the best level for both selection and regeneration.

Optimization of inoculum (bacterial cells) density is also need for an efficient *Agrobacterium*-mediated transformation. The inoculum density of the bacteria affects the infection ability of the bacteria, thus influence the transfer of a foreign gene into the host cells (Li et al., 2017). Research showed that plants turn on their defense system when they are attacked by plant pathogens (Zhang and Finer, 2016). They concluded that higher inoculum

densities of *Agrobacterium* up-regulated the expression of genes related to the defensive system, which inhibits an effective infection of plant cells by *Agrobacterium*. Thus, to develop an efficient transformation system, various levels of inoculum density should be tested.

Determining appropriate co-cultivation time of *Agrobacterium* and explants is important for improving transformation efficiency. During a co-cultivation time, *Agrobacterium* infects host explants to transfer the T-DNA region to plant cells (Birch, 1997; Zhang and Finer, 2016). This implies that an optimized co-cultivation time can ameliorate the ability of the bacteria to transfer a foreign gene into the host genome. In the transformation of soybean and sunflower, the transformation efficiency was improved when the co-cultivation time was set to 15 days (Li et al., 2017; Santarem et al., 1998; Zhang and Finer, 2016). However, many transformation studies have adopted 2-3 day-co-cultivation time due to the overgrowth of *Agrobacterium* occurring during a long co-cultivation time. Overgrowth of the bacteria will damage plant tissue and inhibit regeneration of transgenic cells (Birch, 1997; Jiang et al., 2015; Mathews et al., 1995; Zhang and Finer, 2016). Research showed that overgrowth of *Agrobacterium* decreased the expression of transgenes and resulted in necrosis of the explants of *Casuarina cunninghamiana* and *Brassica rapa* (Jiang et al., 2015; Sanimah et al., 2010). In nature, *Agrobacterium* infects a plant over a long co-cultivation time with a low inoculum density of the bacteria without overgrowth (Bourras et al., 2015). By taking advantage of this feature, Zhang and Finer (2016) reported that the combination of a long co-cultivation (15-days) with a low inoculum density (6×10^2 bacteria ml^{-1}) resulted in the highest transformation efficiency (23.7 %) of sunflower transformation. Therefore, optimization of the co-cultivation time and inoculum density is necessary to develop an efficient transformation system.

Iron is the second most abundant metal in the earth crust and has a critical role in the redox system within cells (Marschner, 2011). However, solubility of iron is extremely low because of alkaline soil conditions in nature (pH > 7.0). The chlorophyll synthesis in a plant is hindered by a low uptake rate of iron from the rhizosphere and this physiological disorder is called iron chlorosis (Marschner, 2011). Iron deficiency is often seen in many wood plants including raspberry species (Black et al., 2009; Jennings, 1988; Pritts, 2016; Smith et al., 2007). The iron uptake mechanism in non-graminaceous species can be summarized as under the acid condition (pH <7) around the rhizosphere, the gene activities in the ferric reductase oxidase (FRO) gene family increase and more Fe^{3+} (the forms plants cannot uptake and transport) was reduced to Fe^{2+} (the form plants can uptake and transport), increasing the iron availability for plants to uptake. Uptake of Fe^{2+} into the root cells is mediated by the iron-regulated transporter (*IRT*) genes (Marschner, 2011).

In previous studies from our group, the *PtFIT* gene (*FER-like iron deficiency-induced transformation factor 1*) was cloned from a European aspen (*Populus tremula*) tree and its functions related to iron uptake were studied (Huang and Dai, 2015). Huang and Dai (2015) confirmed that the expression of *FRO* and *IRT* is controlled by the *PtFIT* gene in *Populus*. The *PtFIT* gene belongs to the basic helix-loop-helix (*bHLH*), family of transcription factors. Their research found that the expression of *PtFIT* in *P. tremula* was up-regulated by iron deficiency and overexpression of the gene increased iron uptake in a few transgenic line of another *Populus* species (*P. canescens* × *P. grandidentata* 'C16') (Huang and Dai, 2015). Thus, our group hypothesized that overexpression of the *PtFIT* gene in raspberries may help mitigate its iron chlorosis.

The purpose of this study was to develop an efficient transformation system for three raspberry (*Rubus* spp.) cultivars 'Amethyst', 'Joan J', and 'Polana' and use the developed system for gene functional analysis in raspberry species. Aldwinckle and Malnoy (2009) reviewed the previous studies of raspberry transformation. They reported that the transformation frequencies varied from 0.37 % to 49.6 % in 'Comet', 'Candy', 'Chilliwak', 'SCR18242EF' (blackberry × raspberry), and 'Meeker' (Faria, 1993; Mathews et al., 1995). Mathews et al. (1995) reported that hygromycin selection at 10 $mg \cdot l^{-1}$ resulted in the effective transformation frequency of 'Meeker' (49.6 %). In contrast, Faria (1993) reported that kanamycin selection at 40 $mg \cdot l^{-1}$ established a stable transformation method for 'Comet' (0.37 %). Comparison of the previous studies showed that a suitable condition for an optimum transformation varies in raspberry genotypes. Limited information is available on how inoculum density and co-cultivation time influencing raspberry transformation frequency for raspberry species. Therefore, we designed a few experiments to determine an optimum selection system including optimum level of antibiotics and to evaluate the influence of inoculum density and co-cultivation time on transformation frequency, and to analyze the expression of the *PtFIT* gene that was transferred into 'Amethyst' raspberry using the developed method.

Materials and Methods

Plant materials and media

In vitro plants of the purple raspberry 'Amethyst' (*Rubus occidentalis* × *R. idaeus*) and red raspberry (*R. idaeus*) 'Joan J' and 'Polana' were maintained in Murashige and Skoog (MS) medium containing 2.5 µM 6-benzyladenine (BA), 0.5 µM gibberellic acid (GA_3), 3 % sucrose, and 0.525 % agar. Their cultures were subcultured every four weeks in GA7 Magenta boxes (Magenta Corp., Chicago, IL) with 40 ml medium in it. The regeneration systems were reported

Chapter III and the study of Lenz et al. (2016). The optimized PGRs were 1.0 µM thidiazuron (TDZ) for 'Amethyst', 2.5 µM BA + 1.0 µM TDZ for 'Joan J', and 2.5 µM BA + 0.1 µM TDZ for 'Polana'. The optimized PGRs were supplemented in Woody Plant Medium (WPM) containing 2 % sucrose, 0.525 % agar, and 200 $mg \cdot l^{-1}$ polyvinylpyrrolidone (PVP). The medium pH was adjusted to 5.2-5.3 prior to autoclaving. Cefotaxime (cef) at 250 $mg \cdot l^{-1}$ and carbenicillin (carb) at 500 $mg \cdot l^{-1}$ were added into the cooled medium at 65 ℃ to prevent growth of *Agrobacterium* during selection and regeneration. An optimized level of selective agent (kanamycin or hygromycin B) was added to selection medium to screen transgenic tissues. The medium (25 ml) was poured into a Fisherbrand™ petri dish with a clear lid (Fisher Scientific International Inc., Pittsburgh, PA). All plates were placed in a drawer with 24 h dark condition at 18 ℃ for four weeks and then transferred to the culture room with a 16/8 (light/dark) photoperiod at 36 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity and a temperature of 25/18 ℃ (day/night).

Preparation of the *Agrobacterium* strain with the binary plasmid vectors

Agrobacterium tumefaciens strain EHA105 was used. Two different binary vectors, pBI121 and pCAMBIA1300 (CAMBIA, Canberra, Australia), were used. The pBI121 is composed of a *nopaline synthase* (nos) promoter (pro)/terminator (ter), a cauliflower mosaic virus (CaMV) promoter/nos-terminator, the *PtFIT* gene, and the neomycin phosphotransferase II (*nptII*) gene (Fig. 4.1A) (Huang, 2015). In the pCAMBIA1300, CaMV promoter/terminator, Super-promoter/nos-terminator, the *PtFIT* gene, and the hygromycin phosphotransferase (*hpt*) gene were inserted into the multiple cloning sites of pCAMBIA1300 (Fig. 4.1B) (Huang, 2015). The *PtFIT* gene was used to determine whether the transformation system is working or not by PCR and RT-PCR. These binary vectors were introduced individually into EHA105 competent

cells by the freeze-thaw method (Chen et al., 1994). Then, the bacteria cells were proliferated in Luria-Bertani (LB) medium with 100 mg·l^{-1} kanamycin.

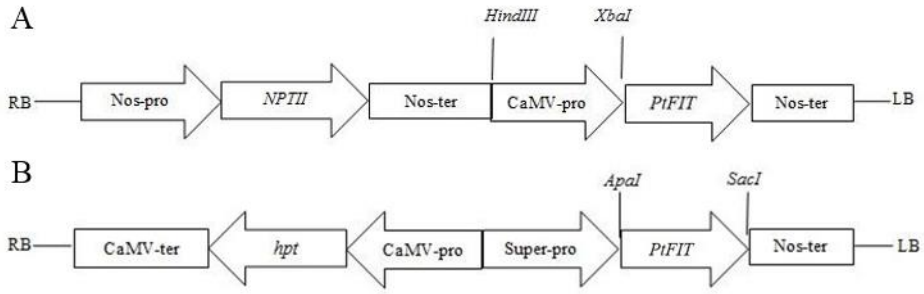

Figure 4.1. Schematic representation of binary vectors (A: pBI121 and B: pCAMBIA1300) (Huang, 2015).

Preparation of infection suspension and plant transformation

Agrobacterium was streaked on LB medium with 100 mg·l^{-1} kanamycin for 2 days at 28 ℃ incubator. A starter culture was initiated from a single colony in 2 ml LB containing 100 mg·l^{-1} kanamycin and was grown for 24 hours with agitation at 200 rpm and 28 ℃. Then, 100 µl of starter culture was added into 25 ml LB medium containing 100 mg·l^{-1} kanamycin for overnight with agitation at 200 rpm and 28 ℃. Once the bacteria growth is achieved to an optical density at 600 wavelength (OD_{600}) = 0.55 (10^8-10^9 bacteria mL^{-1}), the bacteria cells were collected by centrifuging at 3,000 rpm and 4 ℃ for 15 min. The cell pallet was suspended in 25 ml liquid of the optimized regeneration medium with 20 µM acetosyringone (AS) and agitated at 28 ℃ and 200 rpm for 1 hour to activate the virulent genes of the bacteria. Seven-day-old leaf segments (0.5 cm^2) and petioles (0.5 cm) were submerged in the infection medium for 30 min with some agitation. Infected explants were blot to dry using the autoclaved paper towel and then inoculated into a co-cultivation medium (the optimized regeneration medium containing 200 µM AS without antibiotics). Co-cultivated explants were transferred to the optimized medium containing 250 mg·l^{-1} cef, 500 mg·l^{-1} carb, and the optimized level of antibiotics.

