ORIGINAL ARTICLE

Agrobacterium-mediated transformation of modified antifreeze protein gene in strawberry

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Abstract

Khammuang, S., Dheeranupattana, S., Hanmuangjai, P. and Wongroung, S.
***Agrobacterium*-mediated transformation of modified antifreeze protein gene in strawberry**
Songklanakarin J. Sci. Technol., 2005, 27(4) : 693-703

The optimum condition for shoot regeneration from leaf explants of strawberry cultivar Tiogar was investigated. It was found that the best regeneration condition was MS medium containing N_6-Benzyladenine (BA) and 2,4-Dichlorophenoxy acetic acid (2,4-D) at concentrations of 1 $mg.l^{-1}$ and 0.2 $mg.l^{-1}$, respectively. Antibiotics sensitivity test found that shoot regeneration from leaf explant was inhibited more than 90% at the concentration of kanamycin (Km) as low as 5 $mg.l^{-1}$. The modified gene encoding antifreeze protein isoform HPLC 6 was successfully constructed using codons which were optimally expressed in the strawberry plant. The antifreeze protein genes, naturally in plasmid pSW1 and modified in plasmid pBB, were transformed to strawberry leaf explants by *Agrobacterium tumefaciens* LBA 4404. The strawberry plants, transformed with both AFP genes, were able to root in MS media containing 50 $mg.l^{-1}$ Km, while no roots grew from nontransformed plant in this condition. Polymerase chain reaction indicated that the transgenes were integrated in the genome of transformants.

Key words : antifreeze protein, codon optimized gene, strawberry transformation, *Agrobacterium*, Tioga

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Received, 23 September 2004 Accepted, 2 November 2004

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การถ่ายยีนแอนติฟรีซโปรตีนดัดแปลงในสตรอเบอรีโดยอาศัยเชื้อ *Agrobacterium*

ว. สงขลานครินทร์ วทท. 2548 27(4) : 693-703

การศึกษาสภาวะที่เหมาะสมในการเจริญกลับเป็นต้นใหม่จากชิ้นใบสตรอเบอรีพันธุ์ Tioga พบว่าสภาวะที่ดีที่สุดคือ อาหาร MS ที่ประกอบด้วยฮอร์โมน N_6-Benzyladenine (BA) และ 2,4-Dichlorophenoxy acetic acid (2,4-D) เข้มข้น 1 mg.l^{-1} และ 0.2 mg.l^{-1} ตามลำดับ การศึกษาผลของยาปฏิชีวนะพบว่า กานามัยซินที่ความเข้มข้นเพียง 5 mg.l^{-1} สามารถยับยั้งการเจริญกลับเป็นต้นใหม่ของชิ้นใบสตรอเบอรีพันธุ์นี้ได้มากกว่า 90% สร้างยีนดัดแปลงของแอนติฟรีซโปรตีน isoform HPLC 6 โดยมีโคดอนเหมาะสมแสดงออกในพืชสตรอเบอรีได้สำเร็จ แล้วถ่ายยีนแอนติฟรีซโปรตีน ทั้งจากธรรมชาติในพลาสมิด pSW1 และยีนแอนติฟรีซโปรตีนที่มีโคดอนเหมาะสมแสดงออกในสตรอเบอรีซึ่งอยู่ในพลาสมิด pBB เข้าสู่ชิ้นใบของสตรอเบอรี โดยอาศัยเชื้อ *Agrobacterium tumefaciens* LBA 4404 พบว่าสตรอเบอรีที่ได้รับการถ่ายยีนสามารถออกรากในอาหาร MS ที่มียาปฏิชีวนะกานามัยซินเข้มข้น 50 mg.l^{-1} ได้ในขณะที่ชุดควบคุมไม่สามารถออกรากในอาหารดังกล่าว ผลจากการทำ polymerase chain reaction พบยีนแอนติฟรีซโปรตีนแทรกในจีโนมของต้นสตรอเบอรีที่ได้รับการถ่ายยีน

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Strawberry (*Fragaria* x *ananassa* Duch.) is a major berry crop around the world. Production of strawberries has been attempted to meet the demands for improved yields, fruit size and quality traits. However, the narrow genetic base of the cultivated strawberry, combined with the polyploid nature of the crop constrain traditional breeding methods. Biotechnological approaches, especially genetic engineering, are an alternative efficient strategy to implement strawberry improvement.

