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Low Co-Cultivation Temperature at 20oC Improved Agrobacterium tumefaciens-Mediated Transformation of Tobacco Leaf Disks

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LOW CO-CULTIVATION TEMPERATURE AT 20^{o}C IMPROVED *AGROBACTERIUM TUMEFACIENS*-MEDIATED TRANSFORMATION OF TOBACCO LEAF DISKS

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I would like to offer my special gratitude to my husband Yongbo Peng whose patient love enabled me to complete this work.

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LIST OF ABBREVIATIONS

AS	Acetosyringone or 3, 5-dimethoxy-4-hydroxy Acetophenone
A.tumefaciens	*Agrobacterium tumefaciens*
CaMV	Cauliflower Mosaic Virus
cv	cutivar
FW	Fresh Weight
g	gram
GUS	β-1, 4-Glucuronidase
kb	kilobase
mg	milligram
T-DNA	Transferred-DNA
tml	tumor morphology large gene
TMV	Tobacco Mosaic Virus
Ti Plasmid	Tumor-Inducing Plasmid
TIP	Tumor-Inducing Principle
vir genes	virulence genes

ABSTRACT

I investigated the effect of co-cultivation temperature and binary Ti vectors on the fresh weight yield of leaf disks of tobacco (*Nicotiana tabacum* L. cv. Xanthi (nn, Smith) after *Agrobacterium tumefaciens*-mediated transformation. I concluded that the temperature at 20 ℃ during co-cultivation of leaf disks with *A. tumefaciens* was the most critical factor on the reproducible enhancement of fresh weight yield.

The highest fresh weight increase and presumably transformation efficiency was achieved under the following experimental conditions: *A. tumefaciens* strain LBA4404 containing pLSU binary Ti vector was grown overnight at 28^{o}C in liquid *Agrobacterium* medium and the concentration was adjusted to $3x10^{7}$ cells/ml (0.3 A_{600} units/ml). Leaf disks were co-cultured at room temperature with bacteria for 60 min in the liquid MS medium containing 200 μM acetosyringone and 0.001% (w/v) Silwet L-77 without antibiotics. Pretreated disks were co-cultivated for 4 days under constant light at 20°C in solid MS shoot medium containing 200 μM acetosyringone without antibiotics. Co-cultured leaf disks were selected for 14 days under constant light at 25°C on MS shoot selection medium containing 300 mg/l kanamycin and 500 mg/l carbenicillin. Selected leaf disks were transferred to freshly prepared selection medium for an additional 14 days under constant light at 25°C. Fresh weight yields were measured at the end of a 28 day growth period.

Of the four tobacco cultivars tested, Xanthi (nn Smith) was found to be sensitive to kanamycin concentrations at 100 mg/L and above, and hygromycin concentrations at 25 mg/L and above. I compared five co-cultivation temperatures from 15, 18, 20, and 22 to 25^{o}C during the 4-day period and found the temperature at 20^{o}C promoted the highest

increase in fresh weight yield. I also tested four new binary Ti pLSU vectors containing the plant-expressible kanamycin resistance gene, and four new pLSU vectors containing the hygromycin resistance gene with or without the β-1, 4-Glucuronidase (GUS) gene. New pLSU vectors were similar to conventional pCAMBIA vectors in promoting the increase in fresh weight yield.

INTRODUCTION

Agrobacterium tumefaciens is a Gram-negative soil bacterium and plant pathogen causing crown gall disease in angiosperms and gymnosperms (Smith &Townsend, 1907). *Agrobacterium*-plant interaction was one of the first model systems in which the molecular mechanism for plant pathogenicity has been elucidated in detail (Zaenen et al., 1974; Chilton et al., 1977). About 20 kb segment of DNA (T-DNA) in a tumor-inducing plasmid (about 200 kb Ti plasmid) is transferred from the bacterium to the host plant genome by molecular machinery closely resembling bacterial conjugal transfer (Zupan & Zambryski, 1995; Sheng & Citovsky 1996; Gelvin 2003). The disease phenotype is a manifestation of expression of bacterial T-DNA genes in infected plant cells, which over-produce the plant growth hormones, cytokinin and auxin.

Agrobacterium tumefaciens-mediated transformation has been generally used for genetic transformation of higher plants since 1983. This natural DNA transfer system has been exploited to introduce genes of agronomic interest into plants, resulting in the production of genetically modified crops by commercial plant biotechnology industries. Initial approaches of gene transfer were to introduce a target gene into the T-DNA region of Ti plasmid after either a single- (co-integration) or double-homologous recombination between an intermediate vector (pRK290) and Ti plasmid (Zambriyki et al., 1983; Murai et al., 1983). A binary plant vector strategy was designed to separate the T-DNA region in a small plasmid from the virulence genes in an avirulent T-DNA-less Ti plasmid (Hoekema et al., 1983). The small plant vectors with the T-DNA region are now called binary Ti vectors (Hellens et al., 2000; Komori et al., 2007).

In a model plant *Arabidopsis thaliana*, gene transfer has become routine using the floral dip procedure (Clough & Bent 1998). However, the floral dip procedure does not work for other plants, and there are major technical barriers in transformation of major crops such as soybean, maize, sugarcane and wheat.

Hard lessons were learned when attempting to generate a large number of transgenic tobacco plants to study the effect of 5'-deletion mutations on the promoter activity of the bean seed storage protein phaseolin gene (Burow et al., 1990; 1992). While trying to generate a minimum of ten independent transgenic plants per each of seven deletion constructs, tobacco transformation experiments had to be repeated eight times since only two experiments worked successfully to generate sufficient numbers of transgenic plants. It has not been clear as to what the problem was at that time, until obtaining the results of the experiments reported here.

The importance of controlled temperature during the co-cultivation period was not taken seriously at all in the above-mentioned transformation experiments in 1989. The small culture room (160 cm wide x 142.4 cm deep x 215 cm high) housed one metal shelf rack with four shelves (45 cm wide x120 cm deep) spaced 40 cm apart. Two fluorescent lamp fixtures each holding two 48 inches-long fluorescent lamps (Philip Westinghouse Lamps F40CW Cool White 40W), were hung from each shelf for constant light. The fluorescent lamps generated the heat in the small culture room resulting in a temperature gradient from the top to bottom shelves. While the room temperature of the building was maintained at around 24.5^{o}C, the temperature of the top shelf in the culture room could have been over 30^{o}C. Accordingly, we observed transformation of tobacco leaf disks was most effective on the bottom shelf, and least effective on the top shelf.

The virulence genes are essential for processing and transferring T-DNA from bacteria to plant cells. This regulon is specifically activated by phenolic compounds including acetosyringone secreted from wounded plant cells (Stachel et al., 1985). The VirA-VirG two component regulatory system controls virulence gene expression. At temperatures above 32^{o}C, the virulence genes are not expressed because of a conformational change in the folding of VirA, which inactivates the protein (Jin et al., 1993).

Riker (1926) showed that tumor formation in the tomato plant was sensitive to the elevated temperatures up to 28^{o}C. No tumor was formed above 30^{o}C. The size of tumors produced on inoculated plants increased as the incubation temperature decreased, with the largest tumors formed on plants maintained at 22 $^{\circ}$C.

Braun (1947) confirmed Riker's observation with the temperature shift experiments from 26 $^{\circ}$C to 32 $^{\circ}$C and showed that the tumor formation requires at least 2 days after inoculation. He called this initial or co-cultivation period as the inception phase. He divided the inception phase into the temperature-dependent induction process of the bacterium and temperature-insensitive conditioning process of host plants. During this phase, some factor was moving from the bacterium to the plant cell to cause the transformation at this point. Braun coined a term "tumor inducing principle" (TIP). TIP was found to be the T-strand of T-DNA of Ti plasmid. Braun also showed the second phase of tumor formation (developmental phase) was insensitive to the temperature shift treatment.