Determination of antibiotics level for selection

Explants (leaf and petiole) collected from the three cultivars were inoculated in the regeneration medium with various concentrations of kanamycin or hygromycin B. Influence of *Agrobacterium* infection on plant resistance to antibiotics was also investigated by infecting the explants with EHA105 competent cells (no plasmid to confer the resistance to the selective agent) using the method described above. Each petri dish contained samples of 10 leaf and 5 petiole explants. The infected or non-infected explants were inoculated into a petridish containing 25 ml of the optimized WPM for each cultivar. The kanamycin concentration was 0 to 50 $mg \cdot l^{-1}$ with 2.5 or 5.0 $mg \cdot l^{-1}$ interval. The hygromycin B level was from 0 to 20 $mg \cdot l^{-1}$ with 2.5 $mg \cdot l^{-1}$ interval. The survival rate of explants, callusing, and shoot regeneration from explants were evaluated. This experiment was conducted using Completely Randomized Design (CRD) with three experiments and three replicates (plate) of each treatment. The treatment number of each cultivar varied due to the differences in their resistance to selective agents.

Optimization of co-cultivation time and inoculum density

Agrobacterium tumefaciens EHA105 harboring either pBI121 or pCAMBIA1300 was used. During the preparation of infection solution, the bacteria suspension with OD_{600} 0.55 were diluted into 1, 10^{-2}, 10^{-4}, or 10^{-7} dilution. 'Amethyst' raspberry leaves and petioles (7-days-old were infected explants were placed in the dark for co-cultivation for 3, 5, 10, and 15 days. Each petri dish as one replicate contained 10 leaves (0.5 cm^2) and 5 petioles (0.5 cm). After co-cultivation, the explants were transferred to the medium with an optimized level of antibiotics determined in the previous experiment. All cultures were subcultured every four weeks. The experimental design was a CRD with split-split plot of 2 binary vectors, 4 dilutions of the

suspension, and 4 co-cultivation times with three experiments and three replicates (plate) of each treatment. Non-infected explants in the medium without selectable agent were used as a control.

***Agrobacterium*-mediated transformation for 'Amethyst', 'Joan J', and 'Polana'**

Based on the results from the previous experiment, a modified method was used to do infection and co-cultivation. In brief, explants (leaf and petiole) of 'Amethyst', 'Joan J', and 'Polana' cultivars were infected in the infection solution at OD_{600} 0.3 for 10 min. The infected explants were paper-blotted dry to remove remaining infection solution. The explants were then placed in an empty petri dish for co-cultivation for 3 day and 5 days. The co-cultivated explants were transferred to the optimized regeneration medium with the optimum level of selectable agent for each cultivar. All cultures were in the dark for the first 4 weeks, then moved to the culture room with 16/8 photoperiod. From the second 4 weeks, a half strength of selectable agent was applied to maximize regeneration of transgenic cells. At the third 4 weeks, a full strength was applied again to select true transgenic shoots. Each petri dish contained 10 leaves (0.5 cm^2) and 5 petioles (0.5 cm) as one replicate. This experiment was designed as a CRD with 12 treatments (3 cultivars × 2 co-cultivation time × 2 binary vectors), three experiments, and three replicates (plate) per treatment. For 'Amethyst' and 'Polana' with pBI121, five experiments were performed to expand the size of experiment. Non-infected explants and explants infected by EHA105 competent cells (empty vector) were used as a control.

Confirmation of transgenic lines

Putative transgenic plants were subjected to in vitro rooting in the medium with cef (250 $mg \cdot l^{-1}$), carb (500 $mg \cdot l^{-1}$) and the determined level of selectable agent. The in vitro rooting media were 1/2 MS with 10 µM indole-3-butyric acid (IBA) for 'Amethyst', 10 µM 1-

naphthaleneacetic acid (NAA) for 'Joan J', and 7-day-pulse of 20 µM NAA for 'Polana'. The rooting frequency was recorded prior to polymerase chain reaction (PCR) confirmation.

To confirm transgenic lines by PCR analysis of the transgenes (*PtFIT* and *nptII*), genomic DNAs of transgenic lines were extracted from 100 mg of frozen leaf segment based on the method of Huang (2015). The concentration of extracted DNA was quantified by NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). PCR amplification was performed in a 25 µl reaction solution containing 5 ng of DNA, 0.4 µM of each primer sets (Table 4.1), 0.2 mM dNTP, 1.5 mM $MgCl_2$, 1× GoTaq® Flexi buffer, and 1 U *Taq* DNA polymerase. The thermal cycler conditions were followed: one cycle of 94 ℃ for 3 min, 35 cycles of denaturing for 30 seconds at 94 ℃, annealing for 40 seconds at 56 ℃, and extension for 50 seconds at 72 ℃, and one cycle of 72 ℃ for 5 min. The amplified products were separated in a 1 % agarose gel stained by EtBr at 110 voltages for 1 hour. The gel images were captured by AlphaImager® Gel Documentation System (Protein Simple Inc., Santa Clara, California, USA) under UV light exposure.

Table 4.1. List of primers used in PCR confirmation, semi-quantitative PCR, and RT-PCR.

Primer Name	Sequence (5'-3')	Application
nptII F1	GGCTATGACTGGGCACAACA	PCR confirmation of transgenic lines
nptII R1	GATACCGTAAAGCAGGAGGAA	
UPtFIT F1	GGACCCCCACCCACGAG	PCR confirmation of transgenic lines
UPtFIT R1	AACCAAGGACCGCAAAGCATA	
GAPDH F1	TGTTCTTCCTCTGCGAGTTG	Semi-quantitative PCR/RT-PCR
GAPDH R1	GCGGAACTCGAAACTAAAAGG	
PtFIT F1	ACCGCCACAACGACTAAGAAGAC	Semi-quantitative PCR/RT-PCR
PtFIT R1	AACCAAGGACCGCAAAGCATA	

Hydroponic culture of transgenic lines for analysis of *PtFIT* function under iron deficiency

A hydroponic culture system was established to determine the expression level of the *PtFIT* gene in the transgenic raspberry plants. The PCR-confirmed transgenic lines were inoculated into in vitro rooting media for 3 weeks and then transferred to the hydroponic culture system. Four transgenic clones from each transgenic line were transplanted into plastic containers (54 × 41 × 17 cm) with 60-hole PVC plate covers, an air pump (TOPFIN Auarium Air Pump, Model: AIR-8000), and air stones (Blue Ribbon® Blu-Mist™ 12'' Air Stone, Model: 206). The containers were filled with 15 liters of Hoagland's solution (Hoagland and Arnon, 1950). The solution was refreshed every two weeks. The containers were covered by a plastic film at the start of hydroponic culture to prevent dehydration of the plants. The film was gradually pierced by an awl to make holes during 6 weeks of acclimation period. After the acclimation, the plastic film was removed from the container.

Acclimated plants were treated by iron sufficient or deficient solution for 6 days. For the iron sufficient condition, 30 µM Fe^{2+}- Ethylenediaminetetraacetic acid (EDTA) was added to the Hoagland's solution. In the iron deficient condition, 200 µM ferrozine was supplemented into the solution in which iron was not supplemented. After the treatment for six days, root tissues were collected from each plant of three biological replicated of each transgenic line. Tissue samples were frozen in liquid nitrogen immediately and stored in −80 °C freezer until use. The experimental design was based on CRD with 28 treatments (2 treatments × 14 lines). Each treatment had 3 replicates.

RNA extraction, cDNA synthesis, semi-quantitative PCR, and RT-PCR

Total RNAs were extracted from the collected root samples using SV Total RNA Isolation system (Promega, Madison, WI, USA) based on the manufacturer's instructions. The

isolated RNAs were quantified by NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). A synthesis of cDNA was conducted using SuperScript™ III Reverse Transcriptase Kit (invitrogen by Thermo Fisher Scientific Inc., Carlsbad, CA, USA) according to the manufacturer's instructions.

The semi-quantitative PCR was conducted to check quality of cDNA prior to real-time quantitative PCR (RT-PCR). Primers used in semi-quantitative PCR corresponded with the *PtFIT* gene and the internal reference gene *GAPDH* (glyceraldehyde-3-phosphate-dehydrogenase) were designed according to Huang and Dai (2015) and Gotame et al. (2014) (Table 4.1). PCR amplification of the cDNA with *GAPDH* specific primers was conducted to evaluate contamination and quality of cDNA for RT-PCR. The PCR amplification was carried out in a 16 µl reaction, which is composed of 5 ng cDNA template, 0.375 µM of forward and reverse primer, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 1× Green GoTaq® Flexi buffer, and 5 Unit *Taq* DNA polymerase. The thermal cycler conditions were followed: one cycle of 94 ℃ for 3 min, 30 cycles of denaturing for 30 seconds at 94 ℃, annealing for 40 seconds at 56 ℃, and extension for 50 seconds at 72 ℃, and one cycle of 72 ℃ for 5 min. The PCR products were separated in a 2 % agarose gel stained by EtBr at 110 voltages for 30 min. The gel images were captured by AlphaImager® Gel Documentation System (Protein Simple Inc., Santa Clara, California, USA) under UV light exposure.