One problem in strawberry production is frost damage. Early spring frost damages the flowers leading to poor yields and erratic fruiting. The transfer of genes encoding antifreeze protein (AFP) from antarctic fish (DeVries *et al*., 1970) is one means of increasing frost resistance. In addition, strawberry fruit is soft and fragile product and has a very short shelf life. Strawberries must be stored at low temperatures for extended shelf life but ice crystals, at freezing temperatures, can severely damage the strawberry fruit. Expression of AFP in strawberry fruit should solve this problem. However, high levels of heterologous protein expression in plants is not routinely achieved. One of the critical factors that limit the expression rate is codon usage, due to limited availability of particular tRNAs (Kusnadi *et al*., 1997). It has been necessary to optimize codon usage of several of the genes encoding the *Bacillus thuringiensis* toxins, in order to achieve high expression level in plants (Holmberg *et al*., 2001). Thus, it may be possible to increase expression of type III antifreeze protein from fish in strawberry, by optimizing codon usage, to mimic highly expressed strawberry genes.

Genetic engineering of strawberry has already been reported (Jame *et al*., 1989; Nehra *et al*., 1990). However, transformation frequencies are greatly influenced by the cultivar and the procedures used, e.g. 0.95% transformation frequency has been reported for the cultivar Rapella (Jame *et al*., 1990) and 6.5% for the cultivar Red Coat (Nehra *et al*., 1990). Tioga is a popular cultivar that has been grown commercially in Thailand for long time. Fruits of this cultivar are small and contain high acid which make it appropriate for industrial processing as well as fresh fruit consumption. Biotechnological approachs may also raise other valuable characters for this cultivar.

The scope of this research investigated the optimal regeneration conditions from cultivar Tioga leaf explants, including the effects of antibiotic on their regeneration. Design and construction of strawberry optimized codons genes, encoding the type III antifreeze protein isoform HPLC 6 (Hew *et al.*, 1988) and the protocol for *Agrobacterium*-mediated transformation were also included.

Materials and Methods

Strawberry cultivar and micropropagation

In vitro plants of *Fragaria* x *ananassa* cultivar Tioga were obtained from Associate Professor Dr. Prasartporn Smitamana, Faculty of Agriculture, Chiang Mai University, and maintained on a MS medium (Murashige and Skoog, 1962) supplemented with 30 $g.l^{-1}$ sucrose and solidified with 0.2 % gelrite (Sigma). The pH of the medium was adjusted to 5.80 with 1 M NaOH and sterilized for 15 min at 121ºC. The cultures were incubated at 22-26ºC under a 16 h photoperiod provided by fluorescent lamps.

Shoot regeneration

Initial young leaf explants, 5-8 mm diameter, were excised from 7-8 week-old strawberry plantlets propagated *in vitro*. Ten explants were cultured, adaxial side in contact with the medium, in 250 ml glass jars containing about 30 ml MS medium, supplemented with a combination of BA (0, 0.5, 1.0 and 1.5 $mg.l^{-1}$) and 2,4-D (0, 0.1, 0.2, 0.5 and 1.0 $mg.l^{-1}$).

Antibiotic sensitivity tests

Leaf explants were placed adaxial side in contact with the best shoot regeneration medium (SRM) containing filter sterilized of carbenicillin (Cb) at 0, 100, 200, 300, and 400 $mg.l^{-1}$ or kanamycin (Km) at 0, 5, 10, 20 and 50 $mg.l^{-1}$.