Pili were considered as transport channel and are required to transfer DNA from bacteria to the plant cell. Pilus formation is temperature dependent. Fullner et al. (1996)

showed that pili formation also needs induction of virulence genes; this was because pili were never seen on cells grown on agar plates without acetosyringone. They also showed the stability of pili is temperature-dependent, and pili were more abundant at 19 ˚C than at 28 ˚C. The *Agrobacterium* pili were shown attached to the tobacco leaf cell surface apparently forming a stable connection (Aguilar et al. 2010). Then the pili create a channel to transfer the complex of T-strand and virulence proteins into the plant cell. Taken together, transfer of T- strands, and the pili channel structure through which T-strand migrate from the bacteria to plant cells are temperature-dependent.

A first objective of my research was to determine which cultivar among the four available cultivars was the most appropriate host to use for *Agrobacterium*-mediated transformation. A second objective was to determine optimal concentrations of antibiotics kanamycin and hygromycin for rigorous selection for transformed calli and shoots. A third objective of this research study was to determine the optimal temperature during the co-cultivation period on increase of the final fresh weight yield of leaf disks. A last objective was to compare the effectiveness of new binary vectors pLSU with the conventional pCAMBIA vectors in *Agrobacterium*-mediated transformation of tobacco leaf disks.

MATERIALS AND METHODS

Tobacco Seeds

Tobacco (*Nicotiana tabacum* L. cv. Xanthi) seeds were obtained from E. Ted Woodlief, Department of Crop Science, North Carolina State University, Raleigh, NC. Two genotypes, nn (Smith) and NN with regard to the resistance to infection by tobacco mosaic virus (TMV) were identified.

Tobacco cv. Xanthi (Tsukuba) seeds were obtained from S. Ohtsuki at the National Institute of Agrobiological Resources, Tsukuba, Japan. The genotype of seeds is not known.

Tobacco cv. Wisconsin 38 seeds were kindly provided by Folle Karl Skoog, Department of Botany, University of Wisconsin, Madison, WI.

Tobacco Plantlets Grown *in vitro*

Tobacco seeds were sterilized by 50% (v/v) Clorox and planted per Petri dish on MS-medium (Murashige and Skoog, 1962) (per L 4.3 g Sigma MS salts (M5524), 0.1g *myo*-inositol, 0.4mg thiamine-HCl, 30g sucrose, and pH 5.6, 10g agar for solid medium). Seeds were germinated and plantlets were grown in the growth room under constant light with 61 $\mu E\ m^{-2}\ s^{-1}$ at room temperature. Plantlets were sub-cultured at four week intervals.

Binary Ti Vectors of *Agrobacterium tumefaciens*

A series of plasmid constructs pCAMBIA were obtained from Center for the Application of Molecular Biology to International Agriculture (VAMBIA, Canberra, Australia). Seokhyun Lee of this laboratory constructed new binary Ti vectors pLSU used in this study. The first class of binary vectors contains the plant-expressible kanamycin resistance gene with or without GUS report gene (Abbreviation: N2S, N2R, N2SGS, and

N2RGS). The second class has the plant-expressible hygromycin resistance gene with or without GUS reporter gene (Abbreviation: HS, HR, HSGS, and HRGS).

Agrobacterium tumefaciens

Agrobacterium tumefaciens LBA4404 strain was purchased from Invitrogen (Carlsbad, CA). The bacteria were maintained at 28^{o}C in *Agrobacterium* media (A-media) (per liter 5.0 g yeast extract, 2.0 g mannitol, 2.0 g $(NH_4)_2SO_4$, 8.570 g K_2HPO_4, 4.192 g KH_2PO_4, 160 mg $MgSO_4.7H_2O$, 5.0 mg $FeSO_4.7H_2O$, 11.0 mg $CaCl_2.2H_2O$, and 2.0 mg $MnCl_2.4H_2O$).

Co-cultivation of Tobacco Leaf Disks with *A. tumefaciens*

Agrobacterium tumefaciens-mediated transformation of tobacco leaf disks is according to Burow et al. (1990), as modified by Park (2006). *A. tumefaciens* LBA4404 strains were grown overnight in the *Agrobacterium* medium at 28℃, 250rpm with appropriate antibiotics. Cells were harvested by centrifugation and resuspended in liquid MS medium (per L 4.3 g MS salts, 30 g sucrose, and pH 5.6) at a concentration of $3x10^7$ cells/ml (0.3 A_{600} units/ml). Leaf disks were cut from mature leaves of 4-week-old tobacco plantlets using a cork borer with 1-cm inner diameter, and mechanically wounded with multiple needles of kanzan (Stone Lantern, Passumpsic, VT). Ten wounded leaf disks were soaked in 10 ml of *A. tumefaciens* inoculum containing 200 μM acetosyringone and 0.005 % (v/v) Silwet L-77 for 60 minutes. Inoculated leaf disks were blotted thoroughly on sterilized brown paper towels. Ten leaf disks were plated per Petri dish on the co-cultivation medium (per L 4.3 g MS basal salts, 1 mg nicotinic acid, 1 mg pyridoxine HCl, 0.1 mg thiamine-HCl, 100 mg *myo*-inositol, 1 mg 6-benzylaminopurine,

0.1 mg α-naphtalene-3-acetic acid, 30 g sucrose, 10 g agar at pH 5.6) containing 200 μM acetosyringone without any antibiotics.

Tobacco Leaf Disk Transformation Mediated by *A. tumefaciens*

After leaf disks were co-cultivated with *A. tumefaciens* at different temperatures (15, 18, 20, 22 and 25^{o}C) for 4 days under constant light with 56μE m^{-2} s^{-1}, they were transferred to the shoot selection medium (the same medium composition as co-cultivation medium) containing 500 mg/L carbenicillin and 300 mg/L kanamycin or 50 mg/L hygromycin. Carbenicillin was used to eliminate the bacterium. A range of kanamycin concentrations were tested (0, 100, 250, 500 and 1000 mg/L) and a range of hygromycin concentrations were also tested (0, 25, 50, 100 and 200 mg/L). Leaf disks were incubated at 25 ± 0.7℃ under constant light for 14 days and then transferred to the fresh shoot selection medium for another 14 days. At the end of 28 days selection, the digital images of lead disks were recorded for later examination, the fresh weights of each leaf disks were measured in mg, and the harvested leaf disks were stored at -80^{o}C in a deep freezer for GUS assays.

Determination of Increase in FW Per Plate over Vectorless Control

I took a conservative estimate for the increase in fresh weight per plate over the vectorless control; i.e. each leaf disk FW value was subtracted by the average of vectorless control plus its standard deviation. I discarded any leaf disks without a FW increase compared to the control since those leaf disks turned the leaf color from green to color-less (yellow or white) and were presumably un-transformed and dying. There are five replicates under each treatment, and each replicate consisted of a single Petri dish containing 10 or less leaf disks with the increase in FW. I added up FW increases from 10

or less leaf disks of one Petri dish to obtain a sum of the FW increases per replicate, and averaged the five replicates for each treatment. I used three digits in calculation and keep two digits in my results.

Statistical Analysis

The data were subjected to one-way ANOVA program of Statistical Analysis System (SAS) software, SAS Online Doc 9.1.3 from SAS Institute (Cary, NC). Means were compared using Tukey's Studentized Range (HSD) test (α=0.05).