The expression of the *PtFIT* gene in the root of the transgenic raspberries in response to iron deficiency and iron sufficiency were evaluated using CFX96 Touch™ Real-Time PCR system (Bio-Rad, Hercules, CA, USA). The amplification was carried out in a 25 µl reaction containing 7.5 ng cDNA template, 0.4 µM of forward and reverse primers, and 12.5 µl PerfeCTa SYBR Green SuperMix, Low ROX (2X) kit (Quanta Biosciences, Inc, Gaithersburg, MD, USA)

according to the manufacture's instruction. *PtFIT* primers for the target gene and *GAPDH* primers for the reference gene were used to perform RT-PCR (Table 4.1). Values of relative expression level were calculated based on a C_t value of the *PtFIT* gene and *GAPDH* gene. Amplification specificity was evaluated by melting curve analysis according to the manufacturer's recommendation. Each biological replicate was replicated three times as technical replicates.

Statistical analysis

Sigmoidal regression was conducted to develop antibiotics-killing curve based on the study of Liu et al. (2004). The regression was performed by SigmaPlot ver. 12.5 (Systat Software Inc, San Jose, CA) with three parameters. Analysis of variance (ANOVA) was conducted to calculate standard errors on the killing curves by SAS ver. 9.4 (SAS Institute Inc, Cary, NC) with the GLM procedure based on type III error due to the loss of samples in the tissue culture. In evaluation of the result of transformation, the shoot regeneration, rooting, and transformation frequency were analyzed by the GLIMMIX (Laplace) method of SAS software to identify a significance of inoculum density and co-cultivation time on the result. In the optimization experiment, mean separation was conducted with Least Square Means of the GLIMMIX method for the unbalanced experiment result in plant tissue culture. Mean separations in the antibiotic tests and transformation of three cultivars were used Least Significant Difference at $P \leq 0.05$ in the GLM method. The standard errors from the result of SAS software was used to bar graphs for indicating the standard deviation of the sampling distribution. Graphs were developed by SigmaPlot Version 12.5 (Systat Software Inc, San Jose, CA).

Results

Hygromycin B and kanamycin tests

Various concentrations of hygromycin B or kanamycin were set up to determine the tolerance of raspberry cultivars ‘Amethyst’, ‘Joan J’, and ‘Polana’ to the selective agents. The influence of *Agrobacterium* infection on the resistance was also investigated. The survival rate, number of regenerated shoots, and callusing were recorded.

For the hygromycin test, the survival rate of ‘Amethyst’ showed 0 % at the 12.5 $mg \cdot l^{-1}$ for non-infected explants and at 10.0 $mg \cdot l^{-1}$ for EHA105 competent cells-infected (Fig. 4.2). No shoot regeneration and callusing were observed in the 10.0 $mg \cdot l^{-1}$ treatment (Table 4.2). The non-infected and infected ‘Joan J’ explants showed the completely inhibited growth at 10 $mg \cdot l^{-1}$ (Fig 4.3). This result corresponded with the no or reduced shoot regeneration at 10 $mg \cdot l^{-1}$ or higher treatments in the result of table 4.3. For ‘Polana’, 17.5 $mg \cdot l^{-1}$ and 15.0 $mg \cdot l^{-1}$ hygromycin B killed all vegetative tissues, non-infected and infected explants, respectively (Fig. 4.4). The evaluation in Table 4.4 showed that the presence of hygromycin B inhibited the regeneration of shoot and callusing in all concentrations. It appeared that the infection by EHA105 competent cells decreased the tolerance to the hygromycin B. The concentration of hygromycin B was optimized as 10.0, 10.0, and 15.0 $mg \cdot l^{-1}$ for ‘Amethyst’, ‘Joan J’, and ‘Polana’, respectively.

In contrast to the results of hygromycin test, the EHA105 infection showed no significant effect on the tolerance to kanamycin. All ‘Amethyst’ explants remained green until concentration of kanamycin reached to 45.0 $mg \cdot l^{-1}$ (Figure 4.5). However, the shoot regeneration and callusing were completely inhibited when the concentrations were equal to or higher than 25.0 $mg \cdot l^{-1}$ (Table 4.5). For ‘Joan J’, infection of EHA105 competent cells showed no differences at the minimum killing level. The non-infected and infected explants showed the completely inhibited

growth at 20.0 mg·l^{-1} kanamycin (Fig 4.6). ‘Joan J’ showed a decreased survival rate, decreased callusing and reduced regenerated shoots as increasing concentrations of kanamycin and the growth of explants was completely inhibited at 20.0 mg·l^{-1} of kanamycin (Table 4.6). For ‘Polana’, infection with EHA105 competent cells did not weaken its resistance to the antibiotics since the minimum killing concentrations for non-infected and infected explants were 25.0 mg·l^{-1} and 30.0 mg·l^{-1}, respectively (Fig. 4.7 and Table 4.7). EHA105 infection showed no difference on the minimum killing level of kanamycin selection. The kanamycin concentration was optimized as 25.0, 20.0, and 30.0 mg·l^{-1} in ‘Amethyst’, ‘Joan J’, and ‘Polana’, respectively.

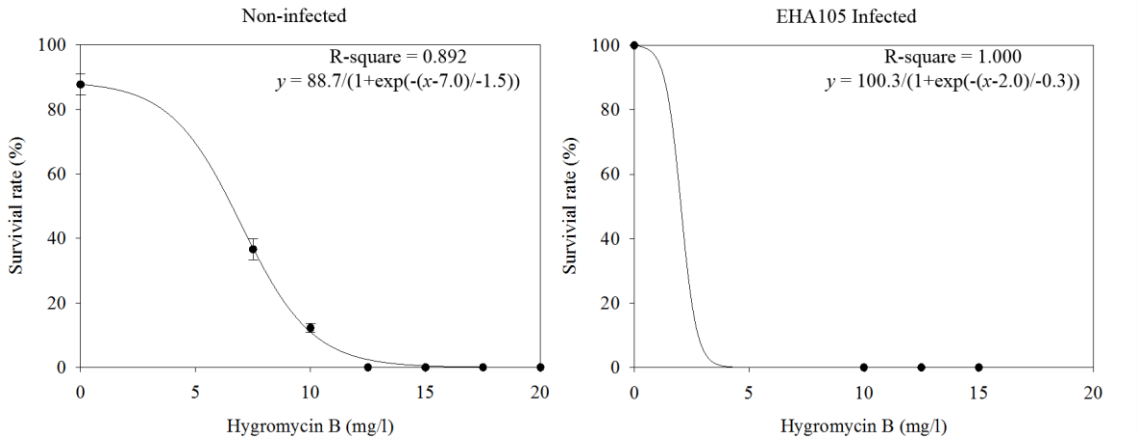

Figure 4.2. Hygromycin B test for ‘Amethyst’ with non-infection and infection explants. The bars on the dots represent standard errors.

Table 4.2. Evaluation of hygromycin B test for ‘Amethyst’.

	Number of regenerated shoots		Callusing[z]	
Hygromycin B ($mg \cdot l^{-1}$)	Non-infected explants	Infected explants	Non-infected explants	Infected explants
0.0	3.1 a[y]	0.0 a	2.3 a	1.9 a
7.5	1.0 b	-	1.1 b	-
10.0	0.0 c	0.0 a	0.0 c	0.0 b
12.5	0.0 c	0.0 a	0.0 c	0.0 b
15.0	0.0 c	0.0 a	0.0 c	0.0 b
17.5	0.0 c	-	0.0 c	-
20.0	0.0 c	-	0.0 c	-
LSD	0.5^{***}	0.0^{ns}	0.4^{***}	0.1^{***}

[z] Callusing was scored 0, 1, 2, and 3 based on no callus, < 1 mm, 1-2 mm, and > 2 mm, respectively.
[y] Mean separation within column and treatments was determined by Least Significant Difference ($\alpha = 0.05$).
[ns] and [***] represent non-significant or significant at $P \leq 0.001$, respectively.

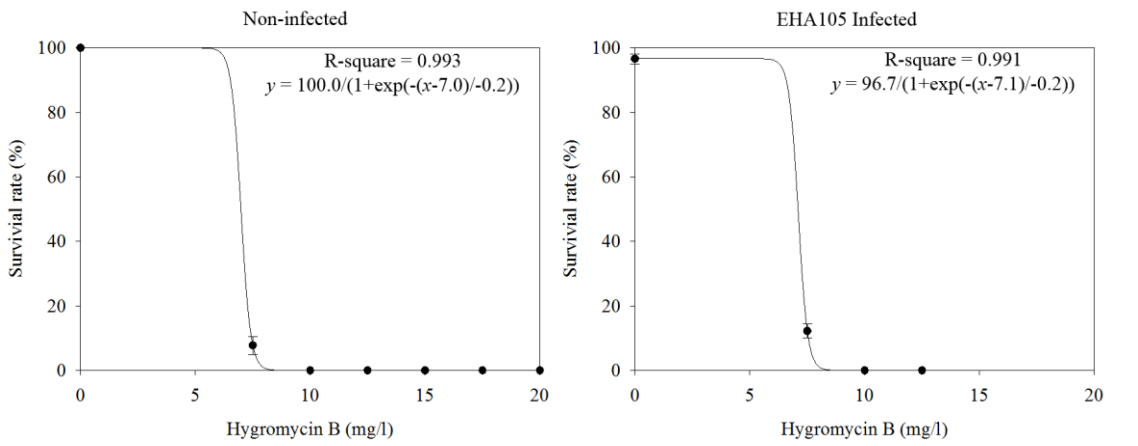

Figure 4.3. Hygromycin B test for ‘Joan J’ with non-infection and infection conditions. The bars on the dots represent standard errors.

Table 4.3. Evaluation of hygromycin B test for ‘Joan J’.

	Number of regenerated shoots		Callusing[z]	
Hygromycin B ($mg \cdot l^{-1}$)	Non-infected explants	Infected explants	Non-infected explants	Infected explants
0.0	3.9 a[y]	0.1 a	3.0 a	0.9 a
7.5	0.4 b	0.0 a	0.2 b	0.0 b
10.0	0.0 b	0.0 a	0.0 c	0.0 b
12.5	0.0 b	0.0 a	0.0 c	0.0 b
15.0	0.0 b	-	0.0 c	-
17.5	0.0 b	-	0.0 c	-
20.0	0.0 b	-	0.0 c	-
LSD	0.7[***]	0.2[ns]	0.1[***]	0.2[***]

[z] Callusing was scored 0, 1, 2, and 3 based on no callus, < 1 mm, 1-2 mm, and > 2 mm, respectively.