Bacterial strains, plasmids, and recombinant DNA techniques

Escherichia coli strain JM 109 was used in all cloning and subcloning procedures. It was grown and transformed using standard techniques (Sambrook *et al.*, 1989). Subcloning vectors were pT7blue and pT7blue2. The binary plant expression vector was pBI121. *Agrobacterium tumefaciens* strain LBA 4404 was used for plant transformation. DNA sequencing was performed by the dideoxynucleotide chain termination method, using bigdye terminator (Applied Biosystem). All other DNA manipulations were performed using standard protocols (Sambrook *et al.*, 1989).

Design and construction of the optimized gene and the plant expression vector

A high frequency of codon usage used in strawberry from Codon Usage Database (www.kazusa.go.jp) was used to design primers AFP-F1, AFP-R1, AFP-F2 and AFP-R2 (Table 1) for construction of strawberry optimized codon gene encoding antifreeze protein by polymerase chain reaction. The modified gene was ligated into pT7blue2 plasmid by TA cloning.

pT7blue2 plasmids containing strawberry optimized codon gene of AFP were digested with *Xba I* and *BamH* I. This fragment was ligated into *Xba* I-*BamH* I digested pBI121 plasmids. The ligation products were transformed to *E. coli* JM109. Plasmids were prepared from random

Table 1. Oligonucleotide used for construction of strawberry optimized codons of the AFP gene.

Primer	Base position in AFP gene	Base sequences
AFP-F1	1-56	5'-ATGAAG CAGTCTGTTGTTGCTACCCAGCTCATTCCAATAACACTGCTTTGACTCC-3'
AFP-R1	55-113	5'-GACATCTCAGCGAATGGGATTCCAATTGGGTTGGTCACCTTTCCCTCCATCATAGCTGG-3'
AFP-F2	85-143	5'-CCAATTGGAATCCCATTCGCTGAGATGTCTCAGATTGTGGGAAAGCAGGTGAACACCCC-3'
AFP-R2	145-204	5'-TTACTTTCCAGCAACGTAGGTCTTCACCATGTTTGGCATGAGGGTCTGTCCCTTAGCCAC-3'

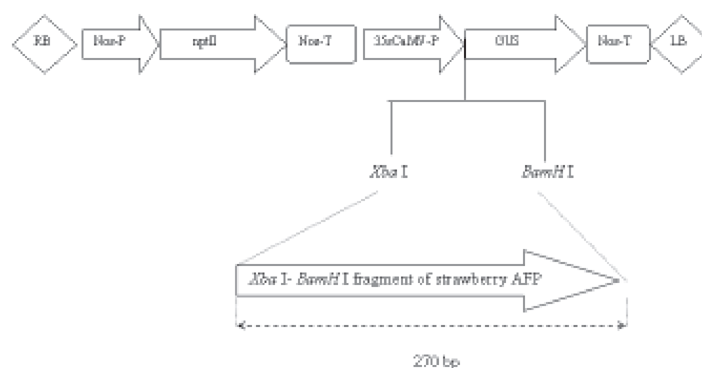


Figure 1. Construction of plant expression vector by insertion of the *Xba* I - *BamH* I fragment of strawberry optimized codon of AFP gene into plasmid pBI 121.

picked colony. Sequence analysis was performed using primer FaAFP-1 (5'-CCTACGTTGCTGG AAAGTAA-3') and FaAFP-2 (5'-CCTACGTTG CTGGAAAGTAA-3'). Plasmid pBI121, containing the strawberry optimized codon sequence, was called pBB (Figure 1). pSW1 was derived from insertion of a natural type III AFP gene from fish into pBI121 at *Xba* I and *BamH* I site (Wongroung, 2000). These two plasmids contained the NOS/NPTII gene for Km resistance and the constitutive promoter, derived from a plant virus, cauliflower mosaic virus. It is called the cauliflower mosaic virus 35S promoter, CaMV35S promoter for short.