RESULTS

I was first interested in choosing an appropriate cultivar of tobacco host and determining an optimal concentration of antibiotics for rigorous selection of transformed cells. Four tobacco cultivars, Xanthi (nn, Smith), Xanthi (NN for the resistance to TMV infection), Xanthi (Tsukuba), and Wisconsin 38 were tested for tolerance to kanamycin. The concentrations of kanamycin tested ranged from 100, 250, 500, to 1,000 mg/L (Tables 1). The lowest concentration of kanamycin at 100 mg/L was sufficient to inhibit the growth of leaf disks of tobacco cultivars Xanthi (nn Smith), Xanthi (NN), and Wisconsin 38 (Figure 1). There was detectable residual growth activity of Xanthi (Tsukuba) at 100 mg/L of kanamycin. On the basis of this result I chose the tobacco cultivar Xanthi (nn Smith) and a kanamycin concentration of 300 mg/L to provide a rigorous selection scheme, so that only transformed cells are selected and survive. I also tested the concentrations of a second selection agent hygromycin ranging from 25, 50, 100, to 200 mg/L (Table 2). Hygromycin was more toxic than kanamycin and inhibited the growth of leaf disks at 25 mg/L and turned the color of leaf disks from green to yellow (Figure 2). I chose the hygromycin concentration of 50 mg/L for rigorous selection.

I observed initially that *A. tumefaciens* cells tended to aggregate into clumps and strings during the liquid co-cultivation period as the incubation time increased up to 60 min, and that the degree of cell aggregation seemed to correlate with the apparent efficiency of tobacco transformation; the higher the aggregation, the greater the recorded increase in fresh weight yield.

Table 1. Growth of tobacco leaf disks from four cultivars were compared in inhibition by kanamycin ranging from 100 to 1,000 mg/L. Plate averages of fresh weight (FW) yield in grams are shown in the table. Tobacco leaf disks were grown at 25 ^{o}C on shooting medium containing various concentration of kanamycin for 4 weeks from 05/08 to 06/05/2008. Each treatment has five plates with ten leaf disks per plate. Numbers in parentheses indicate standard deviations. The experiment was performed once.

Nicotiana tabacum L. cultivars	Kanamycin, mg/L				
	0	100	250	500	1000
	mean FW/plate, g				
Xanthi (nn Smith)	11.51 (6.50)	0.30 (0.05)	0.31 (0.04)	0.14 (0.02)	0.10 (0.03)
Xanthi (NN)	15.06 (4.39)	0.58 (0.08)	0.47 (0.06)	0.31 (0.05)	0.37 (0.06)
Wisconsin38	12.14 (5.51)	0.29 (0.08)	0.43 (0.27)	0.13 (0.03)	0.16 (0.09)
Xanthi (Tsukuba)	18.30 (2.38)	1.01 (0.27)	0.45 (0.07)	0.53 (0.06)	0.59 (0.09)

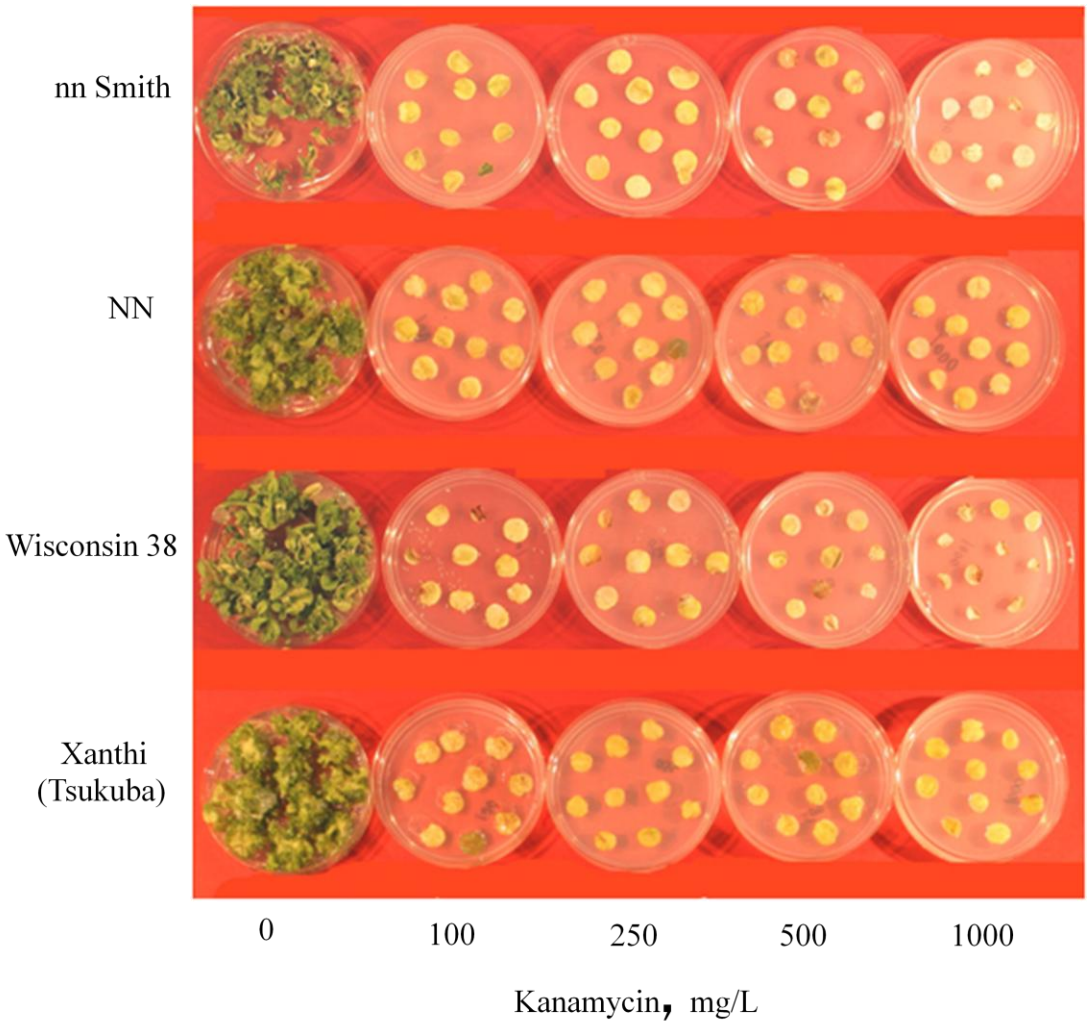


Figure 1. Growth of tobacco leaf disks from four cultivars were compared in inhibition by kanamycin ranging from 100 to 1,000 mg/L. Tobacco leaf disks were grown at 25 ^{o}C on shooting medium containing 0 to 1000mg/L of kanamycin for 4 weeks from 05/08 to 06/05/2008. Each treatment has five plates with 10 leaf disks per plate.

Table 2. Growth of leaf disks of tobacco cultivar Xanthi (nn Smith) were inhibited by hygromycin ranging from 25 to 200 mg/L. Plate averages of FW yield in grams are shown in the table. Tobacco leaf disks were grown at 25 ^{o}C on shooting medium containing 25 to 200mg/L of hygromycin for 4 weeks from 07/16 to 08/13/2008. There are five replicates under each treatment. Numbers in parentheses indicate standard deviations. The experiment was performed once.