[y] Mean separation within column and treatments was determined by Least Significant Difference ($\alpha = 0.05$). Means associated with different letters represent significant differences.

[ns] and [***] represent non-significant or significant at $P \leq 0.001$, respectively.

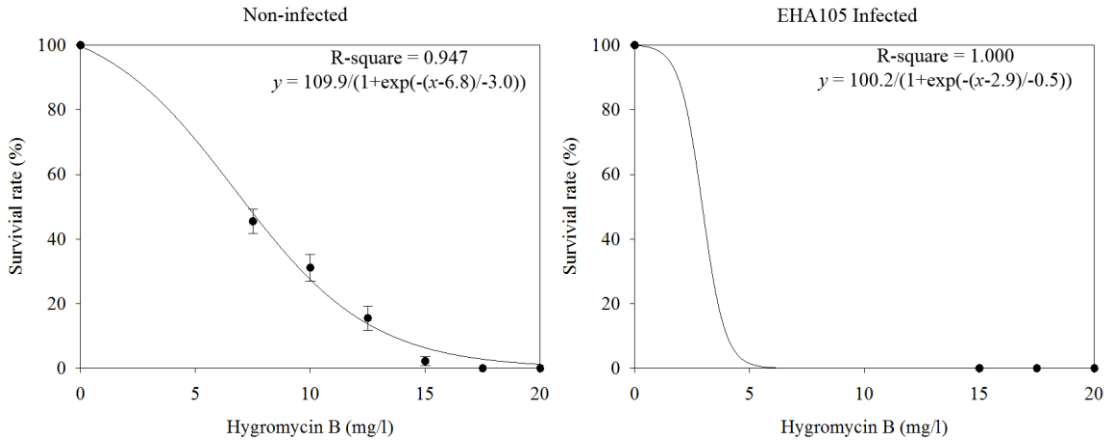

Figure 4.4. Hygromycin B test for ‘Polana’ with non-infection and infection conditions. The bars on the dots represent standard errors.

Table 4.4. Evaluation of hygromycin B test for ‘Polana’.

	Number of regenerated shoots		Callusing[z]	
Hygromycin B ($mg \cdot l^{-1}$)	Non-infected explants	Infected explants	Non-infected explants	Infected explants
0.0	4.2 a[y]	0.1 a	2.9 a	1.9 a
7.5	2.1 b	-	1.0 b	-
10.0	1.9 b	-	1.0 b	-
12.5	0.4 c	-	0.8 b	-
15.0	0.0 c	0.0 a	0.1 c	0.0 b
17.5	0.0 c	0.0 a	0.0 c	0.0 b
20.0	0.0 c	0.0 a	0.0 c	0.0 b
LSD	0.8^{***}	0.2^{ns}	0.2^{***}	0.1^{***}

[z] Callusing was scored 0, 1, 2, and 3 based on no callus, < 1 mm, 1-2 mm, and > 2 mm, respectively.
[y] Mean separation within column and treatments was determined by Least Significant Difference ($\alpha = 0.05$). Means associated with different letters represent significant differences.
[ns] and [***] represent non-significant or significant at $P \leq 0.001$, respectively.

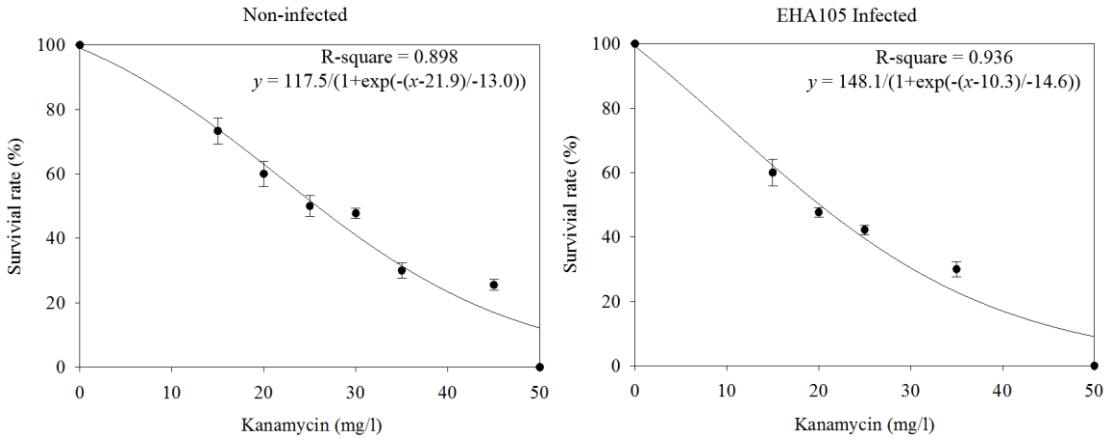

Figure 4.5. Kanamycin test for ‘Amethyst’ with non-infection and infection conditions. The bars on the dots represent standard errors.

Table 4.5. Evaluation of kanamycin test for ‘Amethyst’.

	Number of regenerated shoots		Callusing[z]	
Kanamycin ($mg \cdot l^{-1}$)	Non-infected explants	Infected explants	Non-infected explants	Infected explants
0.0	2.5 a[x]	1.2 a	1.8 a	1.9 a
15.0	0.0 b	0.0 b	1.1 b	1.1 b
20.0	0.0 b	0.0 b	1.0 b	0.8 c
25.0	0.0 b	0.0 b	0.0 c	0.0 d
30.0	0.0 b	-	0.0 c	-
35.0	0.0 b	0.0 b	0.0 c	0.0 d
45.0	0.0 b	-	0.0 c	-
50.0	0.0 b	0.0 b	0.0 c	0.0 d
LSD	0.2***	0.3***	0.2***	0.2***

[z] Callusing was scored 0, 1, 2, and 3 based on no callus, < 1 mm, 1-2 mm, and > 2 mm, respectively.
[y] Mean separation within column and treatments was determined by Least Significant Difference ($\alpha = 0.05$). Means associated with different letters represent significant differences.
*** represents significant at $P \leq 0.001$, respectively.

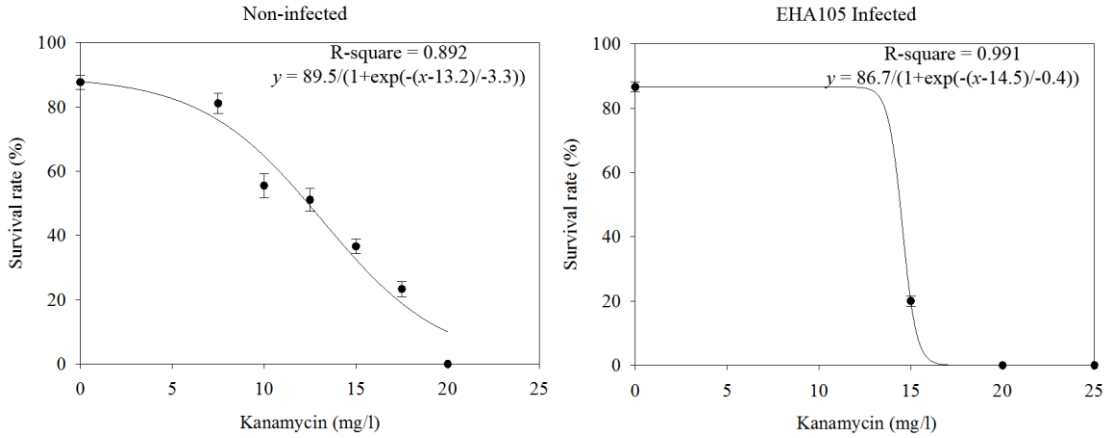

Figure 4.6. Kanamycin test for ‘Joan J’ with non-infection and infection conditions. The bars on the dots represent standard errors.

Table 4.6. Evaluation of kanamycin test for ‘Joan J’.

	Number of regenerated shoots		Callusing[z]	
Kanamycin ($mg \cdot l^{-1}$)	Non-infected explants	Infected explants	Non-infected explants	Infected explants
0.0	2.7 a[x]	0.3 a	2.6 a	0.9 a
7.5	0.1 b	-	2.2 ab	-
10.0	0.2 b	-	1.9 bc	-
12.5	0.1 c	-	1.5 c	-
15.0	0.1 c	0.1 a	0.8 d	0.0 b
17.5	0.1 c	-	0.3 e	-
20.0	0.0 c	0.0 a	0.0 e	0.0 b
25.0	-	0.0 a	-	0.0 b
LSD	0.6^{***}	0.4^{ns}	0.4^{***}	0.1^{***}

[z] Callusing was scored 0, 1, 2, and 3 based on no callus, < 1 mm, 1-2 mm, and > 2 mm, respectively.
[y] Mean separation within column and treatments was determined by Least Significant Difference ($\alpha = 0.05$). Means associated with different letters represent significant differences.
[ns] and [***] represent non-significant or significant at $P \leq 0.001$, respectively.

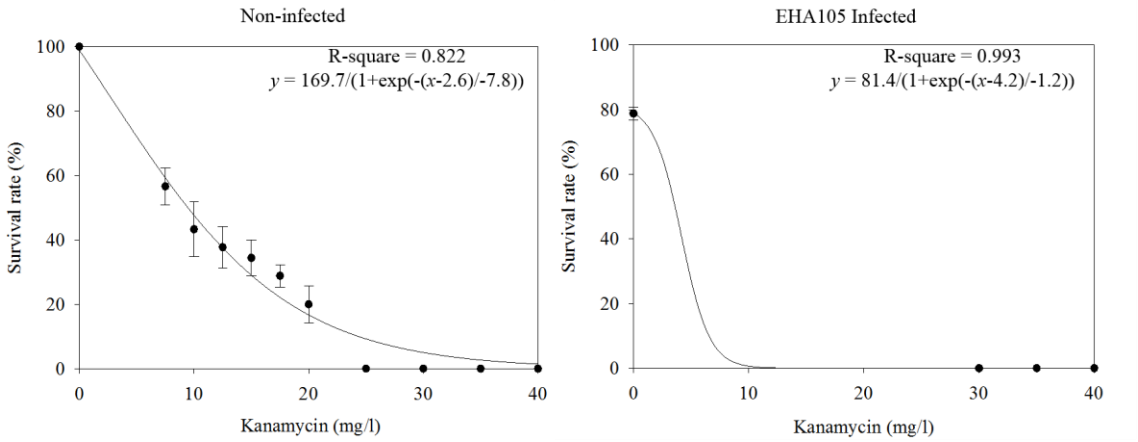

Figure 4.7. Kanamycin test for ‘Polana’ with non-infection and infection conditions. The bars on the dots represent standard errors.