Plasmids pSW1 and pBB were introduced into *A. tumefaciens* LBA4404 via triparental mating (Bevan, 1984).

Transformation

A. tumefaciens strain LBA 4404, containing the non-oncogenic plasmid pAL4404 and plant expression vector pSW1 or pBB was employed in the transformation experiments.

A. tumefaciens strain LBA 4404 with desired plasmid was prepared by culture in 10 ml of Luria-Bertani medium (LB) containing 100 $mg.l^{-1}$ Km and 50 $mg.l^{-1}$ rifampicin, until the optical density at 600 nm was 0.4 then the culture was centrifuged at 4,000 rpm for 15 min. The pellet was resuspended in 15 ml of MS basal media, containing 20 $g.l^{-1}$ sucrose (MS20) with 0.1 mM acetosyringone and 1 mM betain hydrochloride added. This solution was shaken at 150 rpm for 5 h at 25ºC for virulence induction.

Strawberry leaf explants, 5-8 mm in diameter, were taken in *A. tumefaciens* LBA4404 solution with desired plasmids, and swirled to facilitate contact between the bacteria and explants for 30 min. The explants were then bloted dry on filter paper and placed in SRM. After incubating in the dark at 25ºC for 3 days, the explants were washed twice in 500 $mg.l^{-1}$ Cb solution, to remove Agrobacterium and returned to SRM with 500 $mg.l^{-1}$ Cb and incubated at 25ºC for 5 days.

Explants were then transfered to SRM, containing 5 $mg.l^{-1}$ Km and 500 $mg.l^{-1}$ Cb, for 1 week. After that, Cb concentration was reduced to 200 $mg.l^{-1}$, to control *Agrobacterium* growth during the regeneration period. Transformants were screened on medium with 20 $mg.l^{-1}$ Km. Regenerating shoots were transferred to MS medium with 50 $mg.l^{-1}$ Km without Cb for rooting.

Analysis of transformants by polymerase chain reaction

Strawberry genomic DNA was extracted by the hexadecyltrimetylammonium bromide (CTAB) method (Doyle and Doyle, 1990). Briefly, young leaves from rooted shoots of transformed strawberry plants were frozen in liquid nitrogen and ground to a fine powder. Leaf powder (0.05-0.1 g) was transfered to a microcentrifuge tube. 600 µl CTAB buffer (100 mM Tris-HCl pH 8, 1.4 M

NaCl, 20 mM EDTA, 2% CTAB, 1% PVP, 0.2% β-mercaptoethanol) was added in each tube. Samples were placed in water bath at 65ºC for 1 h, followed by extraction twice with phenol : chloroform : isoamyl alcohol (25:24:1). DNA was precipitated by adding 0.3 volume of 7.5 M ammonium acetate and 2 volume of cold 80% ethanol. The pellets were washed with 1 ml of 70% cold ethanol and allowed to dry briefly before being dissolved in 100 μl TE buffer (10 mM Tris-HCl pH 8.0). Samples were treated with RNase and extracted with phenol and once with chloroform. Finally the DNA was precipitated with ethanol, allowed to dry and dissolved in an appropriate volume of TE buffer.

PCR reaction consisted of 1xPCR buffer, 1.9 mM $MgCl_2$, 100 μM each of dNTP, 10 pmol each of primer FaAFP-1 and FaAFP-2, 1 unit of Taq DNA polymerase and 70 ng of strawberry genomic DNA. The volume was adjusted to 10 μl with sterile water.

The PCR condition was 1 cycle at 94ºC for 5 min, then 35 cycles at 94ºC for 30 s, 54ºC for 30 s and 72ºC for 2 min and 1 cycle at 72ºC for 7 min. The estimated length of PCR product was 204 bp.