Nicotiana tabacum L. variety	Hygromycin mg/L				
	0	25	50	100	200
	mean FW/plate, g				
Xanthi(nn Smith)	17.15 (2.17)	0.52 (0.11)	0.24 (0.07)	0.09 (0.02)	0.08 (0.03)

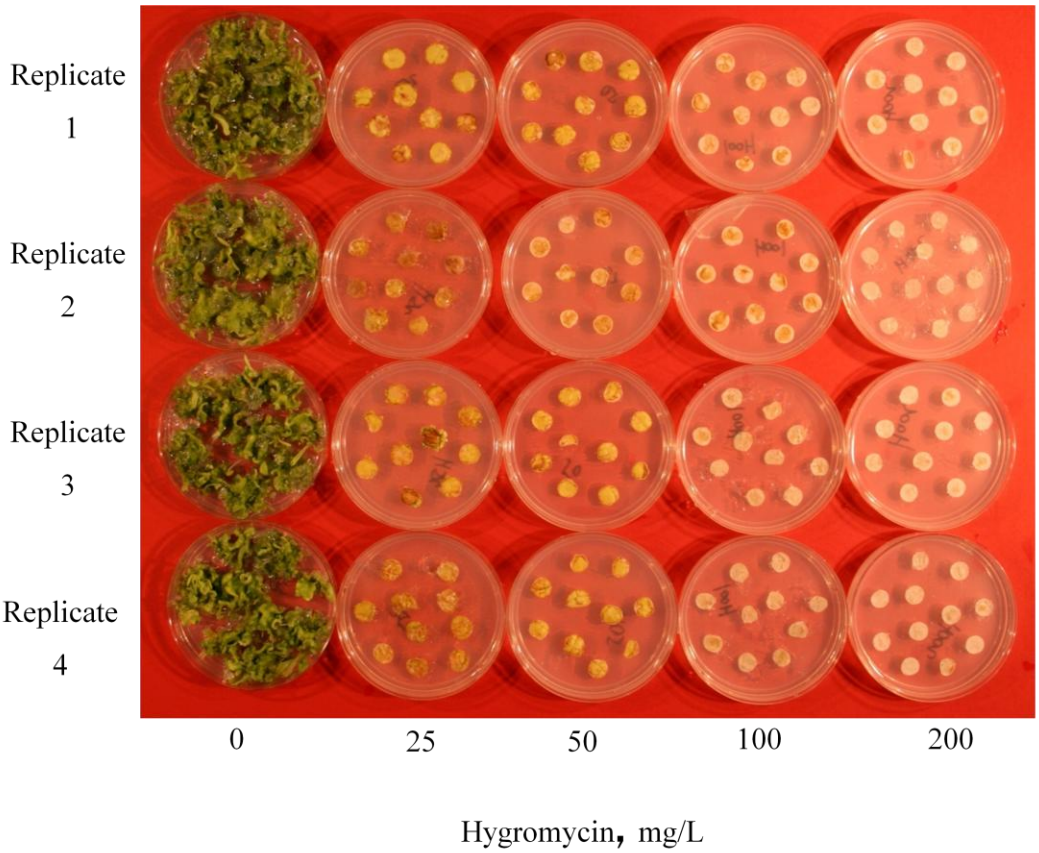


Figure 2. Growth of leaf disks of tobacco variety Xanthi (nn smith) were inhibited by hygromycin concentrations ranging from 25 to 200 mg/L. Tobacco leaf disks were grown at 25 ^{o}C on shooting medium containing hygromycin for 4 weeks from 07/16 to 08/13/2008.

I observed more cell aggregation during the liquid co-cultivation as the number of leaf disks increased from 10 to 20 in 10 ml of co-cultivation medium (20×leaf disks), and the concentration of acetosyringone increased from 200 to 400 μM (2×AS). Five-fold increase in the detergent Silwet concentration from 0.005% to 0.025% reduced the apparent cell aggregation when tested singly (5×silwet) or in combination with the 400 μM acetosyringone (2×AS+5×silwet). However, these modifications during the liquid co-cultivation period did not change the outcome in the final fresh weight yield of transformed calli and shoots (Table 3, Figure 3). Therefore, I decided to maintain the original conditions during the liquid co-cultivation period, as described above.

The importance of temperature during the 4-day co-cultivation period was tested under the most physiologically relevant conditions using identical tissue culture chambers (Model CU-36L5, Percival Scientific). The temperatures during the 4-day co-cultivation period were 15, 18, 20, and 22 to 25^{o}C with the temperature range (variation) of 0.7^{o}C under constant light (56 μE $m^{-2}s^{-1}$). The results from the temperature experiments are summarized in Figures 4 and 5, and Table 4. Initially, I tested each temperature treatment with a corresponding vectorless control and observed no detectable difference in the fresh weights among the negative controls of five temperature treatments. Thus, I had one vectorless negative control of 20^{o}C in this experiment. A 4-day co-cultivation at 20^{o}C resulted in the highest increase in the plate average of fresh weight yields. The 20^{o}C treatment was statistically significantly better than the 15 and 18^{o}C treatments. The 20^{o}C co-cultivation provided higher FW leaf disk increase than 22 and 25^{o}C even though the differences were statistically distinguishable (A vs AB) but not significant.

Table 3. Evaluation of the modification of liquid co-cultivation medium components. Leaf disks were co-cultivated for 4 days with *A. tumefaciens* strain LBA4404 with or without new binary Ti vector pLSU-N2SGS. Co-cultivation was performed from 1/11 to 1/15, the first selection from 1/15 to 1/29 and the second selection from 1/29 to 2/12/2010. Each treatment had five plates with 10 leaf disks per plate. The experiment was performed once. Numbers in parentheses indicate standard deviations.

pLSU binary Ti vectors in different liquid co-cultivation conditions	Mean FW/plate, g	Increase in FW in g per plate over vectorless control	% leaf discs with increased FW
Vectorless negative control	0.91 (0.20)	0	0
N2SGS(+) positive control	12.06 (5.57)	10.96 (5.54)	92
20×leaf disks	14.89 (2.73)	13.78 (2.73)	98
2×AS	10.08 (5.17)	8.98 (5.16)	94
5×silwet	6.91 (4.60)	5.90 (4.55)	71
2×AS+5×silwet	7.21 (0.99)	6.11 (1.01)	94

