IMPROVING GENE TRANSFER IN APPLE

MSc Thesis Report

B.D. MARIANA

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Wageningen University – Plant Sciences Group – Laboratory of Plant Breeding

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IMPROVING GENE TRANSFER IN APPLE

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Cover image: fruits of apple cv. 'Gala' (picture by BD Mariana, 2012)

Abstract

Gene transfer in apple is largely dependent on the efficiency of tissue culture technique, selection of transformants and the recovery of the transgenic plants. There are several factors contributing to a successful gene transfer. To observe these factors, studies based on experiments and on literature study were conducted. In the experiments, the effects of different types of light, in which the plants were grown *in vitro*, and of optical density (OD) of the *Agrobacterium* suspension on regeneration and transformation efficiency of apple cv. 'Gala' as well as kanamycin sensitivity of 'Gala' were studied. In the literature study, comparisons of several apple transformation methods were conducted to find any specific treatment that may lead to higher transformation efficiency. For experiments, gene transfer was evaluated by counting the number of regenerated shoots expressing the *gus* gene. Culture under blue LED light showed a positive effect later on both regeneration and transformation efficiency. Continued dark treatment after inoculation during further culture appeared to be more effective for shoot regeneration than transfer back into the light. On the other hand, no significant difference was observed among the different OD treatments. The optimal transformation efficiencies obtained were 25% and 12% for blue light treatment and OD 1.2 respectively. A kanamycin concentration of 100 mg/l which completely inhibited the growth of non-transformed explants proved to be appropriate for selection. From the literature study, preculture of explants may contribute to higher transformation efficiency. This study revealed promising applications of blue light and that the use of higher OD is potentially beneficial to obtain more efficient transformation using the already established procedures for apple transformation.

Preface

This thesis entitled “Improving Gene Transfer in Apple” is submitted in partial fulfilment of the requirements for a Master Degree in Plant Sciences specializing in Plant Breeding and Genetic Resources. It contains work done from July to December 2011 which was mainly conducted in the cell biology laboratories of Wageningen UR Plant Breeding. The thesis was supervised by Dr. Frans Krens and Iris Tinnenbroek-Capel.

The thesis was made possible by support of thoughtful and generous persons. I would like to thank my supervisor, Dr. Frans Krens, who has provided guidance, knowledge and some perspectives within the topics of gene transfer. I thank Iris Tinnenbroek-Capel for her supervision and assistance in my work and also her kind support to introduce me with all things related to my thesis. I would like to thank Dr. Henk Schouten who made it possible for me to work on this topic and helped me with my data analysis. I would like to express my gratitude for the people working on apple research, Jos Brinkhuis, who kindly assisted me in the lab, Jan Schaart, Sabaz, Stefano and Miguel for nice discussions in apple group meetings. I thank the nice people I encountered during my lab work especially Bernadette, Marjan, Isolde, which were open to any question and Shu who was kind to offer some help.

The work of the thesis was enjoyable not only because of my interest in the project but also because there were many friends and colleagues who supported and lifted up the mood. I thank Jayne who kindly arranged the weekend escape at the right time, the cheerful Anita, Judith, Basak, Maáli, and those who occupied room E. 2.108. I did enjoy our late coffee time and photo shots. I am having a good time in Wageningen also because there are many Indonesian friends whom I shared many nice experiences with. Thanks to Windu, Elfa, Tiara, Halida, Ananti, Novi, Astrid, Eliya, Dewi, Atik, Tika, Esti, Yuni, Evie, Anita, Yahya, Agus, Reva and those that I couldn’t mention here.

I acknowledge NUFFIC for the scholarship that made it possible for me to pursue higher education here at Wageningen University. My appreciation goes to the Director and colleagues at Indonesian Citrus and Tropical Fruits Research Institute for all support and suggestions during the process of the scholarship and my master program.

My gratitude is highly dedicated to my parents who are always supporting and appreciative, no matter what decision I make. I thank my sisters, my lovely nephew, granny and all my big families in Lombok for their love and support so that I could come this far.

Finally, alhamdulillah, my deep gratefulness is devoted toward Allah that provides me chance, health and strength to go through all these things in order to gain one more achievement in my life.

Baiq Dina Mariana

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List of Abbreviations

Abbreviations	Meaning
2ip	N6(2-isopentenyl)adenine
4CPU	*4*-chlorophenylurea
AS	Acetosyringone
BA	Benzyl adenine
BAP	Benzylaminopurine
FeEDDHA	(Fe(3+) ethylenediamine-N,N'-bis(hydroxyphenylacetic acid)
GA	Gibberellic acid
GFP	green fluorescent protein
GUS	beta-glucuronidase
IBA	Indole-3-butyric acid
LEDs	Light Emitting Diodes
LS	Linsmaier & Skoog
MEPM	Meropenem Hydrate
MS	Murashige and Skoog
NAA	Naphthaleneacetic acid
npt II	Neomycin Phosphotransferase II
OD	Optical Density
PGR	Plant Growth Regulator
QL	Quorine and Lepoivre
pmi	Phosphomannose isomerase
SEM	Shoot Elongation Medium
SIM	Shoot Induction Medium
SPM	Shoot Propagation Medium
TDZ	Thidiazuron
TIBA	Triiodobenzoic acid

INTRODUCTION

Apple is one of the most important fruits in the world, ranked third after watermelon and banana in world production in 2009 (http://faostat.fao.org). The fruit production of apple is hampered by diseases, therefore, the most common objective for apple breeding is to integrate high fruit quality with disease and pest resistance in new cultivars with resistance to scab and powdery mildew being most desired (Brown and Maloney, 2003).

Breeding apples faces several constraints: a long juvenile period, self-incompatibility, and its highly heterozygous nature (Brown and Maloney, 2003; Szankowski *et al.*, 2003). These result in slow genetic improvement of the crop. As an alternative, transformation techniques offer the possibility to introduce new genes which are beneficial without changing the genetic background and may, therefore, speed up the breeding process (DeBondt *et al.*, 1996).

Transformation involving *Agrobacterium tumefaciens* is the key method for obtaining transgenic plants in apple (Bhatti and Jha, 2010). There are two essential requirements for successful gene transfer: the ability to stably introduce a desired gene into the plant genome and the ability to regenerate a fertile plant from the transformed cells (DeBondt et al., 1994). A number of factors could influence efficient *Agrobacterium*-mediated transformation in apple, including plant materials (genotype, age, physiology) (Puite and Schaart, 1996; Yepes and Aldwinckle, 1994), culture medium (type, hormone, gelling agent) (Bolar *et al.*, 1999; Li *et al.*, 2011), bacterial strain, construct, and cell density (DeBondt *et al.*, 1994; DeBondt *et al.*, 1996), additional compound in bacterial culture medium (James *et al.*, 1993), preculture (Li *et al.*, 2011), selective agent (Degenhardt *et al.*, 2007), cocultivation period and leaf orientation (Seong and Song, 2008). The transformation efficiencies reported ranged from 0.03-20% for scion cultivars and even reached 80% for an apple rootstock variety (reviewed by Aldwinckle and Malnoy, 2009).

In plant transformation, the use of a selectable marker is crucial to recover a high proportion of transgenic cells from untransformed cells (Dominguez *et al.*, 2004). Selection strategies can be classified into two categories: an advantageous (positive) or a disadvantageous (negative) selection. In positive selection, the growth of transformed cells is promoted while in the negative selection their growth is inhibited (causing death) due to the presence of certain compound in the medium (Malnoy *et al.*, 2010) In apple transformation, selection systems are mostly based on the use of the neomycin phosphotransferase II (*npt*II) gene, conferring resistance to aminoglycoside antibiotics, such as kanamycin, neomycin and geneticin (Bolar *et al.*, 1999; Borejsza-Wysocka *et al.*, 1999; Degenhardt *et al.*, 2006; Schaart *et al.*, 1995; Xu *et al.*, 2009). For 'Gala', kanamycin concentration of 50-100 mg/l was reported to effectively inhibit adventitious shoot formation (Puite and Schaart, 1996).

As one of factors that influence efficient *Agrobacterium*-mediated transformation in apple, bacterial density may have important effect. However, there is lack of study that has been done to observe the effect of bacterial density on apple transformation. Debondt *et al.* (1994) found

that there was no significant effect of the bacterial growth phase on the efficiency of DNA transfer.

Tissue culture has an important role in transformation as it is required for recovery of entire, intact plants (Hansen and Wright, 1999). Improvement of tissue culture techniques to be more efficient in regeneration may also improve the transformation efficiency increasing the number of transgenic plants ultimately obtained. For example, improving culture conditions related to light or photoperiod have a significant effect on the morphogenesis (Kim *et al.*, 2004b; Smith, 1982). The effect of light on plant growth in tissue culture has been reported for several crops such as lettuce (Kim *et al.*, 2004a), chrysanthemum (Kim *et al.*, 2004b), potato (Seabrook, 2005), cattleya (Cybularz-Urban, 2007), and *Calanthe* (Baque *et al.*, 2011). In apple, the effect of light on regeneration and growth in tissue culture was observed in cv. 'Golden Delicious' (Liu *et al.*, 1983), M26 (Predieri and Malavasi, 1989) and MM106 (Muleo and Morini, 2006).

Light quality is an essential factor which regulates plant development through photoreceptors active under specific wavelengths of light (Lee *et al.*, 2007). Plants require light for morphogenesis which lies in spectrum near-ultra-violet (300-380 nm), blue (430-490 nm), red (640-700 nm), and far-red (700-760 nm) and for photosynthesis between 400-700 nm (Hart, 1988). In tissue culture, fluorescent lamps (white light: 400-700nm) are generally used as a source of light (Kim *et al.*, 2004b). LEDs recently have been suggested as an alternative source of light for tissue culture because of their advantages including smaller mass and volume, longer life and specific, limited wavelength range (Kim *et al.*, 2004b). According to U.S. Department of Energy, a study in 2009 showed that the lifetime of high-power LEDs may reach 50.000h (http://apps1.eere.energy.gov/buildings/publications/pdfs/ssl). Improvement of the setting of light for specific purposes in terms of the phase of plant growth and development (i.e. shoot or root initiation or inhibition) is possible using LEDs.

Previous studies on several crops showed the effect of different light spectra on the *in vitro* growth and development. Red and blue light showed effect on chlorophyll content of chrysanthemum (Kim *et al.*, 2004b), grape (Poudel *et al.*, 2008), strawberry (Nhut *et al.*, 2003; Samuoliene *et al.*, 2010), Indian ginseng (Lee *et al.*, 2007) and enhanced adventitious bud formation on cattleya (Cybularz-Urban, 2007). Far red light delayed tuberization of potato (reviewed by Seabrook, 2005), inhibited axillary bud formation in *in vitro* cultured tomato and rhizogenesis of *Prunus mahaleb* (reviewed by Morini and Muleo, 2003). In apple, short exposure to red light suppressed adventitious shoot formation by 80% in cv. 'Golden Delicious' and the effect was annulled by immediate exposure to far red after the red light (Liu *et al.*, 1983). On M26, dark treatment and red light were found to be the most effective for shoot regeneration (Predieri and Malavasi, 1989). Dark treatment on MM106 rootstock showed the lowest number of shoots while white light gave the opposite result (Muleo and Morini, 2006).

Effect of light on transformation has been described in *Arabidopsis thaliana* and *Phaseolus acutifolius* (Zambre *et al.*, 2003). The results there showed that the presence of light during coculture was positively affecting GUS expression which was inhibited by darkness. To our knowledge, there is no report on the effect of light on apple transformation. Thus, in this study, the objectives were to examine the effect of different types of culture light as well as the effect

of Agrobacterium optical density on apple transformation. In addition, to confirm that kanamycin concentration used in already established apple transformation method from Plant Research International (PRI) is still relevant for apple cv. ‘Gala’, an experiment to test kanamycin sensitivity of ‘Gala’ was also included.

MATERIALS AND METHODS

The following methods with slightly modifications were done according to the methods which were established by Plant Research International (PRI), unless stated otherwise:

General Tissue Cultures

Plant materials

Shoots of wild type (WT) 'Gala' were used as source of explants. The shoots were grown on shoot propagation medium (SPM) containing MS salts and vitamins, 3.1 µM BAP, 96 mg/L FeEDDHA solidified with 0.9% (w/v) Daishin agar at pH 5.8. The cultures were maintained at 24^{o}C under a 16-hour photoperiod with light supplied by cool white light fluorescence lamps at an intensity of 87 µmol $m^{-2}s^{-1}$.