Table 4.7. Evaluation of kanamycin test for ‘Polana’.

	Number of regenerated shoots		Callusing[z]	
Kanamycin ($mg \cdot l^{-1}$)	Non-infected explants	Infected explants	Non-infected explants	Infected explants
0.0	3.1 a[y]	0.9 a	3.0 a	1.9 a
7.5	0.4 b	-	1.0 c	-
10.0	0.3 b	-	1.3 b	-
12.5	0.0 b	-	0.3 d	-
15.0	0.0 b	-	0.0 e	-
17.5	0.0 b	-	0.0 e	-
20.0	0.0 b	-	0.0 e	-
25.0	0.0 b	-	0.0 e	-
30.0	0.0 b	0.0 b	0.0 e	0.0 b
35.0	0.0 b	0.0 b	0.0 e	0.0 b
40.0	0.0 b	0.0 b	0.0 e	0.0 b
LSD	0.5^{***}	0.5^{**}	0.2^{***}	0.3^{***}

[z] Callusing was scored 0, 1, 2, and 3 based on no callus, < 1 mm, 1-2 mm, and > 2 mm, respectively.
[y] Mean separation within column and treatments was determined by Least Significant Difference ($\alpha = 0.05$). Means associated with different letters represent significant differences.
** and *** represent significant at $P \leq 0.01$ and 0.001, respectively.

Optimization of inoculum density and co-cultivation time

Various combinations of co-cultivation times, inoculum densities, explant types, selection systems were tested for ‘Amethyst’ cultivar. The regeneration frequency, rooting frequency, and PCR analysis for putative transgenic lines were investigated to evaluate an influence of the treatments.

The shoot regeneration frequency of ‘Amethyst’ leaves, which were infected with *Agrobacterium* EHA105 harboring pCAMBIA1300 at the cell density of 10^{-7} dilution of OD_{600} 0.55 and co-cultivated for 15 days in hygromycin B (10.0 $mg \cdot l^{-1}$) medium, was 22.2 % (Fig. 4.8);

however, PCR verified these putative transgenic lines were non-transgenic (data not shown). In addition, the non-infected 'Amethyst' leaves were regenerated with the frequency of 24.4 %, which indicated that the putative transgenic plants were escaped from hygromycin selection. Furthermore, overgrowth of *Agrobacterium* during the entire experiment was observed. Therefore, we concluded that long (> 5 days) co-cultivation times and high inoculum densities resulted in no positive transgenic plants when hygromycin was used for selection.

The transformation with pBI121 under the kanamycin selection (25.0 $mg \cdot l^{-1}$) developed the transgenic lines from leaf explants at the 3-days co-cultivation with OD_{600} 0.55 and 10^{-2} dilution of OD_{600} 0.55 (Fig. 4.10). 'Amethyst' had no regeneration in the non-infected explants in the kanamycin selection medium. In addition, Fig. 4.9 showed that the infection by 10^{-2} diluted *Agrobacterium* with 5-day co-cultivation had 13.3 % shoot regeneration rate, which was relatively higher regeneration rate than other treatments. The combination of OD_{600} 0.55 and 10-day-cocultivation also showed the 13.3 % of putative transgenic regeneration. In the test of root initiation under kanamycin selection, the 10^{-2} diluted *Agrobacterium* with 5-day co-cultivation showed 6.6 % of rooting frequency, which is the significantly highest result in the Table 4.8. Table 4.8 showed that the inoculum density, co-cultivation time and the interaction significantly affected root regeneration since the treatments with higher *Agrobacterium* densities (OD_{600} 0.55 or 10^{-2} dilution of OD_{600} 0.55) developed the rooted transgenic plants with higher rooting frequency (1.1 to 6.6 %). At the same time, lower concentrations (10^{-4} or 10^{-7} dilution of OD_{600} 0.55) showed 1.1 % of rooting frequency in a few plants.

PCR verified that the 3-day co-cultivation treatment with OD_{600} 0.55 or 10^{-2} dilution could develop positive transgenic lines (Fig. 4.10). Within the treatment of co-cultivation times, only 3-day co-cultivation significantly developed transgenic plants since the longer co-

cultivation times (10 or 15 days) showed 0 % of transformation efficiency and overgrowth of *Agrobacterium* was also observed (Table 4.9). Putative transgenic lines from 5-, 10-, and 15-day co-cultivation treatments were verified as non-transgenic plants. Although the mean separation in the results indicated no significant differences from the treatments, the co-cultivation time and the interaction of co-cultivation and inoculum density significantly influenced the transformation frequency (Table 4.9); therefore, the following conditions for 'Amethyst' transformation in this experiment; *Agrobacterium* EHA105 density at OD_{600} 0.55, 3-day co-cultivation time, and kanamycin at 25 $mg \cdot l^{-1}$ for selection, this transformation protocol gave rise to 4.4 % transformation frequency (Table 4.9).

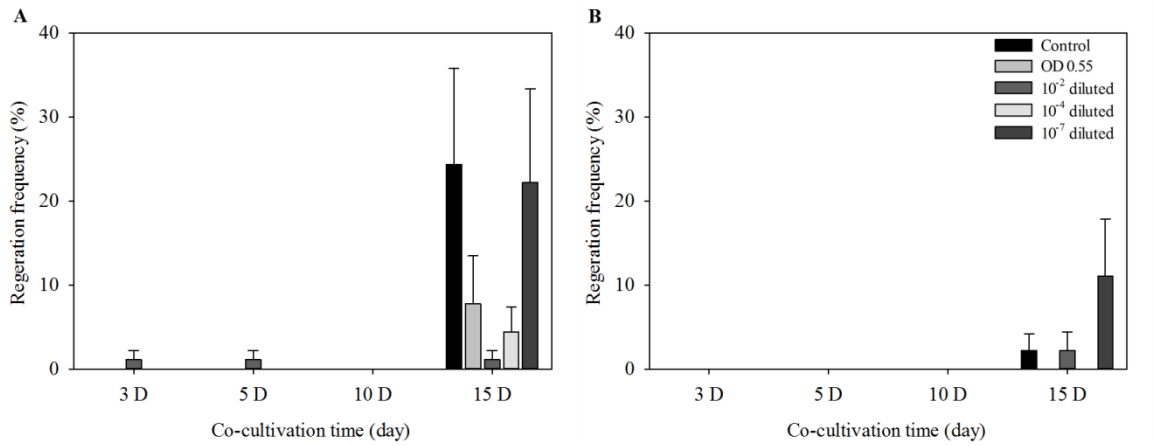

Figure 4.8. Regeneration frequency of the 'Amethyst' explants infected with EHA105 harboring pCAMBIA1300 on hygromycin selection (10.0 $mg \cdot l^{-1}$; A: leaves and B: petioles). The bars on the graph represent standard error among the replications.

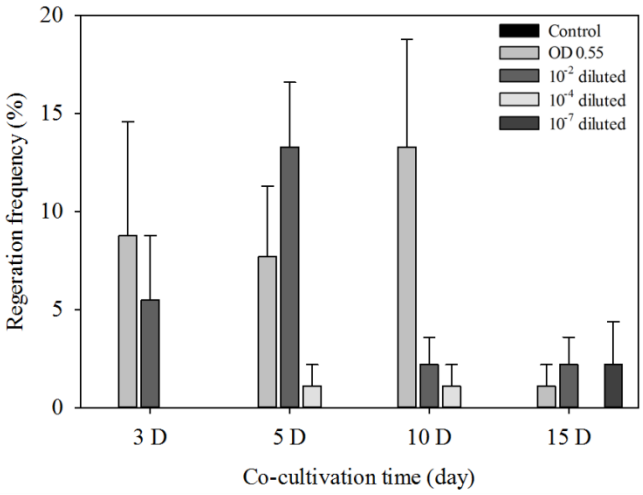

Figure 4.9. Effects of co-cultivation time and inoculum density on the regeneration efficiency of 'Amethyst' leaf explants with pBI121 under kanamycin selection (25.0 mg·l^{-1}). The bars on the graph represent standard error among the replications.

Table 4.8. Effects of inoculum density and co-cultivation time on rooting frequency of transgenic 'Amethyst' with pBI121 under kanamycin selection.

	Rooting frequency (%)			
Co-cultivation time OD_{600} value	3 D	5 D	10 D	15 D
Control	0.0 D e[z]	0.0 C e	0.0 A e	0.0 B e
Original (0.55)	4.4 D a	3.3 C a	5.5 A a	1.1 B a
10^{-2} diluted	3.3 D b	6.6 C b	1.1 A b	1.1 B b
10^{-4} diluted	0.0 D c	0.0 C c	1.1 A c	0.0 B c
10^{-7} diluted	0.0 D d	0.0 C d	0.0 A d	1.1 B d
Significant effects[y]	Cocul***, Conc***, and Cocoul×Conc***			

[z] Mean separation was analyzed by Least Square Means at α = 0.05. Capital letter indicates significance of Co-cultivation time (Cocul) treatment and small letter indicates significance of inoculum density (Conc) treatment.

[y] GLIMMIX method was conducted to test the significant effect of Co-cultivation time, inoculum density, and the interaction of them.