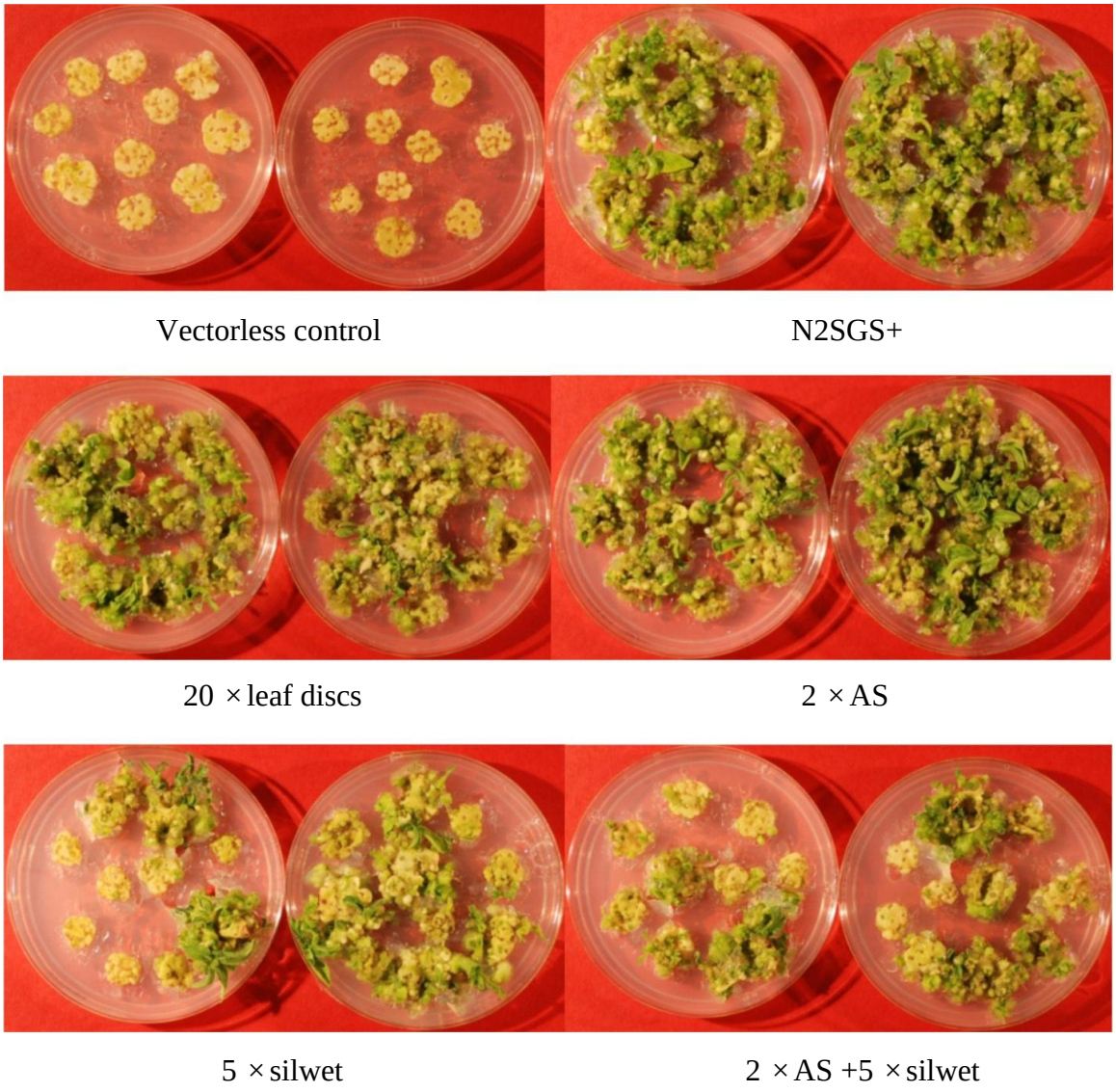


Figure 3. Evaluation of the modification of liquid co-cultivation medium components. Leaf disks were co-cultivated for 4 days with *A. tumefaciens* strain LBA4404 with or without new binary Ti vector pLSU-N2SGS. Co-cultivation was performed from 1/11 to 1/15, the first selection from 1/15 to 1/29 and the second selection from 1/29 to 2/12/2010. Each treatment has five plates with 10 leaf disks per plate, and the experiment was performed once.

The percentage of leaf disks with the increased FW yield indicated the proportion of leaf disks with presumably transformed calli and shoots. The 20^{o}C treatment showed 82% of leaf disks with the increased FW yield which is significantly higher than 50 and 68% by the 15 and 18^{o}C treatment, respectively (Table 4). The 22 and 25^{o}C treatments produced 78 and 98% leaf disks with increase FW, respectively.

After I demonstrated the co-cultivation temperature at 20 ^{o}C was best for *Agrobacterium*-mediated transformation. I applied this treatment to compare the effectiveness of new binary Ti vectors with the conventional pCAMBIA vector. Seokhyun Lee constructed four new binary Ti vectors pLSU containing the plant-expressible kanamycin resistance gene with or without the GUS reporter gene (Table 5). I tested these four binary vectors in comparison with the conventional pCAMBIA 2301. I found the pLSU vectors were similar to the pCAMBIA vector with regard to FW yield increase and the percentage of leaf disks transformed (Table 6 and Figure 6). The pLSU vectors did not perform well when the kanamycin concentration was raised to 500 mg/L (data not shown), while the pCAMBIA vector showed the good selection activity. This may be due to the difference in the promoter activity, i.e. the tml promoter in pLSU and the CaMV 35S promoter in pCAMBIA.

Mr. Lee also constructed four new binary Ti vectors pLSU containing the plant-expressible hygromycin resistance gene with or without the GUS reporter gene (Table 7). I tested these four binary vectors in comparison with the conventional pCAMBIA 1305.2. I found the pLSU vectors were similar to the pCAMBIA vector with regard to the increased FW yield increase and the percentage of leaf disks transformed (Table 8 and Figure 7).

Table 4. Effect of co-cultivation temperature (15, 18, 20, 22 and 25^{o}C) on the final fresh weight (FW) of transformed tobacco leaf disks. Leaf disks were co-cultivated for 4 days with *A. tumefaciens* strain LBA4404 with or without new binary Ti vector pLSU-N2SGS. Co-cultivation was performed from 11/9 to 11/13, the first selection from 11/13 to 11/27 and the second selection from 11/27 to 12/11/2009. Each treatment has five plates with 10 leaf disks per plate. Numbers in parentheses indicate standard deviations. The experiment was performed five times.

Co-cultivation temperatures	Mean FW/plate, g	Increase in FW in g per plate over vectorless control	% of leaf discs with increased FW
15 ^{o}C N2SGS	2.62 (1.30)	1.99 (1.19)	50
18^{o}C N2SGS	3.31 (1.87)	2.59 (1.85)	68
20 ^{o}C N2SGS	10.48 (5.91)	9.74 (5.91)	82
22 ^{o}C N2SGS	6.97 (2.76)	6.24 (2.74)	78
25 ^{o}C N2SGS	7.23 (3.85)	6.48 (3.88)	98
20^{o}C vectorless control	0.58 (0.21)	-	-

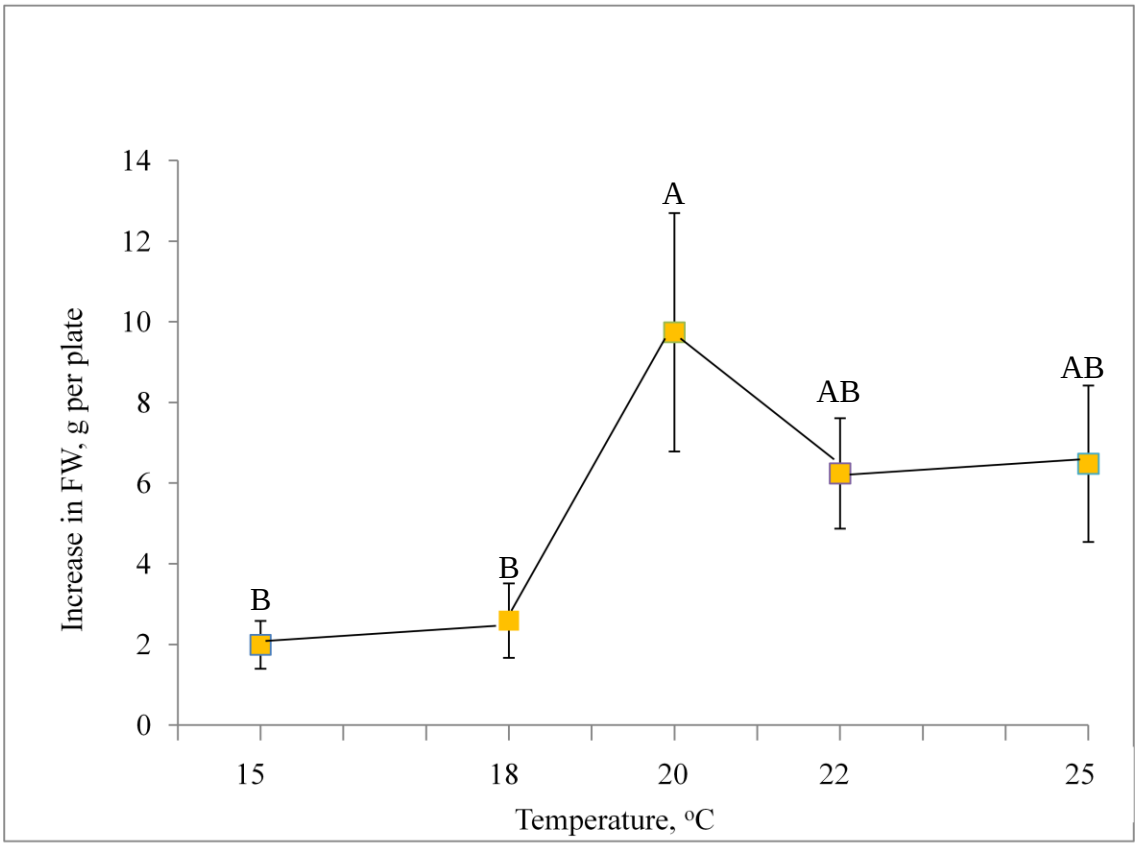


Figure 4. Effect of co-cultivation temperature (15, 18, 20, 22 and 25°C) on the final fresh weight (FW) of transformed tobacco leaf disks. Leaf disks were co-cultivated for 4 days with *A. tumefaciens* strain LBA4404 with or without new binary Ti vector pLSU-N2SGS. Leaf disks were selected at 25 °C on shooting media containing 300 mg/L of kanamycin and 500 mg/L of carbenicilin for 2 weeks. Fresh medium was prepared for additional 2 weeks of selection. Bars indicate standard deviation. Means with the same letter are not significantly different at α=0.05 level. Co-cultivation was performed from 11/9 to 11/13, the first selection from 11/13 to 11/27 and the second selection from 11/27 to 12/11/2009. Each treatment has five plates with 10 leaf disks per plate.