Regeneration

Leaf explants were obtained from 4 week-old *in vitro* cultured shoots. Leaf segments were cut transversely to the midrib into 2-3 mm wide explants and placed with adaxial surface on shoot induction medium (SIM) for 'Gala'. SIM Gala contained MS salts and vitamins, 10 µM TDZ, 13.3 µM BAP, 0.54 µM NAA and 3% (w/v) sorbitol, solidified with 0.3% (w/v) gelrite at pH 5.8. The cultures were maintained in the dark at 24^{o}C. After 4 weeks, callus formation and adventitious shoots were monitored and scored.

General Transformation

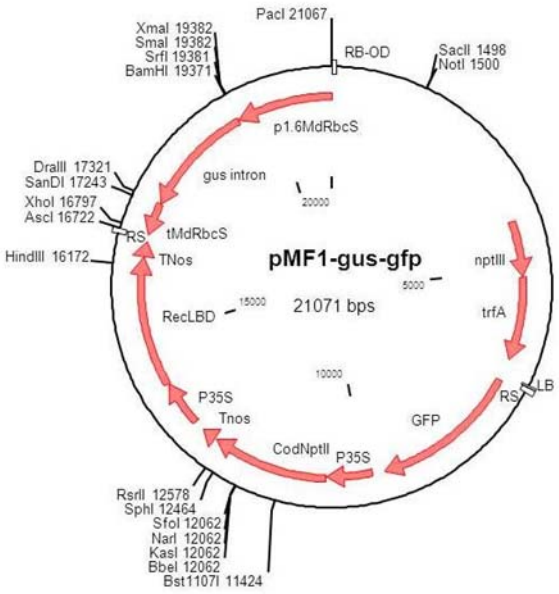

Figure 1. Schematic representation of vector of pMF1-GUS-GFP

Preparation of A. tumefaciens *cultures*

This experiment used *A. tumefaciens* strain AGL0 carrying plasmid pMF1 containing a kanamycin resistance gene as selectable marker and an intron-containing β-glucuronidase (*gus*) gene

(Figure 1, kindly provided by Jan Schaart). The cultures were prepared as follows. 10 ml liquid culture (LB medium + 50 mg/l Kanamycin + 50 mg/l Rifampicin) was made fresh by taking a 100 µl sample from a –80°C bacterial glycerol stock and resuspending it in the afternoon. The cultures, subsequently, were put on a rotary shaker at 150 rpm at 28°C and grown overnight. After centrifugation (3000 x g, 10 min), the pellet of overnight cultures of *A. tumefaciens* were resuspended in MS medium with 3% (w/v) sucrose (pH 5.2) and 100 µM acetosyringone until the OD600nm was 0.4.

Transformation and cocultivation

The explants were cut from the first four unfolded leaves of 4-week old shoots, transferred and cut transversely in *A. tumefaciens* suspension. Inoculation time in total was 30 minutes including 10 minutes cutting in *A. tumefaciens*. After inoculation, the explants were blotted dry on sterile filter paper, before placing them with adaxial side on SIM Gala without selection. The Petri dishes were sealed with household foil and stored in the dark by 24°C for four days (cocultivation). For each transformation, two controls were included: control experiments which were transformation using empty AGL0 and regeneration controls which were cultures of untransformed leaf segments on SIM Gala, SIM Gala with 250 mg/l cefotaxime, and SIM Gala with 100 mg/l kanamycin and 250 mg/l cefotaxime.

Selection

After cocultivation, the explants were transferred to new medium which was SIM Gala with 100 mg/l kanamycin and 250 mg/l cefotaxime and kept in dark at 24°C. The explants were transferred to new medium every three weeks. For the second light experiment (replicate), half of the plates were placed in the lights which were used for shoot propagation after the first subculture, while the other half was kept in dark as is in the standard protocol.

GUS assay

GUS assays were conducted two times; 4 weeks after transformation (preliminary observation) and 12 weeks after transformation (final observation). For preliminary observation, one or two plates were used from each light type. Explants were incubated in GUS staining buffer with the substrate X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid cyclohexyl-ammonium) at 37°C overnight. Stained tissues were washed and placed in 80% (w/v) ethanol before examining under microscope. Explants, calli and shoots which showed GUS expression were observed and counted. Controls for positive GUS expression were included.

Kanamycin sensitivity

In two replications, five concentrations of kanamycin were tested: 0, 50, 75, 100, and 150 mg/l respectively. Plant materials and regeneration were as described previously. Callus and adventitious shoots were observed in the fifth week after cultured. As positive controls, five transgenic lines of 'Gala' were included in the experiment. These lines were maintained previously in SPM. Each of them is carrying a kanamycin resistance gene in different constructs: PBIN V25A-1, PBIN V25A-4, PBIN V25B-3, PBIN V25C-2 and PBIN V25C-5.

Effect of type of light

There were six different light types tested in this experiment (Table 1). The average light intensities were measured with a data logger LI-COR 1400 (courtesy of PRI) and their corresponding wavelengths (Table 2, Appendix 3) were measured using a spectroradiometer LI-COR 1800 (data were kindly provided by Chris Tebrats and Arjo Meijering).

Table 1. Type of light and light intensity

Lights	Specification	Average light intensity (μmol $m^{-2}s^{-1}$)
Red and Blue (two tubes on the shelf: 2x)	PHILIPS Green Power LED HF deep red and blue	69.8
Red and Blue (three tubes on the shelf: 3x)	PHILIPS Green Power LED HF deep red and blue	97
Blue	PHILIPS Green Power LED HF blue	63.8
Red/Blue/Far Red (set on full: Dimmable)	PHILIPS Green Power LED HF deep red/blue/far red	156.7
TL	PHILIPS Master TL-D 840	87.3
Zurich	PHILIPS Master TL-D 865	50

Table 2. Lights wavelengths

Lights	Wavelengths [main peak(s), nm]
Blue LED	456
Far red LED	734
Red LED	662
TL	436, 490, 546, 586, 612
Zurich*	436, 488, 546, 588, 613

*source: *www.mv.helsinki.fi*

Effect of optical density

Materials and methods for this experiment was similar to those of light experiment except that only materials grown under TL light were used and there were 5 ODs tested (0.4, 0.6, 0.8, 1.0, 1.2).

Data collection and analysis

For the experiment testing kanamycin sensitivity, data of callus and shoot formation were collected and presented as averages of plates examined. For the light type and OD experiments data collected were the number of explants forming callus and shoots, the number of explants showing GUS expression (transformed explants), the number of explants showing GUS expression in callus formed (transformed callus) and finally the transformation efficiency (expressed as the number of explants showing GUS expression in shoots divided by total number

of inoculated explants). All data were presented as a percentage from the total number of explants.

Genstat 14th Edition was used to analyse all data. Because numbers of samples were not balanced, Unbalanced Structure Treatment was chosen for variance analysis. Means comparisons were conducted using Least Significant Differences (LSD) at 5%. Further analysis using regression was also employed on OD experiment to see whether there is a relation between the variables that were monitored and the different treatments.

RESULTS

Kanamycin sensitivity

The presence of kanamycin in the medium made explants stress showing curling and browning especially in high concentration 150 mg/l (Figure 2). Explants on medium without kanamycin formed callus after 10 days of culture, while on 50 mg/l kanamycin, WT Gala explants started to form callus after three weeks of culture. On medium with 75 mg/l kanamycin, callus appeared only after five weeks of culture, while on medium with 100 and 150 mg/l kanamycin there was no callus observed. Colour difference was observed between callus derived from explants grown on medium without kanamycin and explants grown on medium with kanamycin. On medium without kanamycin, callus looked slightly yellow while on medium with kanamycin callus looked white.

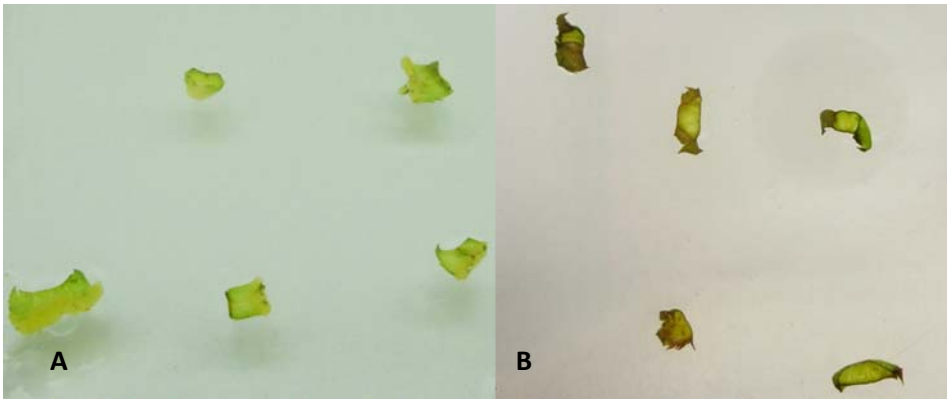

Figure 2. Effect of kanamycin on callus formation in WT 'Gala' after two weeks of growth on shoot induction medium (A: 0 mg/l, B: 150 mg/l)

Shoots started to appear on the third week and only on medium without kanamycin. To check whether the callus formed in the medium with kanamycin can develop shoots, explants from the second replication were allowed to continue growing up to the eighth week. In week eight, the number of explants which formed callus increased by 10% in kanamycin concentration of 50 mg/l and 75 mg/l compared to those on week five. There was no shoot developed from callus in these concentrations. There was no callus and shoot formed in kanamycin concentration of 100 mg/l and 150 mg/l as well. This result showed that on WT Gala, kanamycin allows callus formation in lower concentration but inhibit its further development in any concentrations which were tested. The results suggest effective selection by kanamycin with concentrations higher than 50 mg/l (Table 3). Positive control explants bearing kanamycin resistant gene showed similar responses on media with any concentration of kanamycin to explants on medium without kanamycin. Even on highest concentration of kanamycin (150 mg/l), all positive controls formed callus while WT 'Gala' explants were curling and no callus was formed (Figure 3). The shoots in the transgenic lines looked similar to that of WT 'Gala' in terms of vigour and colour.

Table 3. Effect of kanamycin concentration on callus and shoot formation of explants after five weeks of culture.

Kanamycin concentration (mg/l)	callus formation[¥]	shoot formation[¥]
0	20 c *	2.75 b
50	13.5 b	0 a
75	0.25 a	0 a
100	0 a	0 a
150	0 a	0 a

[¥] Average from four plates, each consists of 20 explants

* Means followed by the same letters in the same column are not significantly different by LSD 5%.

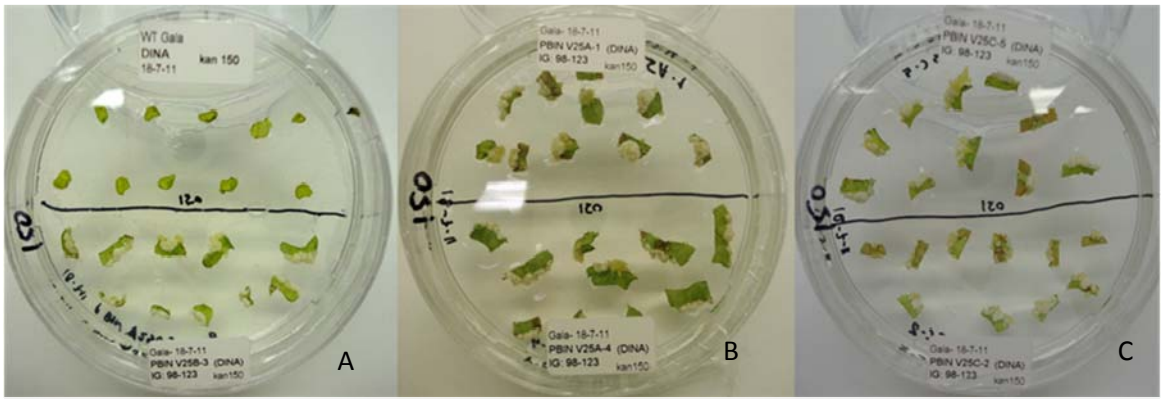

Figure 3. Shoot formation of WT 'Gala' and five transgenic lines of 'Gala' on SIM Gala + 150 mg/l kanamycin after five weeks of culture (A: WT 'Gala' and 'Gala' + PBIN V25B-3; B: 'Gala' + PBIN V25A-1 and PBIN V25A-4; C: 'Gala' + PBIN V25C-5 and BIN V25C-2)

Effect of light types

General performance and appearance in propagation.

Shoots grown under different light types showed similar vigour, except for the 2x material (Figure 4). Blue and TL showed comparable numbers of shoot multiplication (Appendix 1). The colour of medium under TL and Zurich light was less intense than that of others light. Most shoots from Dimmable appeared reddish brown on some parts of the leaves indicative of stress.