Results and Discussion

Shoot regeneration

Combinations of BA and 2,4-D were tested. Explants began callusing at their wounded edges after 1 week and began to regenerate shoots after 3 weeks of culture. All treatments gave callusing even in MS without phytohormone. The combination of these two hormones had a significant effect on shoot regeneration. Shoot regeneration occurred at a 2,4-D concentration ranking from 0 to 0.2 $mg.l^{-1}$ and BA concentration ranking from 0.5 to 1.5 $mg.l^{-1}$. This indicated that BA was required for shoot regeneration. By 8 weeks, maximal shoot regeneration (90%) from leaf explants occurred on a medium supplemented with 0.2 $mg.l^{-1}$ 2,4-D and 1.0 $mg.l^{-1}$ BA (Table 2.). This optimum condition was used for further study and the medium at this condition was called shoot regeneration medium (SRM).

Table 2. Shoot formation from leaf explants of strawberry cultivar Tioga after culturing 8 weeks on MS medium supplemented with various combinations of BA and 2,4-D.

BA ($mg.l^{-1}$)	2,4-D ($mg.l^{-1}$)	Percentage of explants forming shoots[a]
0	0.0	0
	0.1	0
	0.2	0
	0.5	0
	1.0	0
0.5	0.0	5
	0.1	35
	0.2	50
	0.5	0
	1.0	0
1.0	0.0	0
	0.1	30
	0.2	90
	0.5	0
	1.0	0
1.5	0.0	10
	0.1	5
	0.2	80
	0.5	0
	1.0	0

[a] **Twenty explants were used in each treatment with ten explants per jar. Values are the means from the two jars in each treatment within an experiment**

Balancing of auxin and cytokinin has long been recognized as a key factor in the control of cell division and organogenesis in most dicot plants. This experiment demonstrated that exogenously applied BA and 2,4-D at 1 $mg.l^{-1}$ and 0.2 $mg.l^{-1}$ respectively, were essential for high frequency shoot regeneration from leaf explants of Tioga strawberry. The difference in regeneration capacity and mode of regeneration at concentrations higher and lower than this optimum may be due to variation in endogenous levels of these growth hormones in leaf explants. Similar work has been reported on regeneration of strawberry

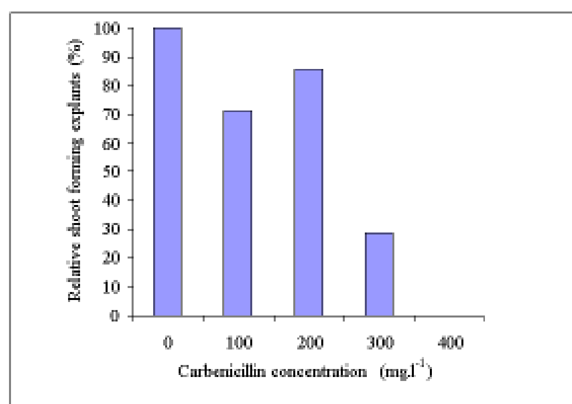
cv. Redcoat (Nehra *et al.*, 1990). The highest percentage calli with shoots (15%) was observed at 2.2 mg.l^{-1} each BA and 2,4-D after 24 weeks (Nehra *et al.*, 1990). In cv. Rapella (James *et al.*, 1990) the optimum conditions for strawberry leaf discs regeneration were a basal medium of MS with 1 mg.l^{-1} BA and 0.2 mg.l^{-1} 2,4-D, which was similar with this experiment. Recent studies (Passey *et al.*,2003) showed a relationship between genetic linkage and regeneration frequency, which our results have matched. Since Tioga is one of the maternal parents of the Rapella it thus shares a similar high regeneration capacity. Variation in ability to regenerate adventitious shoots from different strawberry cultivars has been observed (Passey *et al.*, 2003; Nehra *et al.*, 1989). For *Agrobacterium*-mediated gene transfer technology to be applied to genetically improve existing commercial strawberry cultivars, it is essential that efficient regeneration systems are available. The Tioga shoot regeneration system that we developed in this experiment with 90% regeneration, proved it was a potential system for future *Agrobacterium*-mediated gene transfer technology.