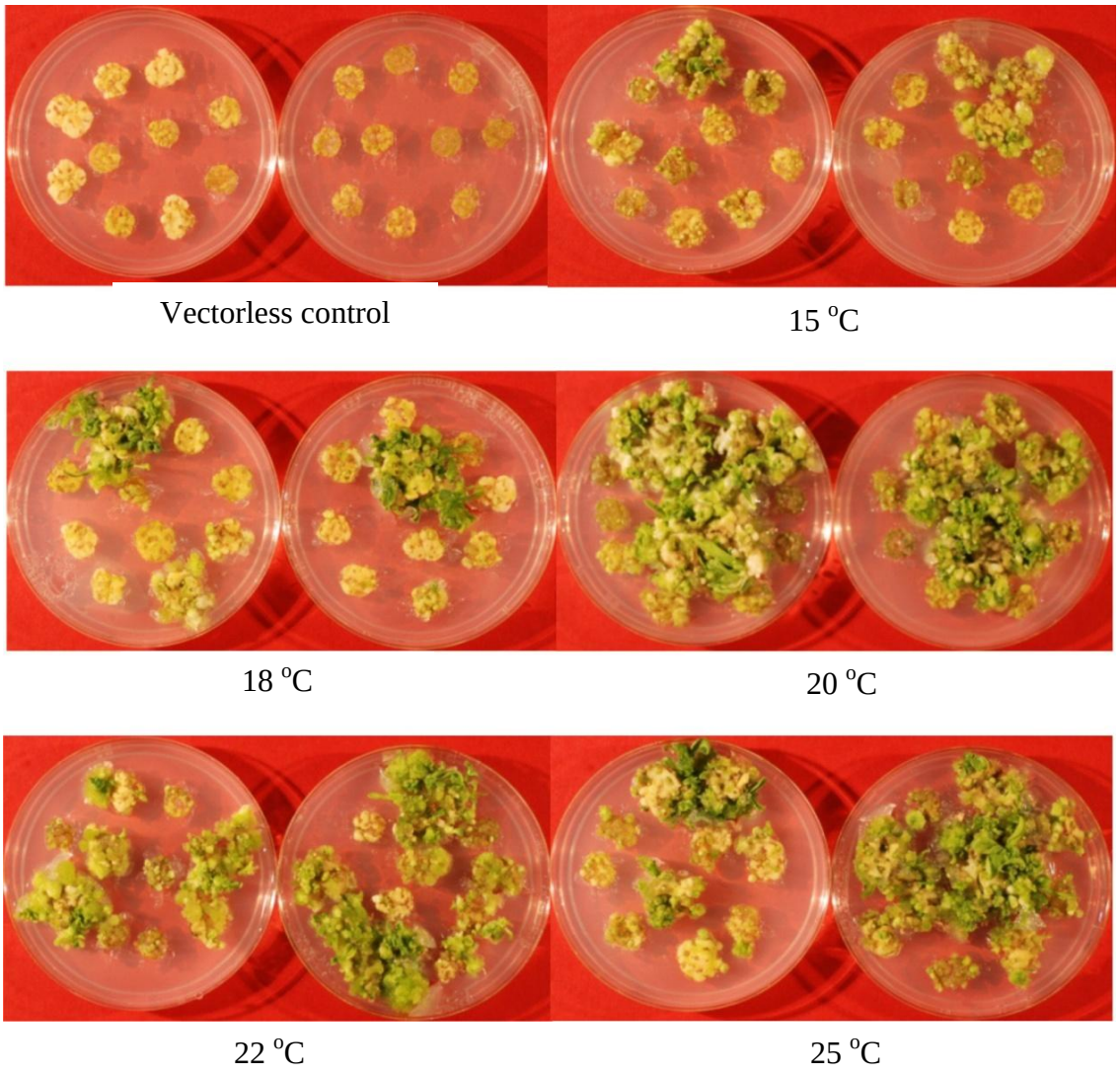


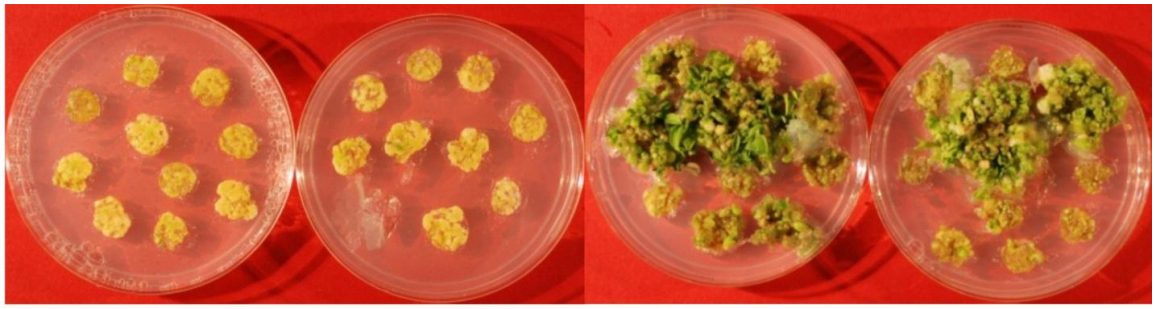
Figure 5. Effect of co-cultivation temperature (15, 18, 20, 22 and 25°C) on the final fresh weight (FW) of transformed tobacco leaf disks. Leaf disks were co-cultivated for 4 days with *A. tumefaciens* strain LBA4404 with or without new binary Ti vector pLSU-N2SGS. Leaf disks were selected at 25 °C on shooting medium containing 300 mg/L of kanamycin and 500 mg/L of carbenicilin for 2 weeks. Fresh medium was prepared for additional 2 weeks of selection. Co-cultivation was performed from 11/9 to 11/13, the first selection from 11/13 to 11/27 and the second selection from 11/27 to 12/11/2009. Each treatment has five plates with 10 leaf disks per plate.

Table 5. T-DNA composition of new binary Ti vectors pLSU and pCAMBIA2301. Seokhyun Lee constructed the four binary Ti vectors pLSU. All binary vectors listed contain the kanamycin resistance gene for selection of transformed cells (+). The presence (+) or absence (-) of the GUS reporter gene is indicated.