Growth and development after transformation

Callus started to appear in the third week, both in Experiment I and II, regardless the type of the light. In contrast, there was no callus formed on explants from the negative control with AGL0 empty in the presence of kanamycin until the fifth week while explants on the regeneration control already formed callus in the second week both on SIM Gala and SIM Gala + cefotaxime but not in SIM Gala + cefotaxime + kanamycin. No further development of callus from experiment control (AGL0 empty) was observed even until final week of observation (week 12). Callus on explants on SIM Gala + cefotaxime looked bigger and brighter of colour than that on SIM Gala. Explants from Experiment I formed callus more than explants from Experiment II irrespective of the type of the light (Figure 5).

Shoot formation was observed in experiment I on the ninth week on explants from TL, Zurich, Blue and Dimmable light while in experiment II, also on the ninth week, shoots were observed on explants from TL, Zurich, Blue and 2x light. Compared to regeneration controls, shoot formation was delayed by 6 weeks indicating the effect of kanamycin on explants, even when they are presumed to be transformed and to be carrying a functional kanamycin resistance gene. Shoot formation showed an opposite response to that of callus formation regarding the explants in Experiment I and II, except for explants for Dimmable and 3x (Figure 6). Explants from Blue light showed highest shoot formation both in Experiment I and II while on the other hand explants from 3x light showed the lowest.

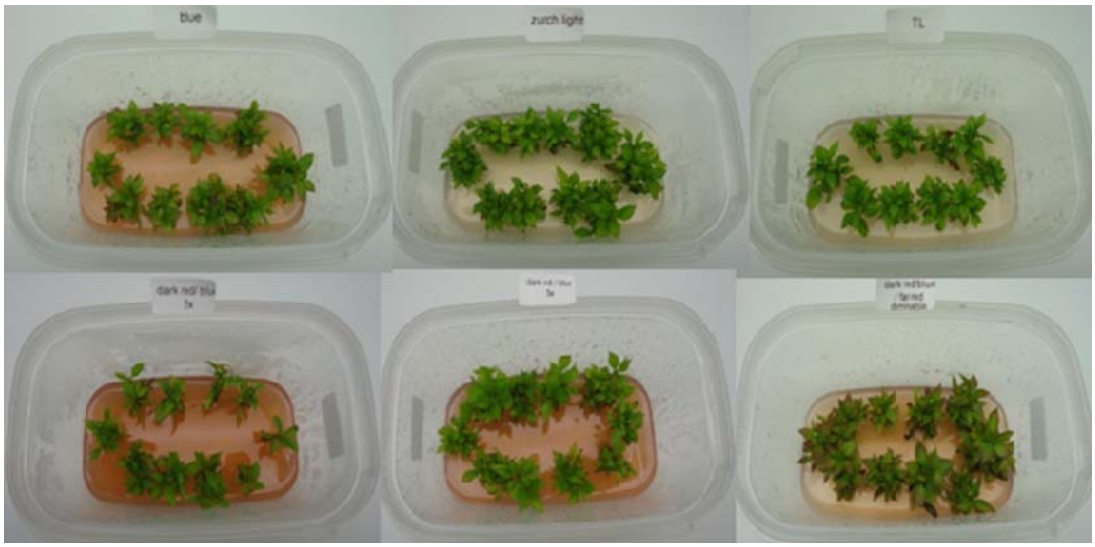

Figure 4. Plant materials grown under different types of light after four weeks of culture

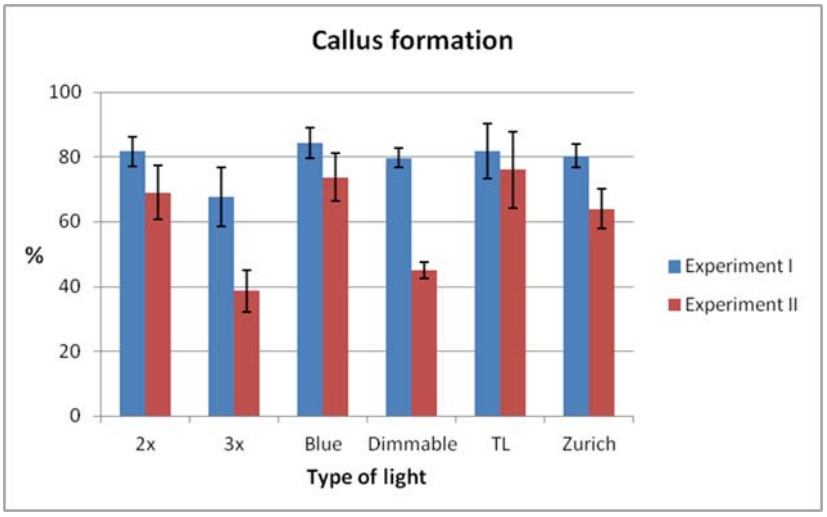

Figure 5. Effect of type of light on callus formation after 12 weeks of culture
(Vertical lines represent standard errors)

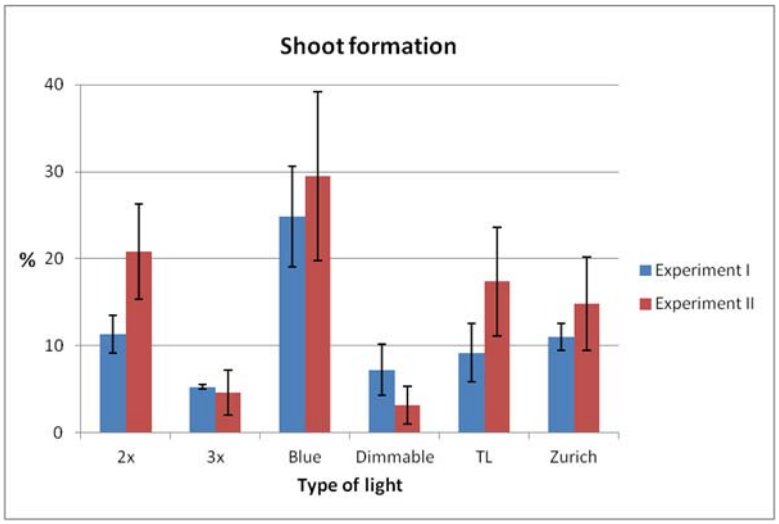

Figure 6. Effect of type of light on shoot formation after 12 weeks of culture
(Vertical lines represent standard errors)

Preliminary result

Table 4. Effect of types of light on gus expression after four weeks of culture

Type of light	Experiment I		Experiment II		Transformed explants (%)	
	No of explants	Transformed explants (%)	No of explants	Transformed explants (%)	Mean	Standard deviation
2x	18	78	21	24	51	38.2
3x	21	57	20	10	33.5	33.2
Blue	20	60	20	65	62.5	3.5
Dimmable	21	57	19	37	47.0	14.1
TL	22	77	21	33	55.0	31.1
Zurich	20	65	20	25	45.0	28.3

The preliminary results gave insight into the effect of the type of light on transformation which was indicated by the number of explants expressing the *gus* gene (Table 4). There were obvious decreasing percentage of transformed explants in all light except Blue which showed a slightly increase from Experiment I to Experiment II. High variability which is showed by high standard deviation may reflect high variability within experiments (plate to plate). Small number of explants which were observed in preliminary result may be one of the causes of variability. Final observation with higher number of explants will show whether such variability can be found as well.

Figure 7 showed obvious GUS expression on calli of explants from 2x, Blue and Dimmable while expression on explants from 3x, TL and Zurich were not that clear. This result showed that only small parts of the explants were transformed and mostly in the edges where the leaf vein lies. Callus formation also developed better in this part. Other parts, like in the middle of the leaf

segment (see Figure 7, red arrow), may also be transformed but further development was rarely observed.

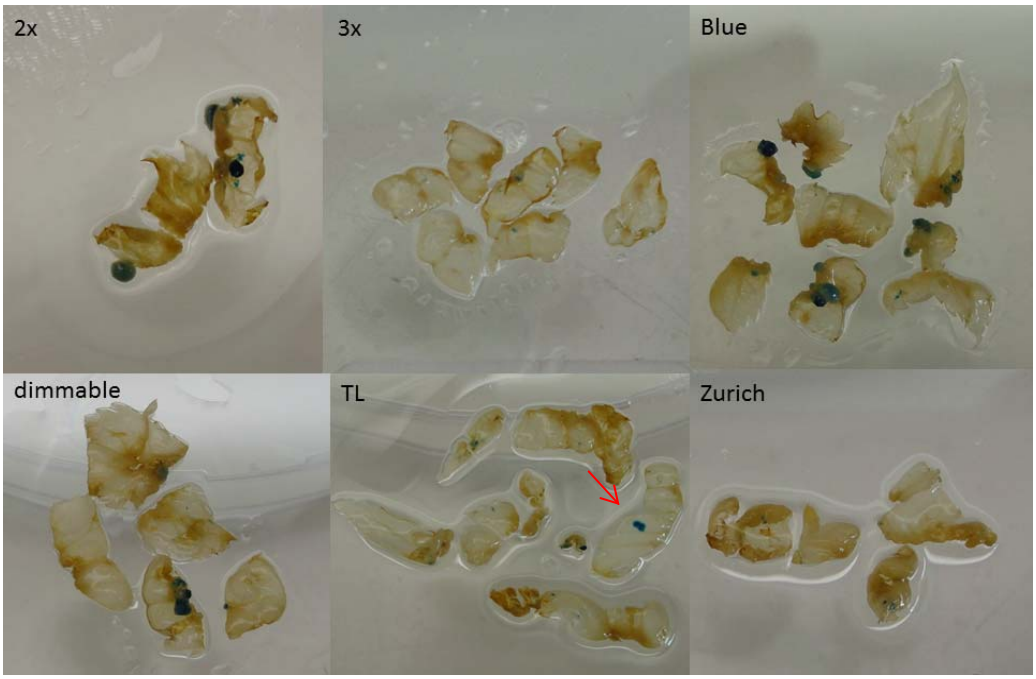

Figure 7. GUS expression on explants from six different light types after four weeks of culture

Final observation

Regardless of the type of light, the percentages of transformed explants observed in Experiment I were generally higher than in Experiment II (Figure 8). This demonstrates the variability that can occur from one experiment to the other with this protocol. This final observation confirmed the preliminary result apart from the outcome that now Blue was also decreasing. The highest decrease was observed from Dimmable light. A similar result was also observed on percentage of transformed callus (Figure 9).

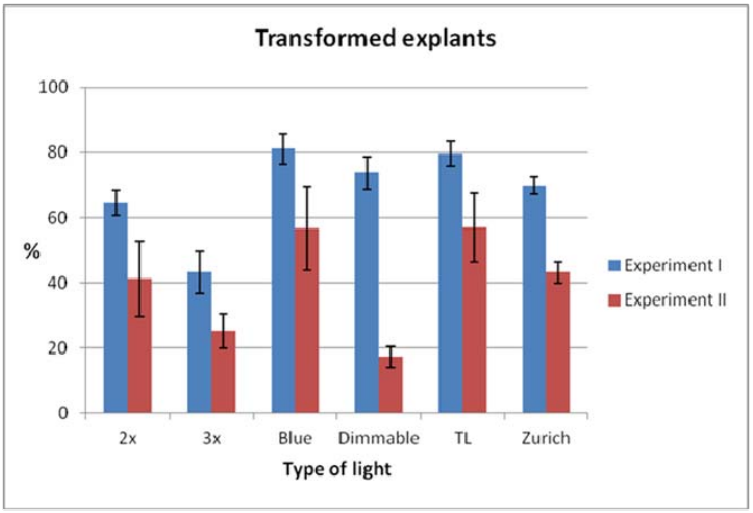

Figure 8. Effect of type of light on the percentage of transformed explants after 12 weeks of culture

(Vertical lines represent standard errors)

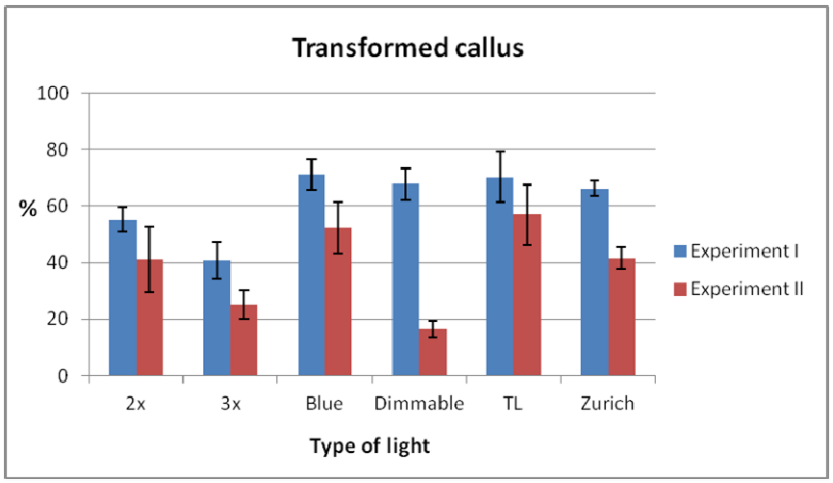

Figure 9. Effect of type of light on percentage of transformed callus after 12 weeks (Vertical lines represent standard errors)