Effects of antibiotics on Tioga leaf explant regeneration

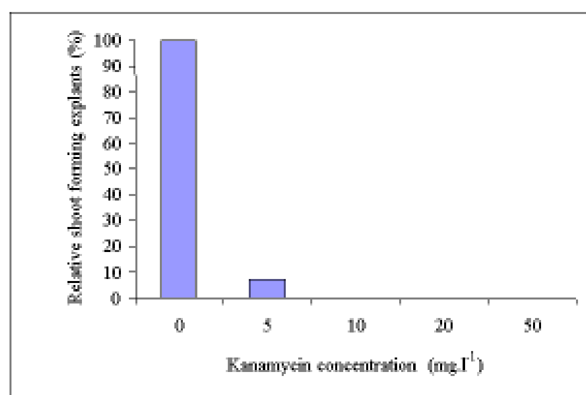
Cb has been used as bacterial control agent in strawberry plant transformation (Alsheikh *et al.*, 2002). It was found that Cb has some effect on cv. Tioga. Figure 2a shows that the number of leaf explants forming shoots in SRM containing Cb, was lower than that in SRM without Cb. At higher concentrations of Cb, the numbers of explants forming shoots were lower except at 200 mg.l^{-1} Cb. This concentration of Cb resulted in a higher number of explants forming shoots than 100 mg.l^{-1}. This result indicated that at a concentration of 200 mg.l^{-1}, the inhibiting effect of Cb on shoot regeneration from strawberry leaf explant cv. Tioga was least.

After 8 weeks, Km strongly inhibited shoot regeneration. At the concentration as low as 5 mg.l^{-1}, shoot regeneration was inhibited by more than 90%, compared with the control. No explants survived in the treatment of Km added at 10 mg.l^{-1} or higher (Figure 2b). Tioga leaf explants were clearly very sensitive to this antibiotic thus it can be used for transformation selection.

For effective *Agrobacterium*-mediated transformation, the antibiotic regime should control bacterial overgrowth without inhibiting regeneration of the plant cells. The result of the Cb test after 8 weeks showed that the carbenicillin concentration of 200 mg.l^{-1} or lower had little effect on strawberry leaf explants regeneration. A previous report on *Agrobacterium* growth inhibition (Alsheikh *et al.*, 2002) found that, among some



a



b

Figure 2. Effects of carbernicillin (a) and kanamycin (b) concentration on regeneration of leaf explants from the strawberry cultivar Tioga.

antibiotic tested, regeneration of Fragaria vesca explants was least inhibited by Cb and this antibiotic was highly effective at LBA4404 control. There were also reports on stimulation effects of Cb on callus growth and organogenesis (Lin *et al.*, 1995; Ling *et al.*, 1998). Shoot regeneration of carnation was 2.55% higher than the control, no Cb, when 500 $mg.l^{-1}$ of Cb was added in the media (Monstserrat *et al.*, 2001).

Tioga was very sensitive to Km, since no survival was recorded at a concentration of 10 $mg.l^{-1}$. It was reported that the presence of Km at 10 $mg.l^{-1}$ drastically decreased the organogenic capacity of strawberry leaf discs. After 6 weeks, 100% of discs remained viable in the absence of Km but only 21% remained viable at 10 $mg.l^{-1}$ in the presence of Km (Barcelo *et al.*, 1998). There was no sign of regeneration and the explants were dead after 10 weeks of culture. They also found that, at 25 $mg.l^{-1}$ Km, all explants were dead after 6 weeks. Thus, they chose this concentration to select transformed plants. For cv. Tioga, the initial use of 5 $mg.l^{-1}$ Km, followed by gradually increases up to 50 $mg.l^{-1}$ was very effective for transformant selection.