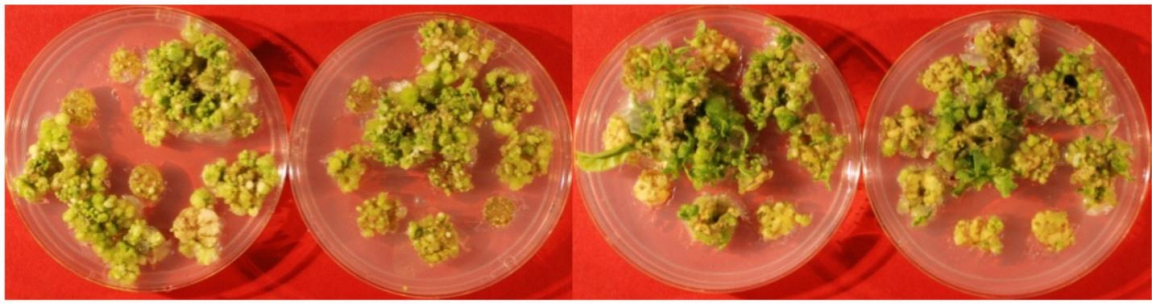
Binary vectors	Kanamycin resistance	GUS reporter gene
pCAMBIA 2301	+	+
pLSU N2S	+	_
pLSU N2R	+	_
pLSU N2SGS	+	+
pLSU N2RGS	+	+

Table 6. Tobacco leaf disk fresh weights (FW) were compared after transformation with new pLSU binary Ti vectors of *A. tumefaciens*. After the co-cultivation with *A. tumefaciens* strain LBA4404 at 20 ^{o}C for 4 days, leaf disks were selected at 25 ^{o}C on shooting medium containing 300 mg/L of kanamycin and 500 mg/L of carbenicilin for 2 weeks. Fresh medium was prepared for an additional 2 weeks of selection. Co-cultivation was from 11/23/09 to 11/27, the first selection was 11/27 to 12/11 and the second selection was 12/11 to 12/25/2009. Each treatment has five plates with 10 leaf disks per plate. Numbers in parentheses indicate standard deviations. Asterisks * indicate that means with the same letter are not statistically different. The experiment was performed three times.

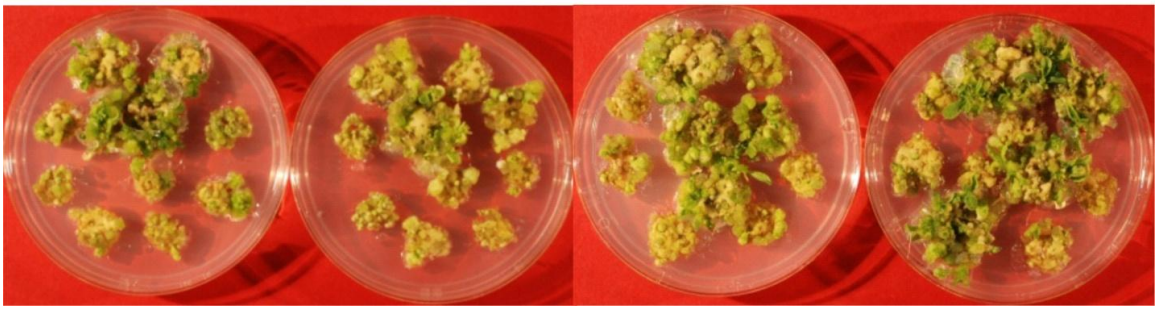
pLSU binary Ti vectors	Mean FW/plate, g	Increase in FW in g per plate over vectorless control	% leaf discs with increased FW
Vectorless control	0.76 (0.07)	0	0
pCAMBIA 2301	8.48 (2.59)	7.68 A* (2.58)	80
pLSU N2S	7.88 (2.65)	7.14 A* (2.65)	66
pLSU N2R	7.74 (3.20)	7.00 A* (3.18)	78
pLSU N2SGS	5.16 (2.66)	4.34 A* (2.66)	92
pLSU N2RGS	8.12 (2.96)	7.33 A* (2.90)	89



Vectorless control pCAMBIA 2301



N2S N2R



N2SGS N2RGS

Figure 6. Tobacco leaf disk fresh weights (FW) were compared after transformation with new pLSU binary Ti vectors of *A. tumefaciens*. After the co-cultivation with *A. tumefaciens* strain LBA4404 at 20 ^{o}C for 4 days, leaf disks were selected at 25 ^{o}C on shooting medium containing 300 mg/L of kanamycin and 500 mg/L of carbenicilin for 2 weeks. Fresh medium was prepared for additional 2 weeks of selection. Co-cultivation was from 11/23/09 to 11/27, the first selection was 11/27to 12/11 and the second selection was 12/11 to 12/25/2009. Each treatment has five plates with 10 leaf disks per plate.

Table 7. T-DNA composition of new binary Ti vectors pLSU and pCAMBIA1305.2. Seokhyun Lee constructed the four binary Ti vectors pLSU. All binary vectors listed contain the hygromycin resistance gene for selection of transformed cells (+). The presence (+) or absence (-) of the GUS reporter gene is indicated.

Binary vectors	Hygromycin resistance gene	GUS reporter gene
pCAMBIA 1305.2	+	+
pLSU HS	+	_
pLSU HR	+	_
pLSU HSGS	+	+
pLSU HRGS	+	+

Table 8. Tobacco leaf disk fresh weights (FW) were compared after transformation with new pLSU binary Ti vectors of *A. tumefaciens*. After the co-cultivation with *A. tumefaciens* strain LBA4404 at 20 ^{o}C for 4 days, leaf disks were selected at 25 ^{o}C on shooting medium containing 50 mg/L of hygromycin and 500 mg/L of carbenicilin for 2 weeks. Fresh medium was prepared for an additional 2 weeks of selection. Co-cultivation was from 12/14/09 to 12/18/09, the first selection was 12/18/09 to 01/01/10 and the second selection was 01/01/10 to 01/15/2010. Each treatment has five plates with 10 leaf disks per plate. Numbers in parentheses indicate standard deviations. Asterisks * indicate that means with the same letter are not statistically different. The experiment was performed three times.

pLSU binary Ti vectors	Mean FW/plate, g	Increase in FW in g per plate over vectorless control	% leaf discs with increased FW
control	1.08 (0.14)	0	0
pCAMBIA 1305.2	9.76 (2.17)	8.57 A* (2.19)	98
pLSU HS	4.54 (1.33)	3.33 B* (1.33)	96
pLSU HR	7.81 (2.70)	6.59 AB* (2.69)	98
pLSU HSGS	9.55 (3.09)	8.35 AB* (3.05)	92
pLSU HRGS	11.19 (3.89)	9.99 A* (3.88)	100