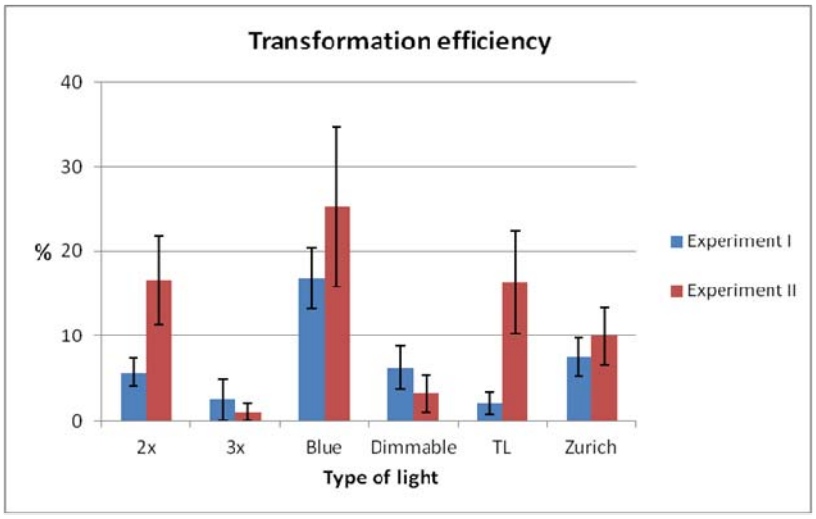

Figure 10. Effect of type of light on percentage of transformation efficiency after 12 (Vertical lines represent standard errors)

Transformation efficiency (Figure 10) showed similar patterns to shoot formation. Since transformation efficiency was calculated based on number of explants with transformed shoots over total explants examined, it was expected to have a similar configuration. Based on the assumption that only shoots derived from transformed cells/callus will be able to develop in the presence of kanamycin, it is expected that the more shoot are formed, the more shoots are transformed. Highest transformation efficiency was also found on Blue light explants which indicated a positive effect of the Blue light on both transformation and regeneration.

Table 5. Effect of type of light on percentage of callus and shoot formation and transformation efficiency observed after 12 weeks of culture.

Type of light	Total explants		Callus formation (%)		Shoot formation (%)		Transformed explants (%)		Transformed callus (%)		Transformation efficiency (%)	
	I	II	I	II	I	II	I	II	I	II	I	II
2x	126	95	82 c*	69 c	11 abcd	21 cde	65 de	41 bc	55 cd	41 bc	6 a	17 bcd
3x	80	100	68 c	39 a	5 ab	5 ab	43 bc	25 ab	41 bc	25 ab	3 a	1 a
Blue	105	92	84 c	74 c	25 de	30 e	81 e	57 cd	71 d	52 cd	17 cd	25 d
Dimmable	70	96	80 c	45 ab	7 abc	3 a	74 b	17 a	68 d	16 a	6 ab	3 a
TL	104	105	82 c	76 c	9 abc	17 bcde	80 e	57 cd	70 d	57 cd	2 a	16 bcd
Zurich	95	130	80 c	64 bc	11 abcd	15 abcd	70 de	43 bc	66 d	42 bc	8 ab	10 abc
Average	97	103	79 b	61 a	11 a	15 a	69 b	40 a	62 b	39 a	7 a	12 b

* Means followed by the same letters within the same variable for both experiments are not significantly different by LSD 5%.

Table 5 provides further information on variables observed within experiments. It also showed that the result can be significantly different over experiments. Explants from Blue light gave consistent positive performances across experiments.

The presence of the light during culture

To observe the effect of light on culture after transformation, three weeks after cocultivation, the second experiment was divided in two where half of the explants was transferred back into the light that were used for shoot propagation initially and the other half was kept in the dark. Explants put under Dimmable light suffered the most showing reddish brown tissues (Figure 11). This indicated that Dimmable light gave a lot more stress to the explants than other lights.

Obvious differences were observed on shoot formation and transformation efficiency (Figure 12) showing culture in dark is better to stimulate shoot formation which is then related to higher transformation efficiency. In contrast, light treatment stimulates callus formation resulting in more transformed callus and transformed explants as well. Effect of light stress can be seen on 3x and Dimmable explants especially in shoot formation and transformation efficiency. There were no shoots formed in spite of relatively high percentages of callus formation. It means that 3x and Dimmable may stimulate callus formation but inhibit shoot formation. Explants from 3x light also showed poor responses and most of them were overgrown by bacteria resulting in half of the plates thrown away.

As in the first experiment, explants from Blue light overall gave better responses regardless the presence or absence of the light (Figure 12). Unfortunately, it was not possible to compare growth under given light between regeneration control and experiment control (with AGL0 empty) with treatments under lights because they were not included.

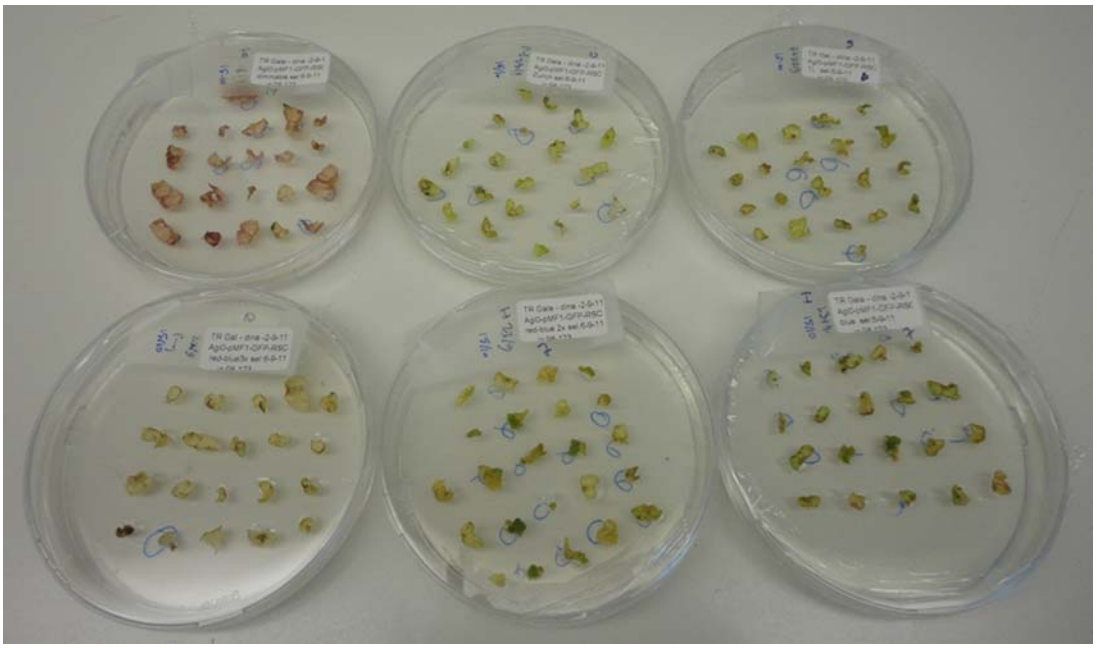

Figure 11. Effect of the presence of light on appearance of explants 18 days after transfer to light treatment
(upper row, left to right: Dimmable, Zurich, TL; lower row, left to right: 3x, 2x and Blue)

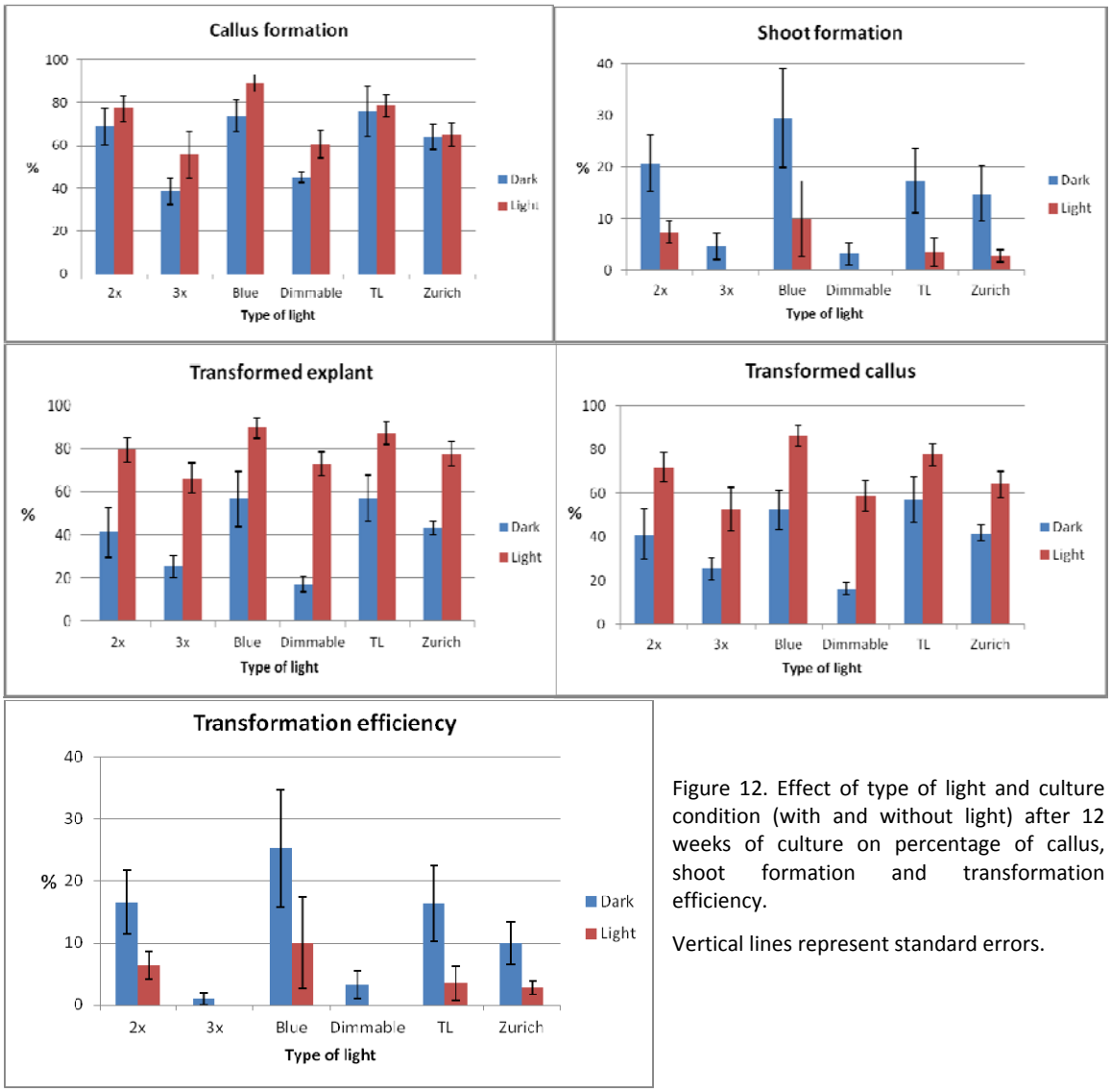

Figure 12. Effect of type of light and culture condition (with and without light) after 12 weeks of culture on percentage of callus, shoot formation and transformation efficiency.

Vertical lines represent standard errors.

Table 6. Effect of type of light and the presence of light during culture on percentage of callus and shoot formation and transformation efficiency observed after 12 weeks of culture.