Construction of plasmid vectors harboring strawberry optimized codons of AFP gene

The purpose of modifying the antifreeze protein gene originally from fish was to create a modified gene that would be expressed strongly in strawberry cells. The sequence of the wild type antifreeze protein gene would be replaced with the strawberry bias codons, without changing the amino acid composition. The strawberry optimized codons of modified gene base on type III antifreeze protein isoform HPLC 6 was successfully constructed. The base sequence of this gene compared to original AFP gene from fish is shown in Figure 3.

HPLC-6 type III AFP was expressed in *E. coli* but at inefficient levels. This low level expression may be due to mRNA secondary structure (Li *et al.*, 1991). A gene with a codon usage different from the target host organism often has poor expression in that particular host. The

Figure 3. A comparison of base sequences between strawberry optimized codon and original fish AFP cDNA. Bases identical between these two sequences are boxed.

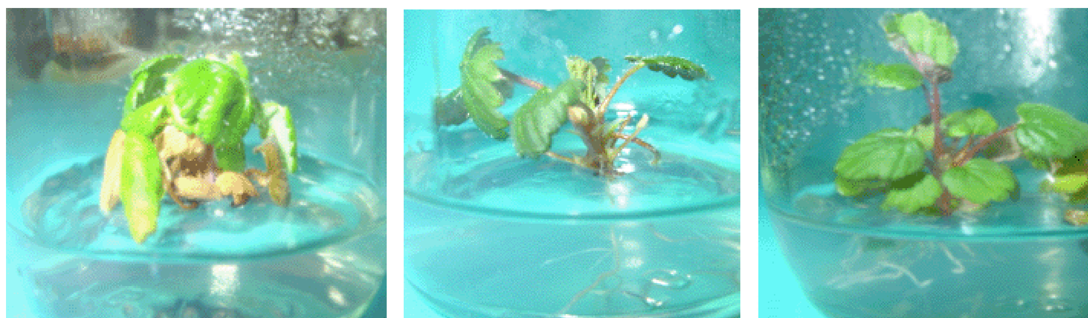


Figure 4. **A comparison of nontransformed (a) and transformed strawberry plants with pSW1 (b) and pBB (c) in MS containing 50 mg.l^{-1} Km. Nontransformed strawberry plants cannot root and show stress, observed by red colour and dried leaves while transformed strawberry plants were able to root and showed normal morphology.**