*** represents significant at $P \leq 0.001$, respectively.

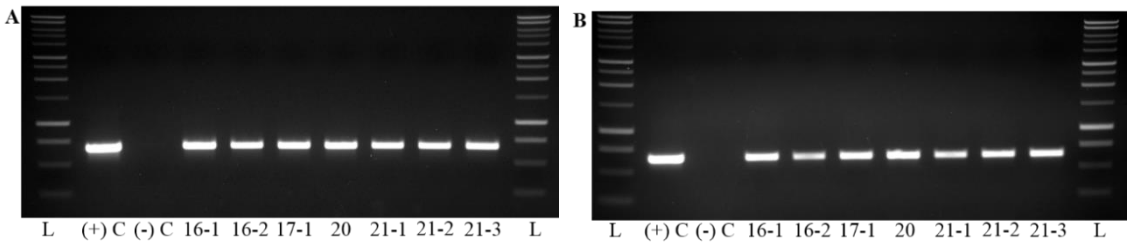

Figure 4.10. Presence of the *PtFIT* and *npt*II genes in the transgenic lines confirmed by PCR analysis. A: the *nptII* gene amplification by the primer of nptII F1 and nptII R1 (700 bp); B: the *PtFIT* gene amplified by the primer of UPtFIT F1 and PtFIT R1 (680 bp). Lane L, lane (+) C, land (-) C, and lane 16-1 to 21-3 indicate 1kb ladder, plasmid DNA (positive control), wildtype (negative control), and genomic DNAs from transgenic lines, respectively.

Table 4.9. Effects of inoculum density and co-cultivation time on transformation efficiency of the transgenic 'Amethyst' with pBI121 under kanamycin selection.

	Transformation efficiency (%)			
Co-cultivation time OD_{600} value	3 D	5 D	10 D	15 D
Control	0.0 A a [y]	0.0 B a	0.0 C a	0.0 D a
Original (0.55)	4.4 A a	0.0 B a	0.0 C a	0.0 D a
10^{-2} diluted	3.3 A a	0.0 B a	0.0 C a	0.0 D a
10^{-4} diluted	0.0 A a	0.0 B a	0.0 C a	0.0 D a
10^{-7} diluted	0.0 A a	0.0 B a	0.0 C a	0.0 D a
Significant effects[z]	Cocul***, Conc^{ns}, and Cocoul×Conc***			

[z] Mean separation was analyzed by Least Square Means at $\alpha = 0.05$. Capital letter indicates significance of Co-cultivation time (Cocul) treatment and small letter indicates significance of inoculum density (Conc) treatment.

[y] GLIMMIX method was conducted to test the significant effect of Co-cultivation time, inoculum density, and the interaction of them.

^{ns} and *** represent nonsignificant and significant at $P \leq 0.001$, respectively.

Optimization of transformation for 'Amethyst', 'Joan J' and 'Polana'

The aforementioned transformation protocol was modified by adjusting OD_{600} to 0.3 and co-cultivation time to 3 or 5 days for all three cultivars 'Amethyst', 'Joan J', and 'Polana'. Co-cultivation times were tested based on the result of transformation efficiency.

The transformation of three cultivars under the hygromycin B selection could not regenerate putative transgenic lines. The explants showed callus growth only. 'Polana' regenerated shoots under the half strength of hygromycin B, but the shoots died in the full strength selection medium at the elongation stage. 'Joan J' produced three transgenic lines from 3 and 5 days co-cultivation treatment, but these plants turned out as the false transgenic lines. No significant differences in callusing in three cultivars were observed. Therefore, we concluded that *Agrobacterium*-mediated transformation of 'Amethyst', 'Joan J', and 'Polana' with pCAMBIA1300 under hygromycin B selection is not efficient for development of transgenic raspberries.

The transformation of 'Amethyst' with pBI121 under kanamycin selection (25.0 $mg \cdot l^{-1}$) showed the transformation efficiency of 3.3 % when 3-day co-cultivation time was used (Table 4.10), while no shoot regeneration was observed for 'Joan J' with pBI121 under the kanamycin selection (20 $mg \cdot l^{-1}$). The shoot regeneration of 'Amethyst' leaves and 'Polana' leaves gave rise to 8.0 and 7.3 % of regeneration frequency in the 3 days co-cultivation time treatment (Fig 4.11, 12, and 13). In vitro rooting under kanamycin selection and PCR were used to confirm the transformation. No amplification the *PtFIT* and *nptII* genes was detected in the putative transgenic 'Polana' plants. These of 'Polana' lines could not develop any roots under the kanamycin selection (30 $mg \cdot l^{-1}$). Fortunately, the *PtFIT* and *nptII* genes were amplified using PCR in the putative transgenic lines of 'Amethyst' and in vitro rooting showed the roots were

produced from cuttings of the transgenic ‘Amethyst’ lines in the kanamycin containing medium (Fig. 4.14 and Table 4.10). Thus, the combination of 3-day co-cultivation time and inoculum density at OD_{600} 0.3 can be used to develop transgenic ‘Amethyst’ plants with *Agrobacterium* EHA105 harboring pBI121 under kanamycin selection (25.0 mg·l^{-1}).

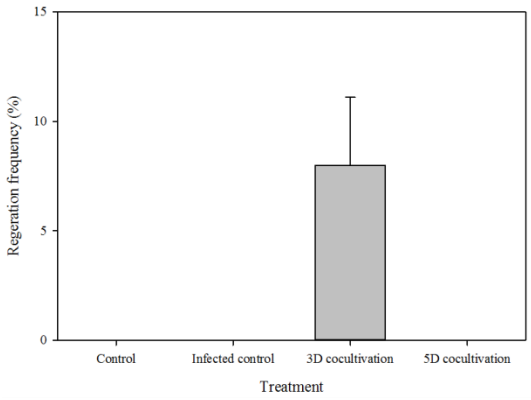

Figure 4.11. Regeneration frequency of the infected leaf explants of ‘Amethyst’ with *Agrobacterium* EHA105 harboring pBI121 under kanamycin selection (25.0 mg·l^{-1}). The bars on the graph represent standard error among the replications.

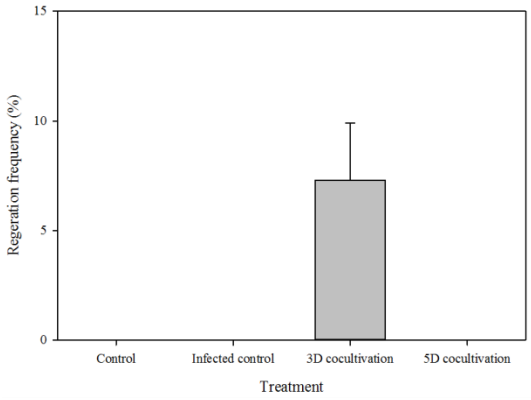

Figure 4.12. Regeneration frequency of the infected leaf explants of ‘Polana’ with *Agrobacterium* EHA105 harboring pBI121 under kanamycin selection (30.0 mg·l^{-1}). The bars on the graph represent standard error among the replications.

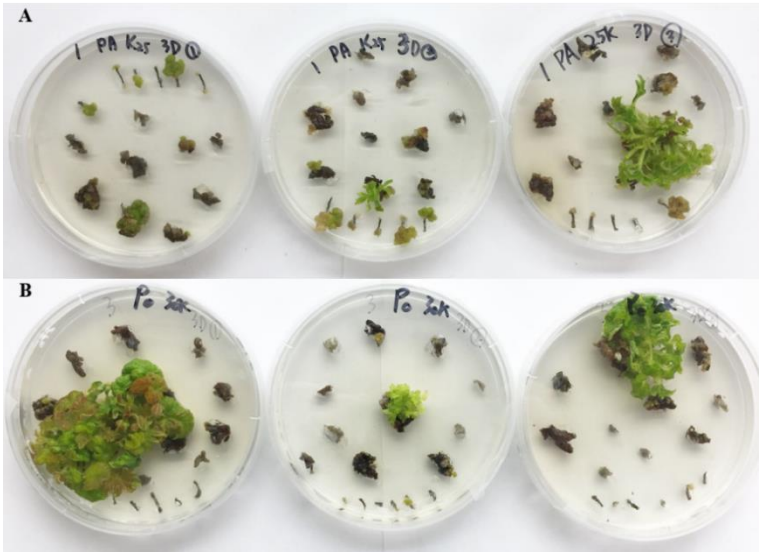

Figure 4.13. Shoot were regenerated from the ‘Amethyst’ (A) and ‘Polana’ (B) explants infected with *Agrobacterium* EHA105 harboring pBI121 under kanamycin selection.

Table 4.10. Effects of co-cultivation times on the rooting frequency, and transformation efficiency of ‘Amethyst’ with *Agrobacterium* EHA105 harboring pBI121 under kanamycin selection (25.0 mg·l^{-1}).

Treatment	Rooting frequency (%)	Transformation efficiency (%)
Control	0.0 b[z]	0.0 b
Infected control	0.0 b	0.0 b
3-day co-cultivation	3.3 a	3.3 a
5-day co-cultivation	0.0 b	0.0 b
LSD	2.1[*]	2.1[*]

[Z] Mean separation within *columns* and *factors* was determined by Least Significant Difference (α = 0.05). Means associated with different *letters* represent significant differences.

[*] represents significant at $P \leq 0.05$.

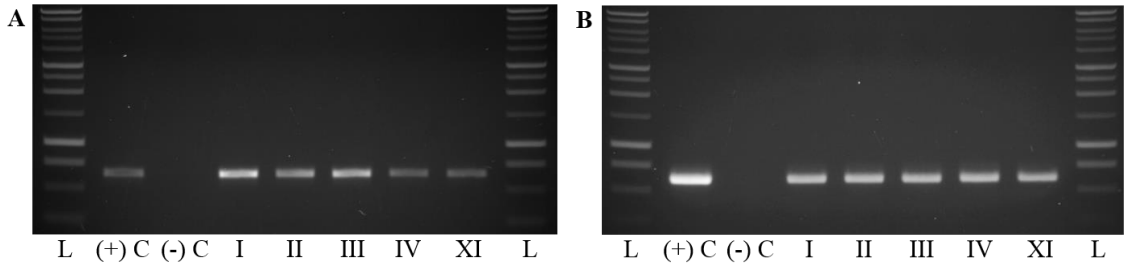

Figure 4.14. Presence of the *PtFIT* and *npt*II genes in the transgenic lines confirmed by PCR analysis. A: the *nptII* gene amplification by the primer of nptII F1 and nptII R1 (700 bp); B: the *PtFIT* gene amplified by the primer of UPtFIT F1 and PtFIT R1 (680 bp). Lane L, lane (+) C, land (-) C, and lane 16-1 to 21-3 indicate 1kb ladder, plasmid DNA (positive control), wildtype (negative control), and genomic DNAs from transgenic lines, respectively.