Type of light	Total explants		Callus formation (%)		Shoot formation (%)		Transformed explants (%)		Transformed callus (%)		Transformation efficiency (%)	
	Dark	Light	Dark	Light	Dark	Light	Dark	Light	Dark	Light	Dark	Light
2x	95	203	69 cde*	77 def	21 de	7 abc	41 bc	79 ef	41 bc	72 def	17 bc	6 ab
3x	100	99	39 a	56 abc	5 abc	0 a	25 ab	66 de	25 ab	53 cd	1 a	0 a
Blue	92	153	74 cdef	89 f	30 e	10 abcd	57 cd	90 f	52 cd	87 f	25c	10ab
Dimmable	96	145	45 ab	61 bcd	3 ab	0 a	17 a	73 def	16a	59 cde	3 a	0 a
TL	105	146	76 cdef	79 ef	17 cde	4 ab	57 cd	87 f	57 cde	77 ef	16 bc	4 a
Zurich	130	231	64 bcde	65 cde	15 bcd	3 a	43 bc	78 ef	42 bc	64 de	10 ab	3 a
Average	103	163	61 a	71 b	15 b	4 a	40 a	79 b	38 a	69 b	12 b	4 a

* Means followed by the same letters within the same variable for both experiments are not significantly different by LSD 5%.

Table 6 showed that culture with and without light had significant different effect on variables observed. The result suggest that for efficient transformation, keeping explants in the dark after transformation is better than treating them with light regardless the type of light tested in this study.

Figure 13 shows GUS expression on calli and shoots of explants with dark and light treatment. There were higher levels of GUS expression in light treatment than n dark treatment. The level of expression is shown by the colour and the coverage of the blue over callus or shoot. The darker the colour and the better the coverage could indicate higher GUS expression. During incubation, after only three hours, explants from light treatment especially Blue and 2x already turned blue. The buffer also turned slightly blue. Explants from 3x and Dimmable grown under light showed more blue coverage on their calli than those grown in dark.

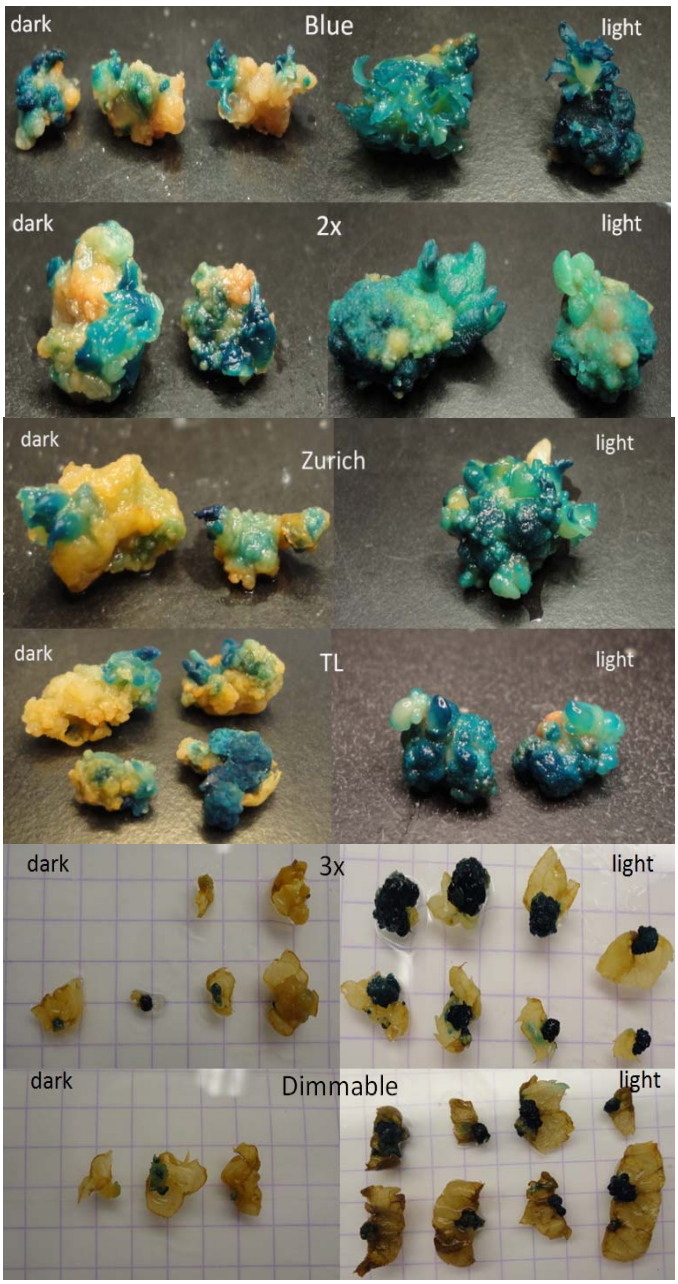

Figure 13. GUS enzyme activity after histochemical staining of shoots and calli from dark and light culture

Effect of ODs

Growth and development after transformation

Callus appeared in the third week, both in Experiment I and Experiment II. Callus showed up in the second week and shoot appeared in the third week on explants of the regeneration control and. Callus formation was also observed in experiment control on medium SIM Gala + Kanamycin + Cefotaxime in the sixth week and they did not develop until the final observation on week 12. Shoot appeared in the ninth week on treated explants. It should be mentioned also that there was no excessive growth of bacteria observed after cocultivation, even with the highest OD.

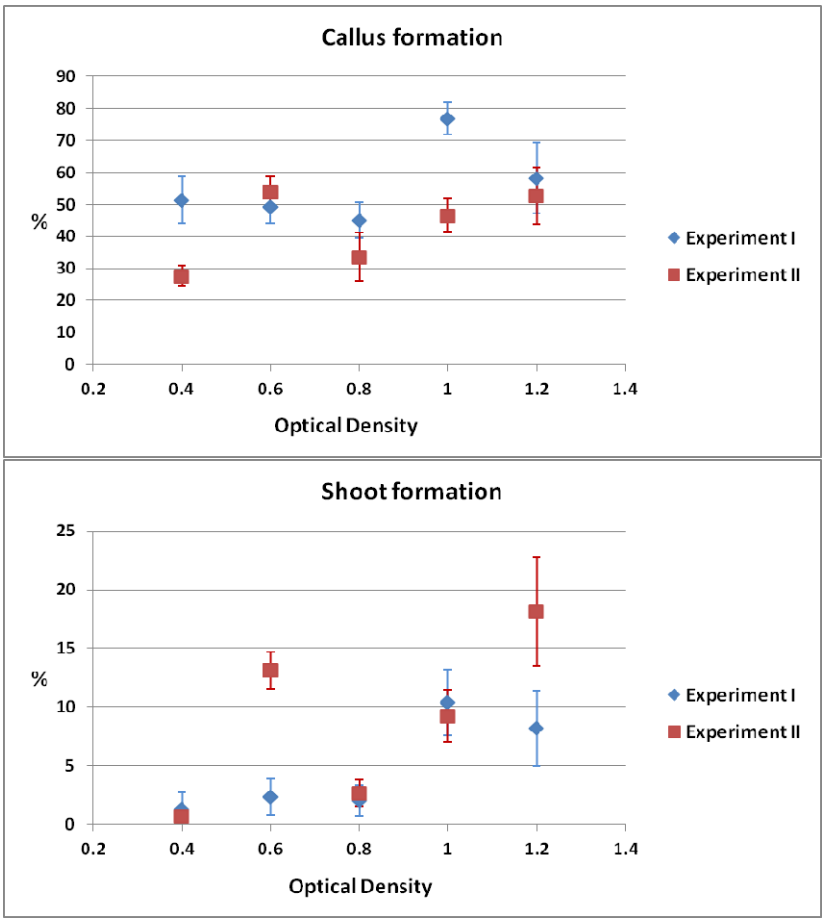

Figure 14. Callus and shoot formation of explants treated by five different ODs after 12 weeks of culture
Vertical lines represent standard errors

In Experiment I, highest formation of callus and shoots was observed in OD 1.0. The pattern for callus looks similar to that of shoot formation for both experiments (Figure 14). Obvious decline from Experiment I to Experiment II is showed in callus formation of explants of OD 0.4 and 1.O. In contrast, high increase is showed in shoot formation of explants of OD 0.6 and 1.2 from Experiment I to Experiment II.

Preliminary result

Table 7. Effect of ODs on gus expression after four weeks of culture

OD	Experiment I		Experiment II		Transformed explants (%)	
	No of explants	Transformed explants (%)	No of Explants	Transformed explants (%)	Mean	Standard deviation
0.4	20	35	34	47	41	8.5
0.6	20	65	19	74	69.5	6.4
0.8	21	24	27	19	21.5	3.5
1	20	35	40	35	35	0.0
1.2	20	45	23	52	48.5	4.9

The order of percentage transformed explants is the same in both experiments starting from OD 0.8 which had the lowest one followed by OD 1.0, 0.4, 1.2, and 0.6 respectively (Table 7). Comparing Exp.I to Exp. II, OD 0.8 showed decreasing percentages while 0.4, 0.6 and 1.2 were increasing. In contrast, observation on 1.0 explants showed steady value even though the number of explants which were observed was twice as much as those in the Experiment I. This result showed that variability within experiments is unavoidable even though other factors (method, plant age, and etc.) are the same.

Final observation

Observations on percentages of transformed explants and transformed callus clearly showed lower values in Experiment II (Appendix 2). Compared to preliminary result, the explants from each treatment in both experiments showed similar tendency.

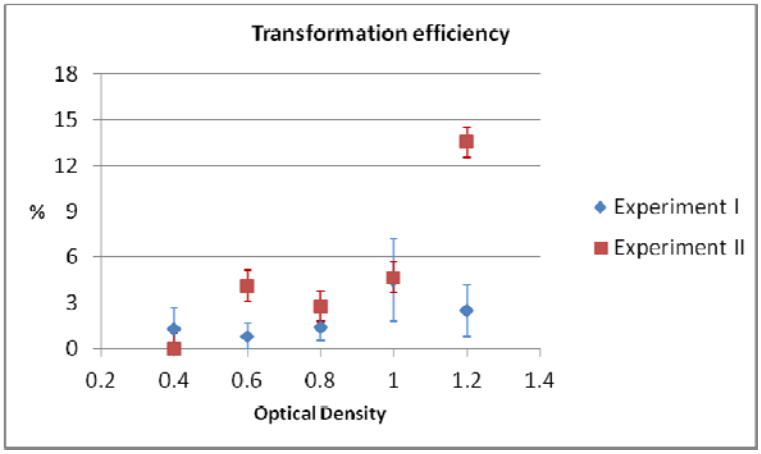

Figure 15. Effect of five different ODs on transformation efficiency
Vertical lines represent standard errors

On the other hand, as expected, transformation efficiency showed similar pattern to shoot formation (Figure 15). The differences are a slightly increase of transformation efficiency of OD 1 from Experiment I to Experiment II aside from the result that shoot formation for OD 1 showed the opposite and there was no shoot observed on Experiment II of OD 0.4. It is also showed that high percentage of shoot formation from OD 0.6 did not result in high transformation efficiency. These shoots may be escapes or the gus activity was very low which makes the expression was not clearly visible.

Table 8. Effect of different ODs on callus and shoot formation and transformation efficiency after 12 weeks of culture.

OD	Total explants		Callus formation (%)		Shoot formation (%)		Transformed explants (%)		Transformed callus (%)		Transformation efficiency (%)	
	I	II	I	II	I	II	I	II	I	II	I	II
0.4	111	137	51 bc*	28 a	1 ab	1 a	44 bc	13 a	29 ab	12 a	1 a	0 a
0.6	135	148	50 bc	54 c	2 ab	13 cd	38 bc	31 abc	34 ab	30 cd	1 a	4 a
0.8	142	176	45 abc	34 ab	2 ab	3 ab	40 bc	18 ab	28 ab	16 ab	1 a	3 a
1	158	175	77 d	47 bc	10 c	9 c	48 c	27 ab	45 c	27 c	5 a	5 a
1.2	119	158	58 c	53 c	8 bc	18 d	35 bc	30 ab	35 bc	30 d	3 a	14 b
Average	133	159	56 b	43 a	5 a	9 b	41 b	24 a	34 b	23 a	2 a	5 a

* Means followed by the same letters in the same variable for both experiments are not significantly different by LSD 5%.

Table 8 showed the effect of OD on variables observed in each experiment. The experiments were significantly different in each variable except transformation efficiency, where only OD 1.2 showed a significant difference within experiments.

Since only transformation efficiency showed no significant different between experiments, regression analysis using pooled data from both experiments only done for transformation efficiency. The slope is significant (p-value= 0.002) which means that there is a linear relationship between OD and transformation efficiency which can be seen on Figure 17.