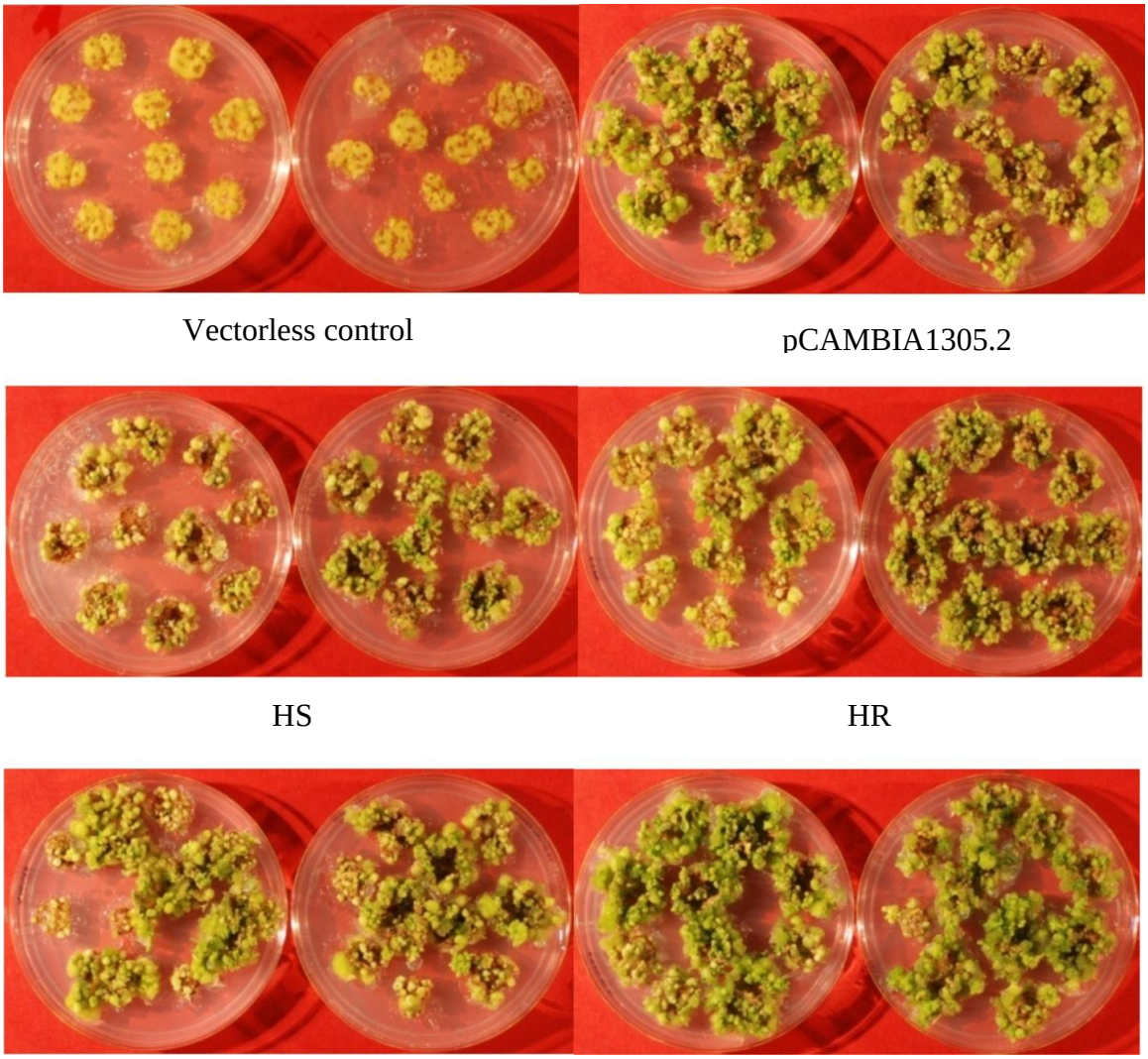


Figure 7. Tobacco leaf disk fresh weights (FW) were compared after transformation with new pLSU binary Ti vectors of *A. tumefaciens*. After the co-cultivation with *A. tumefaciens* strain LBA4404 at 20 ^{o}C for 4 days, leaf disks were selected at 25 ^{o}C on shooting medium containing 50 mg/L of kanamycin and 500 mg/L of carbenicilin for 2 weeks. Fresh medium was prepared for an additional 2 weeks of selection. Co-cultivation was from 12/14/09 to 12/18/09, the first selection was 12/18/09to 01/01/10 and the second selection was 01/01/10 to 01/15/2010. Each treatment has five plates with 10 leaf disks per plate.

DISCUSSION

I demonstrated in this study that the co-cultivation temperature at 20 ℃ was the most significant experimental condition so far tested to achieve the reproducibility and consistency of the increase in the fresh weight yield after *A. tumefaciens*-mediated transformation of tobacco leaf disks. Sunjung Park (2006) also examined co-cultivation temperature (using GUS expression as the criterion for transformation), as well as other experimental conditions, such as co-cultivation days, bacteria concentrations, surfactant silwet, vacuum infiltration, bacteria pre-culture conditions, AS concentrations, wound timing, and selection agents. Among the five temperatures from 15, 18, 20, 22 to 25 ℃ tested, co-cultivation at 20 ℃ resulted in statistically greater growth than that at 15 and 18 ℃. The 20^{o}C co-cultivation provided higher FW leaf disk increase than 22 and 25^{o}C treatments, and the differences were statistically distinguishable (A vs AB) but not significant. The results demonstrated that 4- day co-cultivation with different temperature treatments determined the outcome of 28-days-long growth of transformed tissues after rigorous kanamycin selection. Shoot regeneration and calli formation involves expression of cell division and differentiation-associated genes in a tobacco genome containing more than 20,000 genes (Gelvin, 2003). The temperature profile of fresh weight yield increase is in agreement with thermo-sensitivity profile of plasmid transfer during transconjugation and of stability of pili that are channel structures responsible for plasmid transfer and probably T-strands (Fullner et al., 1996, Aguilar et al., 2010).

Co-cultivation temperature has been an important factor affecting *Agrobacterium*-mediated transformation. Dillen et al. (1997) reported that co-cultivation temperature plays an important role in the T-DNA transfer system. They demonstrated no difference

between 19 to 22^{o}C for T-DNA delivery in tobacco leaves. The GUS (β–glucuronidase) reporter gene was used to detect transient expression, after co-cultivation. The levels of transient β–glucuronidase were decreased dramatically when the temperature increased above 22 ^{o}C. Sunjung Park (2006) reported that the β–glucuronidase activity was reduced dramatically at a co-cultivation temperature of 25 ^{o}C, but was high at 18, 20 and 22 ^{o}C.

In contrast to above reports, Salas et al. (2001) found that co-cultivation at 25 ^{o}C produced the highest number of transformed plants of tobacco, although 19 ^{o}C was optimal for T-DNA delivery.

Uranbey et al. (2005) reported different optimal co-cultivation temperatures. They used *Agrobacterium tumefaciens* strain GV 2260 carrying p35S GUS-INT to determine the effect of different co-cultivation temperatures. The highest transformation frequency was achieved at co-cultivation temperatures of 22 ^{o}C or 24 ^{o}C for 48h, with the tobacco leaf disk explants co-cultured with *A.tumefaciens* in liquid medium.

In conclusion, I selected a tobacco cultivar with a known genotype (nn Smith), concentrations of kanamycin and hygromycin for rigorous selection for transformed calli and shoots, an optimal co-cultivation temperature, and determined the effectiveness of new binary Ti vectors pLSU in *A. tumefaciens*-mediated transformation of tobacco leaf disks. I have shown that the co-cultivation temperature of 20℃ was one of the most critical factors to achieve the reproducibility and consistency of the fresh weight increases of leaf disks. The current findings are consistent with the previous results from investigations of the importance of the co-cultivation temperatures in *A. tumefaciens*-mediated transformation. The current findings should be beneficial for the transformation of Louisiana's major crops such as rice, sugarcane, wheat, soybean, and cotton.

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APPENDIX: LIST OF PENDING PUBLICATIONS

1. Su G, Park S, Lee S, Murai N (2010) Low co-cultivation temperature at 20^{o}C improved the efficiency of *Agrobacterium tumefaciens*-mediated transformation of tobacco leaf disks (To be submitted to *Plant Cell Report*)

2. Lee S, Su G, Aghazadeh MA, Murai N (2010) Smaller high-yielding binary Ti vectors pLSU for efficient *Agrobacterium tumefaciens*-mediated transformation of higher plants (To be submitted to *Plant Physiology*)

3. Lee S, Su G, Lasserre E, Murai N (2010) New binary Ti vectors pLSU-tet adapted for gateway technology for efficient *Agrobacterium tumefaciens*-mediated transformation of higher plants (To be submitted to *Plant Biotechnology Journal*)

VITA

Guiying Su was born in Shandong Province, China, in June, 1979. Her hometown is renowned for peony. She graduated from Yantai Normal University in 2003 with a Bachelor of Science degree in biological sciences.

After graduation, she went to work as a research assistant at National Center of Biomedical Analysis (NCBA) in Beijing. She published her work as a second author in the manuscript entitled "Identification of Ubiquitin Target Proteins Using Cell-Based Arrays" in Journal of Proteome Research 2007, 6, 4397-4406. She also participated in a second work published in the manuscript entitled "Ubc9 interacts with SOX4 and represses its transcriptional activity" in Biochemical and Biophysical Research Communications 344 (2006) 727–734.

Ms. Su enrolled at Louisiana State University in August 2007. She pursued a master degree program in the Department of Plant Pathology and Crop Physiology. She worked as a Graduate Research Assistant in the laboratory of Dr. Norimoto Murai.