Expression of the *PtFIT* gene in response to iron deficiency in transgenic 'Amethyst plants

Transgenic plants were grown in a hydroponic culture system. After 6 weeks acclimation, plants were treated with two different iron conditions, sufficiency (Fe^{+}) and deficiency (Fe^{-}) for six days (Fig. 4.15A). The root tissues were collected from the transgenic lines. RNAs were extracted and cDNAs were synthesized for each of individual lines in two iron treatments. The transgenic plants were maintained in the Fe^{+} or Fe^{-} condition after sample collection to induce iron chlorosis. The chlorosis symptom was apparently observed at the 14^{th} days after Fe^{-} treatment (Fig. 4.15B).

A semi-quantitative PCR was conducted to evaluate quality and quantity of the cDNA samples for RT-PCR (Fig. 4.16). The result of the semi-quantitative PCR confirmed the expression of the *PtFIT* and *GAPDH* genes under the Fe^{+} and Fe^{-} conditions (Fig. 4.16). Variations in the band intensity of the *PtFIT* gene in different transgenic lines were observed, indicating the differential expression of the *PtFIT* gene in different transgenic plants (Fig. 4.16A and C).

The qualified cDNA samples from 12 transgenic lines and 2 control lines (wild type) were subjected to RT-PCR analysis with specific primers for the *GAPDH* and *PtFIT* genes. To evaluate the primer specificity of the *PtFIT* and *GAPDH* genes, melting curve analysis was performed (Fig. 4.17). The result indicated that each of two primer pairs showed the specificity since the melt temperatures for the *GAPDH* gene and *PtFIT* gene were 79.5 – 80 ℃ and 75.0 – 75.5 ℃, respectively.

The result of RT-PCR of 12 transgenic lines and 2 wild type lines showed no significant difference within the experiment. Figure 4.18 showed the relative expression level of transgenic lines under Fe^{+} and Fe^{-} conditions. To normalize C_t value of transgenic lines from RT-PCR data, differences between the C_t value of *PtFIT* and C_t value of *GAPDH* within the same line and same treatment was calculated as the delta C_t value. Then, this delta C_t value was subtracted by the averaged delta C_t value of the wild type under the same iron condition, which was named as delta-delta C_t value and powered negatively on 2 to calculate relative expression level. Two wild type lines of 'Amethyst' showed no significantly different at Fe^{+} and Fe^{-} conditions, respectively. The relative expression levels showed a variant expression tendency in the transgenic lines. In T3 and T5, the expression level in Fe^{-} treatment was slightly higher than that in the Fe^{+} treatment (about 10 units of relative expression level). The expression of T7 and T8 at iron deficiency was considerably increased (15.2 and 4.8 times) than that in the Fe^{+} treatment. However, the other lines (T1, T2, T4, T6, T9, T10, T11, and T12) showed the increased expression in the Fe^{+} treatment and great standard deviations within the biological replicates were also observed.

Figure 4.15. Transgenic ‘Amethyst’ plants were treated with iron sufficient or deficient conditions in a hydroponic culture system. A: the hydroponic culture system used in the research. B: transgenic plants showed iron chlorosis leaves after 14 days of iron deficiency treatment. C: transgenic plants showed no iron chlorosis symptom on leaves in the iron sufficient solution.

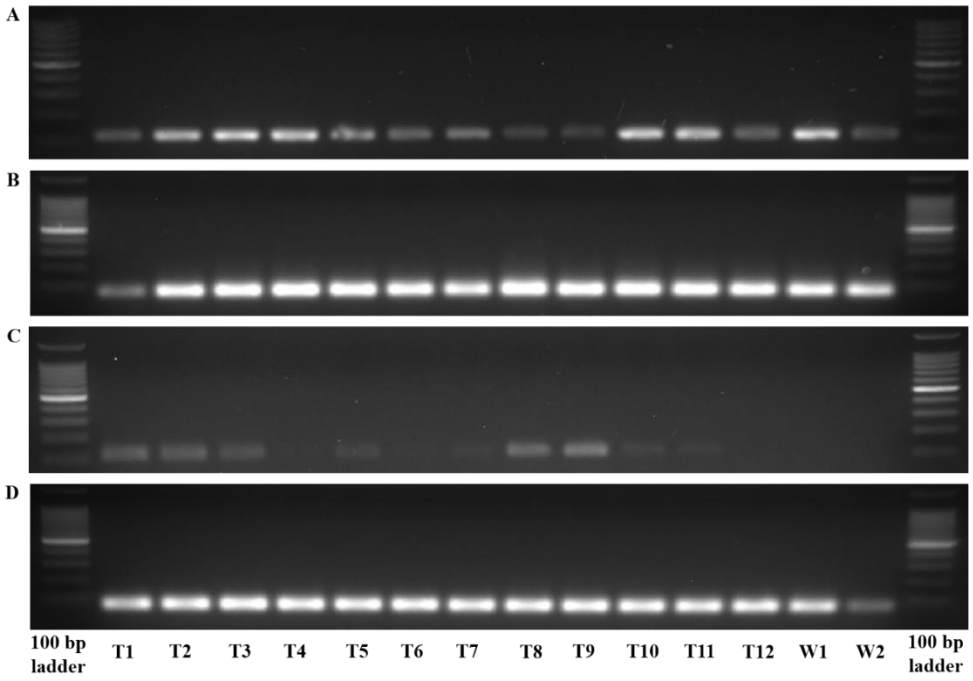

Figure 4.16. Expression of the *PtFIT* gene in the transgenic lines under iron sufficient and deficient conditions. The expression was determined using semi-quantitative PCR with the *GAPDH* gene as a control. A and B: the expression of the *PtFIT* and *GAPDH* genes in response to iron sufficiency (Fe^{+}), respectively. C and D: the expression of the *PtFIT* and *GAPDH* genes in response to iron deficiency (Fe^{-}), respectively.

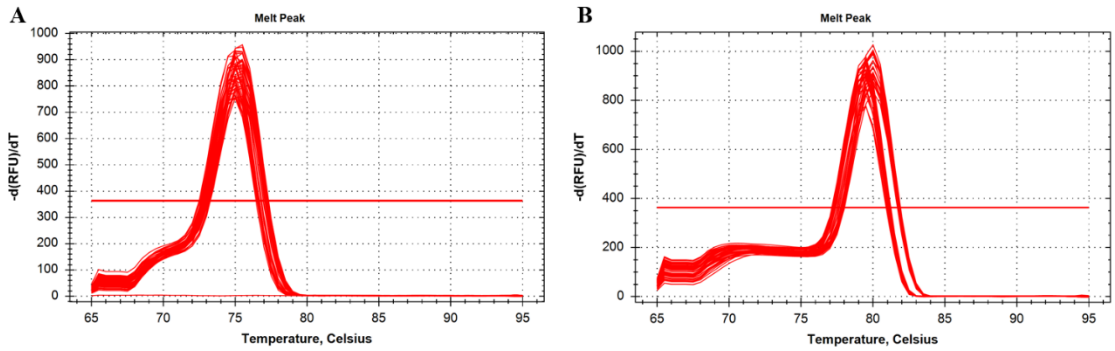

Figure 4.17. Melting curve analysis for evaluation of primer specificity. A: the *PtFIT* and B: the *GAPDH* gene.

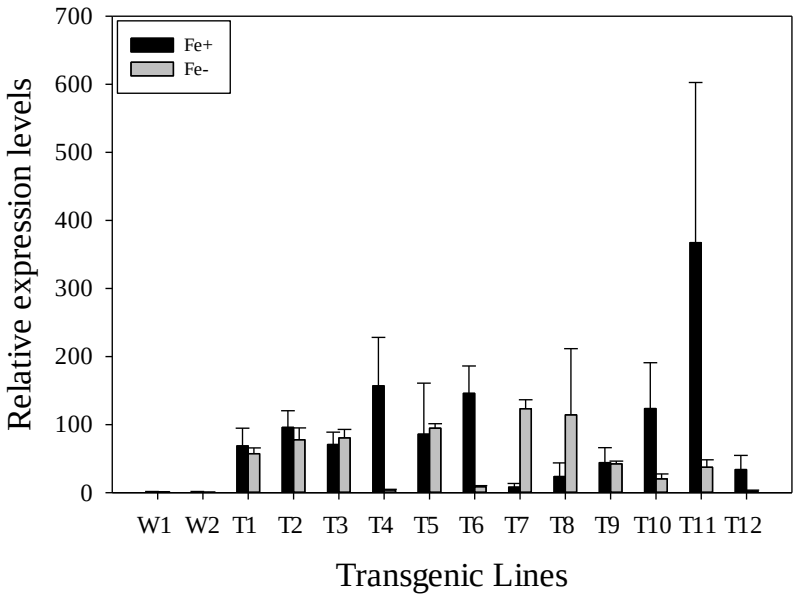

Figure 4.18. Quantified relative expression levels of the *PtFIT* gene in response to iron sufficiency and deficiency in the transgenic 'Amethyst' lines. The relative expression of transgenic lines was normalized to the delta C_t value (C_t value of the *PtFIT* – C_t value of the GAPDH) of the wild type under iron deficiency or sufficiency treatment. The bars on the graph represent standard error within the replications.

Discussion

Optimizing conditions of *Agrobacterium*-mediated transformation is a crucial step to develop transgenic plants with an efficient and stable manner (Birch, 1997). In this research, the influence of inoculum densities, co-cultivation time, explant types, and levels of selectable

agents (antibiotics) on transformation efficiency was evaluated for three raspberry cultivars. The expression of the *PtFIT* gene in response to iron deficient and sufficient conditions was also investigated to verify whether the transformation is working or not.