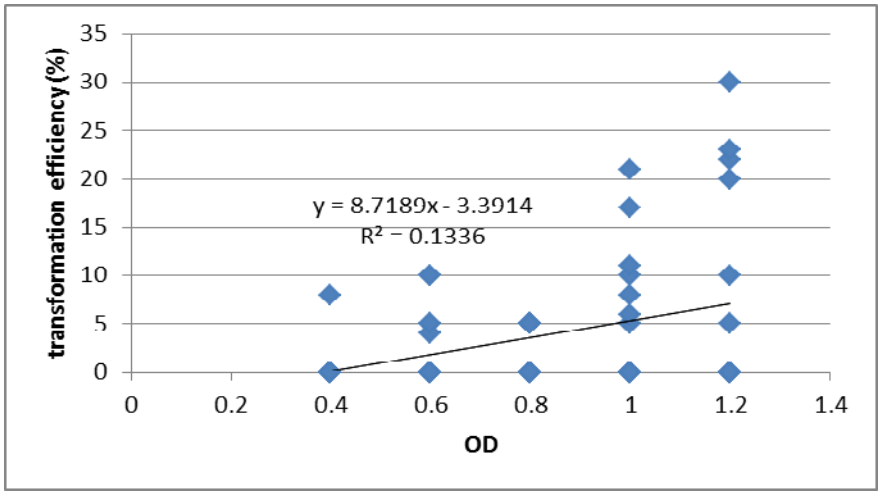

Figure 16. Relation between percentage of transformation efficiency vs. OD

GUS expression on the explants from each treatment is shown in Figure 17. The pattern of the expression is comparable for all treatments. Some of the calli were not expressing the GUS gene which may indicate cells which escape kanamycin selection or simply because of low enzyme activity.

There were several things commonly observed in both experiments (light and OD). First, GUS assays on both experiment controls with AGl0 empty and regeneration controls showed no GUS expression which confirmed that the blue shoots and calli were transgenic. Second, several shoots directly regenerated from explants without callus formation. These shoots were found to be non-transformed, escape shoots (Figure 18A). Third, the presence of chimeric tissues (non-transformed cells and transformed cells) was also observed in both experiments (Figure 18B). The chimeric tissue was especially observed in the OD experiment. A blue dark expression could indicate higher gus expression (Figure 18C).

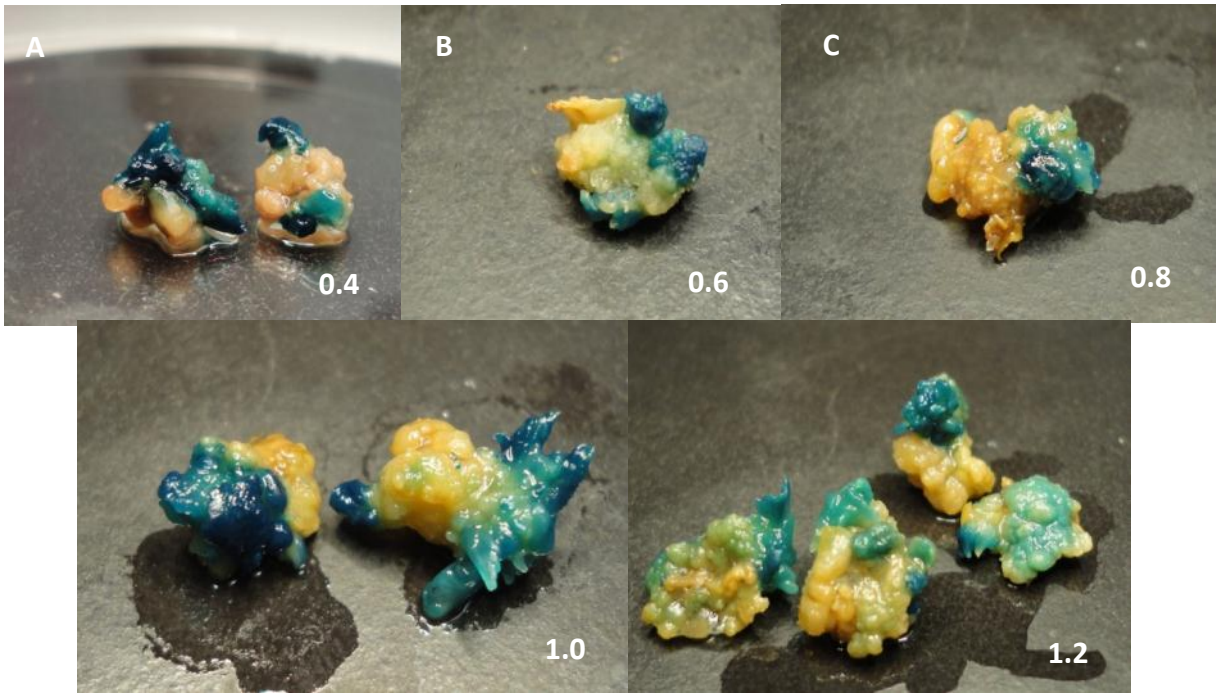

Figure 17. GUS expression of explants treated by five different ODs

Figure 18. A: shoot regenerated directly from explant (red arrow); B: a chimeric explant, C: transverse excision of dark blue expressing explant (left) and less intense blue expressing explant (right)

Other factors contributing to efficient transformation in apple.

As mentioned above, beside the culture of plant materials, there are a lot of factors contributing to a successful transformation in apple, especially with Agrobacterium-mediated techniques. Here, I compared several transformation studies in apple which gave at least 5% transformation efficiency (Appendix 4).

MS is the medium used commonly in these studies either with or without the vitamins and at full or half strength. As carbon source, sucrose, and as gelling agent, agar, are used mostly in propagation medium while sorbitol and gelrite are preferably used in callus or shoot induction medium. Most common PGRs used are BA or BAP, NAA, IBA and TDZ for callus and shoot regeneration. *A. tumefaciens* strain EHA105 is a popular choice as bacterial strain among these studies. While kanamycin is used widely, pmi/mannose as a selection system might provide a promising alternative for the recovery of the plants. Unfolded youngest leaves of 4 week-old

micropropagated shoots are commonly used as the source of explants. Preculture effect varies greatly which makes it difficult to make a general conclusion. Inoculation is generally conducted by immersion with or without shaking treatment in the bacterial solution for at least 2 minutes up to 30 minutes. Cocultivation period varies between 2 to 5 days and is conducted in dark with room temperature 24-26 °C. Selection is conducted for at least two weeks in dark before the explants are transferred to light with or without low light treatment for acclimatization in between. Subculture to new medium during regeneration medium with selective agent is done between 2 to 6 weeks. After shoot appearance, the shoot is transferred to elongation medium or proliferation medium with or without selective agent. Besides these variables, the cultivars used also have an important role since it is showed from these studies that successful transformation has strong tendency to be genotype dependent. Transformation efficiencies range from 5% to 24%.

DISCUSSION

Kanamycin sensitivity

Kanamycin is an aminoglycoside antibiotic which can inhibit plant growth by binding to the ribosome 30S subunit in the chloroplast and mitochondria and interfere with protein synthesis which then leads to chlorosis and death of plants (Duan *et al.*, 2009; Zhang *et al.*, 2001). Therefore, colour difference observed between callus of WT Gala on medium without kanamycin (yellowish) and on medium with kanamycin (whitish) is not unexpected. Even though the explants were kept in dark, which means distinct green and white callus will not be found, the colour difference was obvious.

The proper concentration for selection with antibiotics is the one that inhibits growth of non-transformed cells without being too toxic to transformed cells. In this experiment, it was found that on kanamycin at a concentration of 50 mg/l, more than half of the explants of WT 'Gala' formed callus indicating that the concentration is not enough to inhibit the growth of the cells albeit that shoot formation was severely hampered at this concentration. For efficient selection, the general approach is to use the lowest concentration that completely inhibits cell growth (Park *et al.*, 1998). Therefore, selection with 100 mg/l kanamycin which showed no callus formation whatsoever will be more appropriate. This result confirmed the result from experiments by Puite and Schaart (1996) which is used as a basis for kanamycin concentration determination for PRI method. The use of 100 mg/l kanamycin for selection is still relevant, at least for 'Gala'. Even if the selection is done with 150 mg/l kanamycin, it was shown here that this was not toxic to the transformed cells and explants as demonstrated by the positive controls.

It was also observed that even though selection for transformants in the other experiments was done using kanamycin 100 mg/l, non-transformed calli and shoots still can be found. It indicates that with certain mechanisms, plants can cope with the stress given by kanamycin. Possible mechanisms proposed are: endogenous non-specific tolerance to kanamycin (Jordan and Mchughen, 1988; Ur-Rahman *et al.*, 2009) cross protection of non-transformed cells by transformed cell (Chen, 2011; Dominguez *et al.*, 2004; Park *et al.*, 1998) and persistence of *A. tumefaciens* in infected tissues (Birch, 1997; Dominguez *et al.*, 2004). In experiment control using empty AGL0 for both light experiment and OD experiment, there were several explants formed callus. Nevertheless, the calli failed to develop further which indicated growth inhibition by kanamycin. Cross protection may be one of the escape mechanisms since it was observed that transformed calli especially from OD treatment were chimeric. Even though cefotaxime is present in the medium, it is possible that Agrobacterium can still exist inside the explants. Consistent presence of bacterial colonies which are resistant to kanamycin could also help surrounding tissues to detoxify the antibiotics resulting in regeneration escapes (Dominguez *et al.*, 2004).

Effect of light and OD

Callus formation in general was found in the edge of explants especially where the vein lies. It is due to the fact that the vein has vascular tissues which included cambial cells. These cells have a role in wound healing in plant by forming new cells (callus). Similar result was also reported by Yepes and Aldwinckle (1994) in apple or Peňa *et al.* (2004) in citrus. Callus was also found in the part where the wound because of forceps presence which indicated that callus is a way of the plants to recover from wounding. Wounding induced division and phenolic compounds such as acetosyringone which then recognized by Agrobacterium to induce *vir* gene expression and activate T-DNA transfer (Saini and Jaiwal, 2007; Zambryski, 1992)

GUS assay in preliminary result for both light and OD experiments were slightly different with that of the final observation. Small number of samples and different response from plate to plate seems to be the cause of the differences. GUS expressions were observed mostly in the part where regeneration started, near the cut edge and where the vein lies. Peňa *et al*. (2004) found that, in citrus explants, GUS expression was localized in callus cells coming from the cambium, which indicating that the development of the cambial callus was also essential to obtain transformation. Thus, they suggested that cells competent for transformation and for regeneration were localized in the same callus tissue. The same case observed in this experiment confirmed their view. In this study, direct shoots regeneration (without callus formation) were also observed, mostly in the edge but not in part with the main veins. Nevertheless, they turned out to be non-transfomed cells.

Effect of light

Spectral quality of light is the relative intensity and range of the different wavelengths emitted by a light source and perceived by photoreceptors within the plant (Seabrook, 2005). In this experiment, the spectral quality was showed to have influence on transformation efficiency. Since the transformation efficiency is calculated based on the number of transformed shoot over number of explants examined, the effect of light to stimulate the transformed cells to regenerate into callus and shoot is essential. The wavelength of TL and Zurich is somewhat similar (Appendix 3). Both lights have wide range of light wavelengths which main peaks are in between 436 to 613 nm but none of them has exactly similar peak with blue, red or far red LED. The difference between these two lights is on the intensity of the light which lies on blue wavelength between 436-488 nm. On Zurich, the intensity is slightly higher (> 0.1 µmol $cm^{-2}s^{-1}$) than that of TL (< 0.1 µmol $cm^{-2}s^{-1}$) which may give slightly blue look of Zurich. Far red, blue and red LED showed the specificity of the wavelength, only one peak was observed. 2x has similar light wavelength to 3x which is not unexpected. However, the blue peaks of these lights are not in exactly the same position as that of Blue. Blue has peak in 456nm, 3x has blue peak in 464 nm and 2x has blue peak in 468 nm. The intensity of the blue light of 2x and 3x is much less than that of Blue. On the other hand, red wavelength (660 nm) shifted is not observed. Only the intensity is less which pure red LED has the highest intensity followed by 3x and 2x, respectively.

In the first experiment, the effect of light 2X, Blue, TL and Zurich were more less similar as observed on callus and shoot formation as well as transformed explants and callus. However, Blue light showed highest transformation efficiency. The lights were used to grow plant materials for the transformation. It means that spectral quality was not directly affected the

process of gene transfer. Instead, it influenced growth (physiology) of the starting plant materials having a possible effect on its ability to cope better with stress due to wounding and transformation and recover quickly forming whole plants again. While recovering, it is expected that the competent and transformed cells will develop further to form callus and then shoot. The plant materials from the lights mentioned previously indeed showed good vigour. In addition, dark green leaves showed by plant material from Blue light indicated rich content of chlorophyll. Blue light is important in chlorophyll biosynthesis, stomatal opening, enzyme synthesis, maturation of chloroplasts and photosynthesis (Kim *et al.*, 2004b; Poudel *et al.*, 2008; Samuoliene *et al.*, 2010).

In photosynthesis, plants have various plant pigments absorb light in overlapping spectral regions, mainly are chlorophyll *a* (Chl *a*) and *b* (Chl *b*). The absorption spectrum of Chl *a* is different from that of Chl *b*. In absorption light within blue wavelength, the peak of Chl *a* is at 429.0 nm, while that of Chl *b* is at 454.0 nm (Porra et al., 1989). By using these pigments and other accessory pigments, plants are able to gather a wider range of sunlight. Taking into consideration that Blue light has a peak in 456 nm that closed to the absorption maximum of Chl *b* and that the presence of Blue light gave positive influence to the growth of explants, presumably, in this study the Chl *b* on explants is more active than Chl *a*.

It should be mentioned here that the tubes of 2x and 3x light have more red LED than blue. Each tube consists of 16 blue modules and 40 red modules. Thus, it was expected that red module would give higher effect than the blue one. Nevertheless, the effect of 2x light which is combination of red and blue LEDs and Blue which is pure blue LEDs is more less comparable. Taking into account that Blue and 2x have similar light intensity in average (Table 1), it may explains comparable effect of those lights. In contrast, high intensity of light as showed in Dimmable gave detrimental effect to the explants.

The effect of the intensity of light may be different according to the state of the explants development. The effect of the light to the shoot explants in propagation and maintenance was not as detrimental as its effect on leaf explants since the shoots were able to grow and multiply with only slight reddish brown on the edge of the leaves. In fact, the rate of the shoot multiplication under Dimmable light is similar to that of 2x and 3x light (Appendix 1). On the other hand, leaf explants under Dimmable light failed to generate shoot from the calli. Light intensity as high as 98 µmol $m^{-2}s^{-1}$ gave similar effect with light intensity 13 µmol $m^{-2}s^{-1}$ on shoot regeneration from leaf explants of rootstock M26, but it has better impact on shoot weight (Predieri and Malavasi, 1989). The authors suggested that dark was more effective in inducing regeneration while high light intensity helped further shoot development. This in agreement with the result from dark and light experiment which showed less shoots formed in light treatment, independent of the type of light. Noe *et al.* (1997) observed that light intensity 80 µmol $m^{-2}s^{-1}$ decreased overall shoot proliferation of apple. Yepes and Aldwinckle (1994) found high light intensity, 100-125 µmol $m^{-2}s^{-1}$, reduced regeneration of apple cv Empire and McIntosh while low intensity 15-30 µmol $m^{-2}s^{-1}$ was optimal for regeneration. They stated, once regeneration had taken place, an increase of intensity from 40-70 µmol $m^{-2}s^{-1}$ had a stimulatory effect on shoot growth and development. The method used to regenerate shoot in this experiment was similar to these studies, dark treatment during shoot induction/initiation followed by light treatment after shoot appeared. Unfortunately, because of limited time for the

experiment, the shoots appeared were not transferred to lights. Thus it was not possible to observe the shoot development under light.

By using Blue light, it was possible to obtain 25% of transformation efficiency which is much higher than was reported for Gala which was grown under white fluorescence light previously (DeBondt *et al.*, 1996; Puite and Schaart, 1996).

Effect of OD

In general, several studies on *Agrobacterium*-mediated apple transformation used an optical density between 0.8-1.0 (James *et al.*, 1989; Li *et al.*, 2011; Seong and Song, 2008; Szankowski *et al.*, 2003; Vanblaere *et al.*, 2011; Wu *et al.*, 2011; Xu *et al.*, 2009), 0.5-0.7 (Debondt *et al.*, 1994; DeBondt *et al.*, 1996) or 0.3-0.5 (Puite and Schaart, 1996; Schaart *et al.*, 1995; Zhang *et al.*, 2006). Here, I tested five ODs to see its influence on the transformation efficiency. The result did not show a clear effect of bacterial density. Only on variable percentage of transformation efficiency in the second experiment of OD 1.2 showed significant difference among other treatments including OD 1.2 on the first experiment. Similar result also obtained by DeBondt *et al.* (1994) which concluded the bacterial growth phase had no significant effect on the efficiency of transformation.

Although it is difficult to make a clear conclusion over the best OD for apple transformation, regression analysis with significant slope for transformation efficiency gives indication that higher OD may give more efficient transformation. Taking into account that no excessive growth of Agrobacterium observed on the explants, OD 1.2 may be applied in apple transformation to obtain higher transformation efficiency. Using OD 1.2, transformation efficiency as high as 12 % can be achieved. It is higher than the result of Puite and Schaart (1996) who used OD 0.3-0.5.

Other factors contributing to efficient transformation in apple

A lot of factors may contribute to efficient transformation. Below, I will highlight several aspects regarding to the method comparison presented and the result of the experiments in this study.

Plant materials

This experiment began with limited materials so the use of very young leaves (unfolded) was unavoidable. I observed that when the explants were taken from a very young leaves smaller than 1 cm in length, they tended to wilt and brown easily. The wound because of cutting and also forceps pressure was inevitably causing stress to the explants. Moreover, the presence of the Agrobacterium also put another stress which in the end caused the explants to brown and die. Yepes and Aldwinckle (1994) observed that small leaves gave the least regeneration rate and required higher BA doses to promote regeneration.

While most studies used non rooted in vitro micropropagated shoot as the source of the leaf explants, Dolgov *et al.* (2000) used leaves of rooted shoots and Wu *et al.* (2011) used shoot tips. It is not clear whether rooted and non-rooted shoots as source of explant has an effect to transformation. Probably for the sake of simplicity, non-rooted shoot explants are much easier to obtain. It is also more practical to use leaves than shoot tips since the explants are much

smaller thus requires high skills to handle them while doing transformation and step afterwards. Because the shoot tips also much younger, they tended to wither, brown faster and more susceptible to be overgrown by bacteria than the young leaves.

DeBondt et al. (1996) observed that explants age of 4 weeks is the optimal age for transformation of 'Jonagold'. On the other hand, Seong and Song (2008) found higher transformation efficiency of 'Fuji' by using explants which were taken from young leaves of 8-week-old rooted shoots instead of 4-week-old rooted shoots. Thus it is suggested that variation in leaf structure or physiology might explain the differences in transformation efficiency of different explants age and genotype (Maximova *et al.*, 1998; Seong and Song, 2008).

Preculture

Preculture treatment is a specific treatment given directly to the explants or to shoots as donor explants prior to transformation. There are two kinds of preculture treatment found in these studies, preculture treatment to leaf explants and preculture treatment to shoots (the donor explants). Li *et al.* (2011) found better transformation efficiency with 7 days preculture treatment to the leaf explants of 'Greensleeves'. Debondt *et al*. (1994) observed higher transient expression as the preculture period getting longer up to 6 days but they found otherwise in the 6 weeks of culture. They concluded higher transient expression might not reflect higher stable expression. Schaart *et al.* (1995) and Puite and Schaart (1996) found that preculture for 2 days for 'Gala' and 'Golden Delicious' was not significant to improve transformation efficiency. Yao *et al.* (1995) also found that preculture up to 3 days did not show significant effect on transformation efficiency while preculture for 5 days decrease the efficiency. In other crops, such as strawberry, preculture period for 7 days gave 23.3 % transformation efficiency compared to 11.9% without preculture (Husaini, 2010), while in citrange, preculture reduced the efficiency to nearly half of that of without preculture (Cervera *et al.*, 1998). These results indicated specificity of cultivars or species to this treatment.

While the effect of preculture of leaf explants on apple transformation has been studied extensively, the case for preculture of donor explants is the opposite. Orlikowska *et al.* (2010) stated that cold treatment to donor explants in dark for several months promoted regeneration of apple rootstock while cold treatment for 1 to 2 months had minimal effect. The cold treatment is expected to encourage tissues and plants to resume active growth after transfer back to higher temperature. Sedira *et al.* (2001) used one week dark and cold treatment for the donor explants. However, they mentioned neither any reason of using it nor effect of the treatment. There was no control (without treatment) included in the experiment as well. Thus, the effect is not known. Probably, they aimed the treatment for reducing browning of the explants since light could increase enzyme activity (thus dark treatment could reduce it) and low temperature could decreased phenolic biosynthesis by decreasing enzyme activity (reviewed by Dobránszki and da Silva, 2010).

In this study, preculture was not part of the method. It is interesting to test the effect of this treatment, especially with preculture of explants for 7 days in dark as suggested by Li *et al.* (2011) in combination with or without cold treatment as Sedira *et al.* (2001) did.

Strain/vector

EHA 101, EHA 105, and LB 4404 were used widely across cultivar or even species with various vectors. EHA was used in citrange (Cervera *et al.*, 1998), blackgram (Saini and Jaiwal, 2007), almond (Miguel and Oliveira, 1999), and various cultivars/species of apple (Bolar *et al.*, 1999; Debondt *et al.*, 1994; James *et al.*, 1989; Seong and Song, 2008; Xu *et al.*, 2009). EHA 101 outperformed LBA 4404 while carrying the same vector in apple (Debondt *et al.*, 1994) while EHA 105 outcompeted the same strain in almond (Miguel and Oliveira, 1999). For the case of EHA 101, the supervirulence is correlated with virG and and 3-virB loci which may enhanced transcription of the vir genes leading to a more efficient transport of the T-DNA through the bacterial cell wall (Vanwordragen and Dons, 1992). In this study, AGL0 was used. AGL0 is comparable to EHA 105 but generated in a different way by a different group (Lazo *et al.*, 1991). EHA105 was developed using EHA 101 (Torregrosa *et al.*, 2002). It is expected that they will have similar characteristic regarding the supervirulence nature.

Vector used also influences the efficiency of transformation. A vector with small size for easy manipulation and has broad host range origin replication to allow plasmid maintenance in a wide range of Gram-negative bacteria is preferred (Hellens et al., 2000). Moreover, compatibility with the Agrobacterium strain, the type of plant which will be transformed, and the size of DNA fragment are also important. Other features, such as selectable marker gene and reporter gene may also be useful.

Inoculation time, inoculation method and coculture

Inoculation time, method and coculture are important to ensure the occurrence of the induction of virulence, attachment and gene transfer. For apple, inoculation time for 2-30 minutes with explants immersion in the inoculation is commonly used. Several studies also included shaking while immersion to ensure proper and even contact between explants and Agrobacterium. Radchuk and Korkhovoy (2005) used a different method for inoculation. Instead of immersion, they put a thin layer of Agrobacterium on explants which then result in higher transformation efficiency than that of five minute immersion. They assumed that higher cell density and better contact between explants and Agrobacterium were the causes of such result. In general, short time of inoculation may not provide enough time for the attachment of the bacteria to the explants while longer time may risk the explants for overgrown by bacteria. It was observed in this study (in experiment control) that explants that kept in the bacterial inoculums up to one hour were severely overgrown by bacteria and cannot be rescued even with immersion for ten minutes in cefotaxime. The explants became more susceptible for wilting and browning.

This study used coculture period of 4 days. Coculture for 4 days was found to be effective to increase apple transformation efficiency compared to 2 or 5 days (Debondt *et al.*, 1994). Similar finding was observed using 'Fuji' (Seong and Song, 2008) which also showed increasing coculture period to 5 days decrease the efficiency. On the other hand, Yao *et al.* (1995) found there was no significant effect of coculture period for 2-5 days while (Xu *et al.*, 2009) observed excessive overgrowth of *A. tumafaciens* in 4 days coculture. In tomato (Sharma *et al.*, 2009), *Dendrobium* (Subramaniam *et al.*, 2009), blackgram (Saini and Jaiwal, 2007), and citrange (Cervera *et al.*, 1998), 3 days coculture found to be the most efficient while in artemisia 3-4 days gave highest

gus expression and in tea, 5 days coculture was the most efficient (Mondal *et al.*, 2001). Generally, cocultivation between 2-5 days is appropriate for apple transformation. Longer cocultivation may pose threat for overgrown of explants by bacteria as well.

Medium and PGR

Yepes and Aldwinckle (1994) observed significant interaction between genotype, medium and BA level on apple regeneration. Since the ability to regenerate whole plants from somatic tissues is a prerequisite for Agrobacterium-mediated transformation (Ibrahim and Debergh, 2001), it is important to establish a well-defined regeneration techniques for corresponding genotype before transformation conducted. Each step of the regeneration (callus, shoot, and root regeneration) may need different medium as well as phytohormones. For basal medium, MS is still preferred, probably because it has complete compositions for macro and micronutrient. Several studies also used QL medium. As for shoot initiation, cytokinins, mostly TDZ and BA, are widely used in apple. Moreover, the addition of a small quantity of auxin, primarily NAA, was found to be beneficial by many authors.