Different plant genotypes have different tolerance to selective agents (antibiotics) used for selection of transgenic cells and plants. Research indicated that the minimum killing level of hygromycin B in *Rubus* species showed the similarity as 10 to 20 $mg \cdot l^{-1}$ (Aldwinckle and Malnoy, 2009; Birch, 1997; Trigiano and Gray, 2011). Our result showed that hygromycin B could completely inhibit cell growth at a relatively low level that is much lower than the concentration of kanamycin. Miki and McHugh (2004) and Sundar and Sakthivel (2008) reviewed that hygromycin B can inhibit protein synthesis of chloroplast and mitochondria is more toxic to plant than kanamycin that can block translation procedures in plant cells. For example, kanamycin at 50 $mg \cdot l^{-1}$ completely inhibited growth of 'Amethyst' explants. Similarly, Faria (1993) reported that 40 $mg \cdot l^{-1}$ kanamycin was the optimized concentration for selection of transgenic 'Comet' cells. Graham et al. (1990) used 50 $mg \cdot l^{-1}$ kanamycin for selection of transgenic red raspberries; however, hygromycin at 10.0 $mg \cdot l^{-1}$ can cause death of non-transformed 'Meeker' explant during the *Agrobacterium*-mediated transformation (Mathews et al., 1995). High concentrations of antibiotic may decrease regeneration of transgenic cells (Birch, 1997; Trigiano and Gray, 2011). In our research, a minimum killing level of antibiotics was used for a less inhibited regeneration capability of explants; therefore, the optimized kanamycin levels were 25.0, 20.0, and 30.0 $mg \cdot l^{-1}$ for 'Amethyst', 'Joan J', and 'Polana', respectively. Our research also found that infection of EHA105 competent cells attenuated raspberry tolerance to hygromycin B. Faria (1993) reported that the bacteria infection led the increased susceptibility to kanamycin selection. However, our results only support the plant responses to hygromycin

because the bacteria infection of raspberry explants did not change their tolerance to kanamycin. It indicates that optimum level of a selective agent should be tested based on the genotype, antibiotics type, and *Agrobacterium* strains.

In the experiment, 'Amethyst' cultivar was used to optimize *Agrobacterium* conditions including inoculum density and co-cultivation time prior to an optimization for each cultivar since 'Amethyst' showed the high frequency in shoot and root regeneration (Lenz et al., 2016). The 7-day old 'Amethyst' explants were infected by the *Agrobacterium* suspension with four-inoculum densities (OD_{600} 0.55, 10^{-2}, 10^{-4}, or 10^{-7} dilution of the original bacteria density) and four co-cultivation times, 3, 5, 10, or 15 days, after a 30 min infection. Our result showed that the 3-day co-cultivation had the best transformation frequency of 'Amethyst'. Shoots regenerated after 15 days co-cultivation under hygromycin B selection were false-transgenic. The main cause of hygromycin-escape might be derived from a relatively long co-cultivation time without selection pressure. Other studies of *Rubus Agrobacterium*-mediated transformation showed that co-cultivation for 1 to 3 days turned to a good result (Faria, 1993; Georgiva et al., 2008; Graham et al., 1990; Mathews et al., 1995). Aldwinckle and Malnoy (2009) also indicated that a short co-cultivation time has presented an efficient transformation frequency in *Rubus* species.

Our experimental design was motivated by the report of Zhang and Finer (2016) in which low inoculum density could increase transformation frequency. We had aimed to identify a low inoculum density that is much lower than the threshold value for a plant defensive system. However, the infection by 10^{-4} and 10^{-7} diluted suspension of OD_{600} 0.55 gave rise to lower or no shoots regeneration (Figure 4.9). Only in the treatments with the OD_{600} 0.55 or 10^{-2} diluted infection solution regenerated the transgenic lines (Fig. 4.10). Previous studies for *Rubus* transformation often adjusted inoculum density to OD_{600} 0.1 to 1.0 (Faria, 1993; Georgiva et al.,

2008; Graham et al., 1990; Mathews et al., 1995). We found that the inoculum density at OD_{600} 0.55 induced overgrowth of *Agrobacterium*, causing necrosis of explants, especially in the treatment of high inoculum densities combined with a longer co-cultivation. Moreover, Song and Sink (2005) and Alsheikh et al. (2002) reported that the infection with over OD_{600} 0.5 had the overgrowth in sour cherry and strawberry transformation. Hence, we optimized the infection condition as OD_{600} 0.3 of inoculum density and 3-day co-cultivation time for raspberries.

Modifications in preparations of *Agrobacterium* were made in our research. *Agrobacterium* solution was prepared via direct cell culture without centrifugation procedure to prevent cell damage. In brief, the EHA105 bacteria harboring pBI121 or pCAMBIA1300 were grown on LB medium for four days until OD_{600} 0.3, which is considered at the early exponential phase of *Agrobacterium* (Prapagdee et al., 2004). We expected that the bacteria culture at the exponential phase without any cell damage from centrifugation would show an improved transformation ability on raspberry explants. At the same time, the infection time was changed to 10 min and co-cultivation was performed in an empty petridish to prevent overgrowth of *Agrobacterium*, which is under the condition in nature for *A. tumefaciens* (Bourras et al., 2015). No overgrowth of *Agrobacterium* was observed.

Five transgenic ‘Amethyst’ lines from leaf explants were developed by the infection of the OD_{600} 0.3 suspension of *Agrobacterium* EHA105 harboring pBI121 with 3-day co-cultivation under the kanamycin selection system (25.0 $mg \cdot l^{-1}$). However, the transformation for ‘Joan J’ and ‘Polana’ resulted in 0% in the transformation frequency under both selection systems. The studies of raspberry transformation by Graham et al. (1990), Faria (1993), Mathews et al. (1995), and Georgiva et al. (2008) reported that the transformation efficiency of some cultivars within *Rubus* species was lower than 1 % or 0% even many trials were conducted for optimization of

conditions. This implies that the genotype of 'Joan J' and 'Polana' may inhibit an activity of *A. tumefaciens* based on their inherited tolerance to the bacteria infection (Birch, 1997).

We hypothesized that the expression of the *PtFIT* gene would be up-regulated in transgenic raspberries under iron deficiency condition since the study of the *PtFIT* gene in transgenic *Populus tremula* showed the increased transcripts in root tissues when the hydroponic solution contains limited iron contents (Huang and Dai, 2015). However, the relative expression of the *PtFIT* gene in the transgenic raspberry lines indicated that no significant difference under Fe^{+} and Fe^{-} conditions (Fig. 4.18). This might be caused by non-equality in growth of transgenic lines and heterozygous in the biological replicates. Although the transgenic plants were hydroponically grown and acclimated for 6 weeks, half of the transgenic plants were still struggling prior to the iron treatments. In addition, some transgenic lines were shown the inhibited growth under six days of the iron deficiency, which may result in relatively high C_t value (over 30; normally in the range of 15 to 30) of the *PtFIT* gene (Lodge et al., 2007). Furthermore, even the C_t value among the technical replicates were in an acceptable range (lower than 0.5 difference), the most delta C_t values within the biological replicates showed significant differences and brought a high standard deviation in the analysis. These reasons may explain the relatively lower expression under the iron deficiency conditions. The expression levels in Fig. 4.18 were corresponded with the gel images from semi-quantitative PCR, which showed the transgenic lines with iron sufficiency treatment had more clear bands on the gel (Fig. 4. 16A). Research showed that *AtbHLH*38/39 (the orthology gene in *Arabidopsis* to the *PtFIT*) was up-regulated under iron deficient condition (Bauer et al., 2007; Huang and Dai, 2015; Yuan et al., 2008). VanBuren et al. (2016) and Jibran et al. (2018) reported that the predicted genes of black raspberries (*Rubus occidentalis*) are similar to *Populus* and *Arabidopsis*, which may support our

hypothesis of an up-regulation of the *PtFIT* gene in raspberry species. Therefore, further studies of the *PtFIT* in transgenic raspberries are needed to identify its functions of the *PtFIT* gene in response to the iron condition in plants.

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CHAPTER V. GENERAL CONCLUSION

Optimization of a genetic transformation system is important in agriculture as a research tool and practical method to improve performance of crops. Since the efficiency of plant genetic transformation with *Agrobacterium*-mediated method varied among crop species and cultivars, factors that affect the transformation efficiency have been optimized for many major crops, such as soybean, rice, wheat, and maize. However, only few studies of raspberry transformation have been reported and the results showed differences based on cultivars. To cope with the limitation of raspberry research, a transformation system of raspberry wad developed through the development of an efficient regeneration system and optimization of transformation conditions including explant type, selection system, inoculum density, and co-cultivation time.

Chapter II states the literature review of the characteristics of raspberries and plant genetic transformation. This section reviewed the previous publications related to raspberry physiology, history of raspberries, and influencing factors on optimization of plant genetic transformation.

Chapter III describes the development of the plant regeneration procedure for 'Joan J' and 'Polana'. Plant growth regulators, explant types, and explant ages were determined for an efficient shoot regeneration of the two cultivars. Results showed that the explant age significantly affect the regeneration frequency and 7-day-old leaf explants performed better than other ages. Result also showed that plant regeneration of 'Joan J' can be achieved using WPM supplemented with 2.5 µM BA + 1.0 µM TDZ for shoot regeneration and ½ MS with 10 µM NAA for in vitro rooting. The plant regeneration of 'Polana' can be achieved using WPM with 2.5 µM BA + 0.1 µM TDZ for shoot regeneration and ½ MS with 7-day-pulsing of 20 µM NAA

for in vitro rooting. Ex vitro rooting with the treatment of 500 μM IBA for ‘Joan J’ and 500 μM NAA for ‘Polana’ can be also used.

Chapter IV characterized the optimized conditions for *Agrobacterium*-mediated transformation of raspberries and the expression of the *PtFIT* gene in transgenic raspberry plants under the iron sufficient and deficient conditions. The influence of selection system, inoculum density, co-cultivation time, and explant type on transformation frequency was determined. The optimized condition for ‘Amethyst’ transformation is that the infection of 7-day old leaves with the OD_{600} 0.3 inoculum density and co-cultivation for 3 days under kanamycin selection at 25 $mg \cdot l^{-1}$, which showed 3.3 ~ 4.4 % of the transformation frequency. The transgenic raspberries with the *PtFIT* gene were grown in a hydroponic culture system to study the function of the gene responding to iron deficiency. The RT-PCR result presented no significant differences in the expression in response to the iron deficient and sufficiency conditions and further studies are needed.

In conclusion, the optimized regeneration and transformation methods could be beneficial to the research in the field of plant biotechnology such as germplasm preservation, molecular breeding, and improvement of performance of raspberry or other *Rubus* species.