As the best medium for regeneration does not necessarily mean the best medium for transformation as well, it is more important to seek for a balance between these two steps which gives high transformation efficiency and a reasonable regeneration. Type of carbon source used in the medium for apple regeneration has been studied as well. While the capacity to metabolise different type of sugar may vary within plant kingdom (Zhu *et al.*, 2001), it was observed that in *Malus*, sorbitol is the primary product of photosynthesis and the major translocation for of carbon (Loescher *et al.*, 1982). Thus, it is expected that using sorbitol as carbon source in the medium is suitable for early regeneration of transgenic cells since it may be easily metabolised. The medium and PGR used for 'Gala' in this experiment is based on a study by Puite and Schaart (1996) which showed MS medium with corresponding concentration of NAA, TDZ and BAP was the most efficient for leaf regeneration and transformation.

Selection marker and antibiotics

The result of kanamycin sensitivity experiment confirmed kanamycin concentration used for selection in this study. Degenhardt *et al.* (2006) revealed the possibility of using mannose as an alternative selectable marker to obtain more efficient transformation. In addition, another method of apple transformation without the use of any selectable marker has been described by Malnoy *et al.* (2007). They obtain 12% and 25% transformation efficiency for 'Galaxy' and 'M.26' respectively. Since both methods result in higher than 20% of transformation efficiency, it is exciting to see whether they can be applied for 'Gala' as well.

Cefotaxime alone (200 mg/l) was reported not able to control growth of Agrobacterium EHA 101 (DeBondt *et al.*, 1996). However, in this experiment using cefotaxime (250 mg/l), severe overgrowth of Agrobacterium AGLl0 was rarely observed. Li *et al.* (2011) observed MEPM at 50 mg/l enhanced transformation efficiency in comparison with cefotaxime 300 mg/l. The used of MEPM was aimed as an alternative antibiotic since cefotaxime prevents shoot regeneration in several cultivars. Since it is not the case for 'Gala' and the control experiment showed cefotaxime promoted regeneration of leaf explant, it can be concluded that the use of

cefotaxime for killing Agrobacterium is appropriate. Yepes and Aldwinckle (1994) also found cefotaxime (250 mg/l) to be the least toxic and also promoting apple leaf segment regeneration.

Method improvement for apple transformation

Each factor described above has an important role to obtain efficient transformation. For a reliable result, a large number of biological replications are required for determining effect of the corresponding factors. This is to avoid variability due to the plant physiology, technical skills, or other unknown factors.

In this thesis project, by using the already well-established method for apple transformation from PRI, higher transformation efficiency could be achieved through light quality improvement and higher OD. Blue light is suitable to grow the plant materials prior to transformation. In this study it was not possible to see the effect of Blue light for further development of the newly regenerated shoot when transferred to light, thus it is interesting to see further effect of this light to obtain transgenic plants. Using OD up to 1.2 give slightly higher result than the generally used OD, 0.4. Further investigation is needed whether this result is consistent in other experiments. The PRI method used kanamycin concentration of 100 mg/l for selection which proved to be appropriate in this study. In addition, explants preculture give potential improvement of apple transformation, therefore may also be included for further examination.

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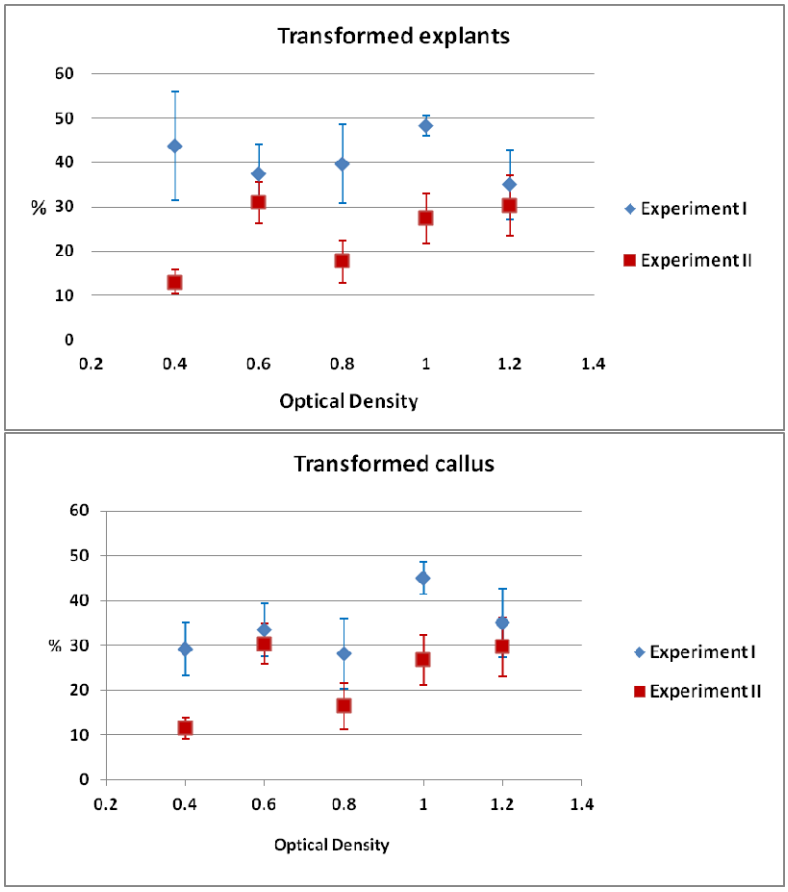
Appendices

1. Shoot multiplication of shoot explants

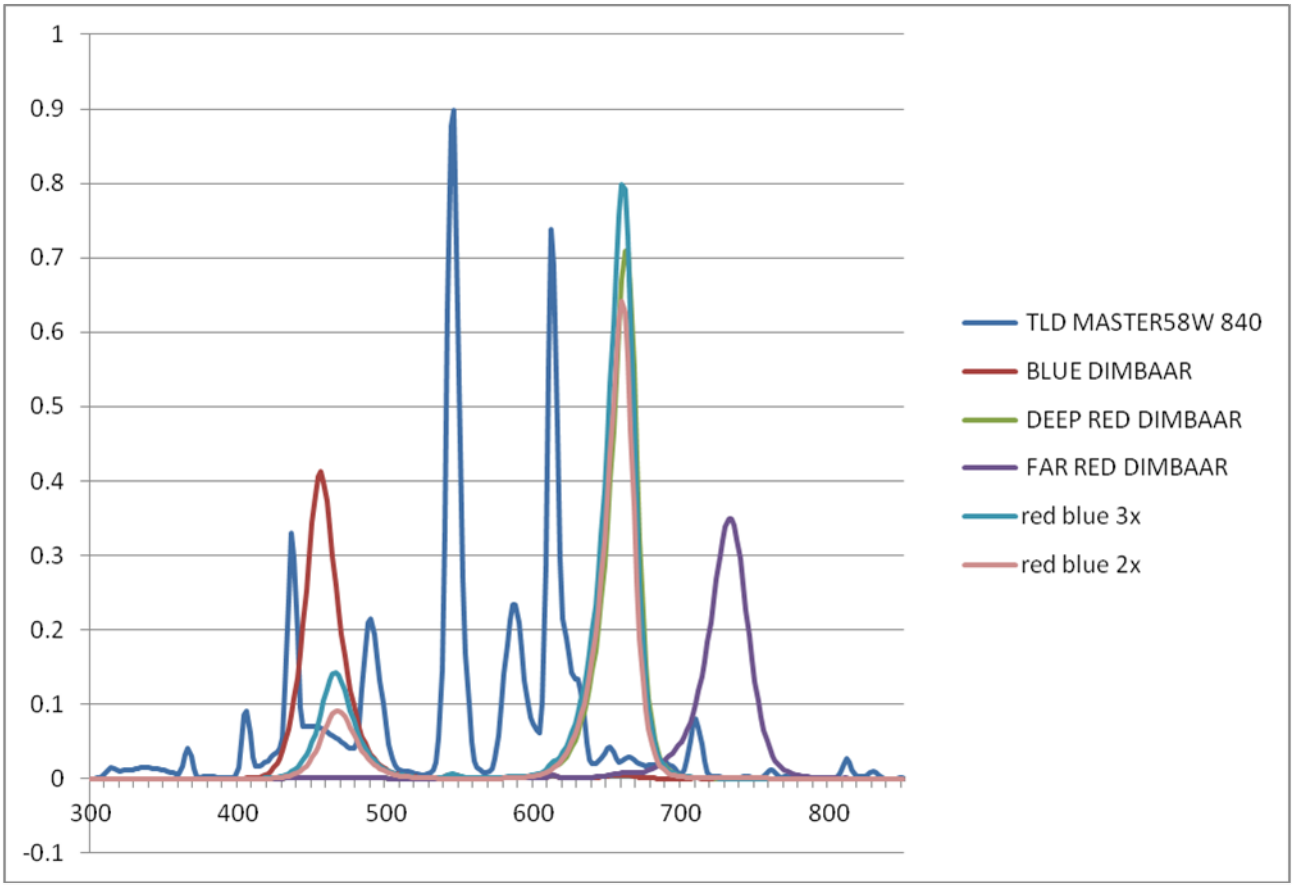
Light	number of shoot/explant		
	Exp I	Exp II	Average
Blue	6	9	8
TL	5	10	8
Zurich	4	9	7
Dimmable	4	7	6
2x	5	6	6
3x	4	7	6

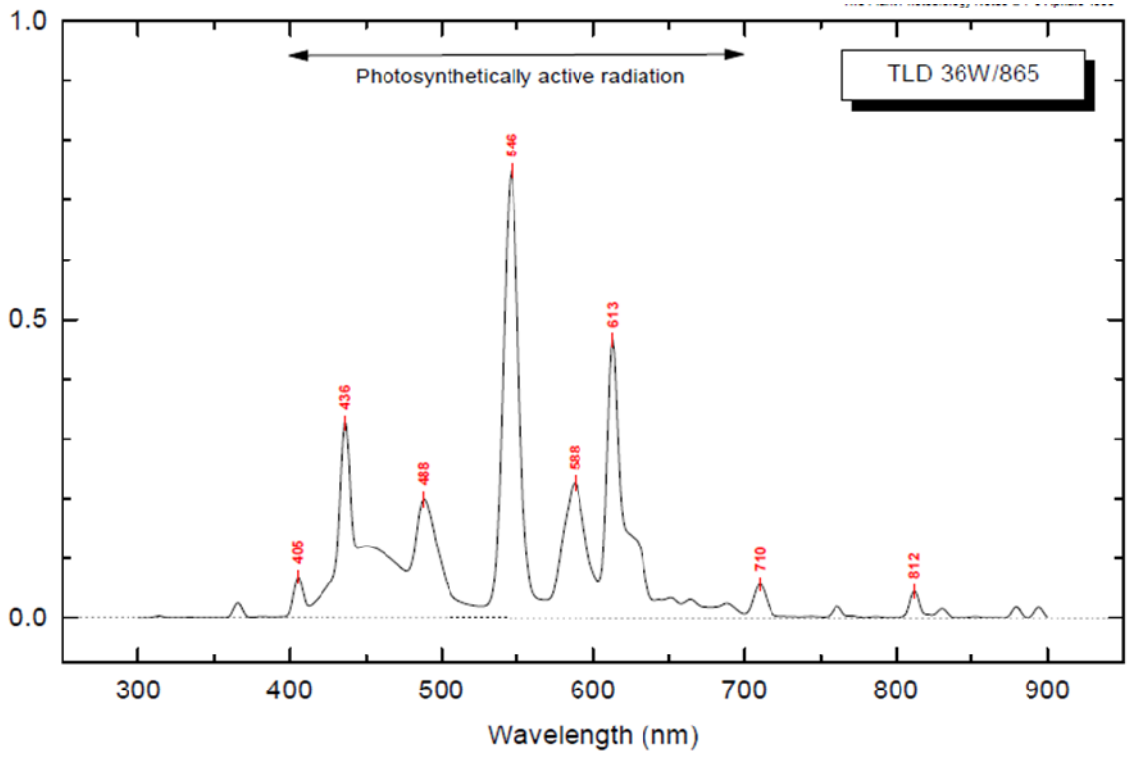
2. Effect of five different ODs on transformed explants and callus



Vertical lines represent standard errors

3. Wavelengths of different types of lights





4. Comparison of Transformation Method in Apple

Microsoft Office
Excel Worksheet