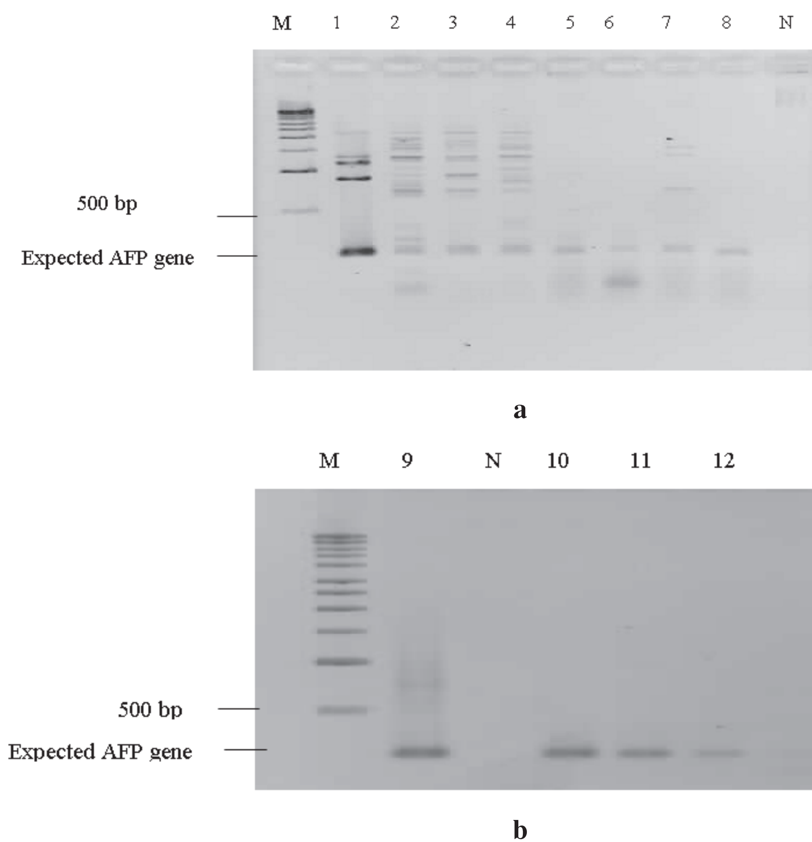


Figure 5. **Detection of the transgene by PCR. Genomic DNA from pSW1 (a) and pBB (b) transformed individual strawberry plants of T0 generation. M, marker; N, nontransformed strawberry plant; 1, pSW1 plasmid as positive control; 2-8, transformants of pSW1; 9, pBB plasmid as positive control; 10-12, transformants of pBB.**

presence of rare codons in cloned genes affects protein expression level and mRNA stability. Excessive presence of rare codons can result in ribosome stalling, slow translation, and translation errors (Kurland and Gallant, 1996; Roche and Sauer, 1999; Zahn, 1996). There are two alternative strategies to circumvent this codon bias (Hannig and Makrides, 1998). One is to alter rare codons in the target gene to the preferred codons of the host without affecting the encoded amino acid sequence. Another is to expand the intracellular tRNA pool by introduction of a plasmid encoding for extra copies of tRNAs for codons rarely used. Thus optimization of codons of the AFP gene for strawberry in our experiment was the former strategy to increase its expression level.

Agrobacterium-mediated transformation

Leaf explants showed better regeneration than petiole in cv. Tioga (data not shown). Although the concentration of Km for selection initially started at only 5 mg.l^{-1}, no regeneration appeared with nontransformed leaf explants tested in SRM containing 5 mg.l^{-1} Km and 500 mg.l^{-1} Cb, while some of the transformed leaf explants began callusing in the same medium. Leaf explants in MS alone began forming shoots after 4 weeks but leaf explants after transformation showed delayed formation of shoots in the selecting medium. This retardation may be due to a synergistic effect between Km and Cb. Explants, transformed with pSW1 and pBB, began forming shoots after 10 weeks. Those shoots were moved to MS with increasing Km up to a final concentration of 50 mg.l^{-1}. Roots were initiated within 2 weeks in MS, containing 50 mg.l^{-1} Km, and sufficient numbers of roots were produced within 4 weeks. No roots were observed in nontransformed strawberry plants, growing in MS with 50 mg.l^{-1} Km. All of the transformants appeared to be morphologically normal, relative to untransformed plants. Figure 4 shows strawberry plants transformed with pSW1 and pBB, compared with nontransformed plants in MS, containing 50 mg.l^{-1} Km. Differences in rooting ability of the transformed and nontransformed plants were clearly observed.

Avoiding use of antibiotics during the early stages of organogenesis seems to be important, since they interfere with developtment, even of transformed cells (James *et al*., 1989; Michelmore *et al*., 1987). In this experiment, rooting was conduced when the concentration of Km was increased to 50 mg.l^{-1} for avoiding development of undesirable number of the escape. According to Draper *et al*. (1988) roots are generally much more sensitive to antibiotics, and thus the ability to root on selection medium containing high levels of selective agent is a strong indication of transformation. All seven clones of strawberry plants transformed with pSW1 and all three clones of strawberry plants transformed with pBB produced band coresponse to the AFP gene. These values of 100% of positive clone chosen for PCR indicated that the selection system using a high concentration of Km was very effective, since there was no escape.

Analysis of transformants by the polymerase chain reaction

PCR analysis revealed that all seven strawberry plants, transformed with the pSW1 gave band coresponseding to antifreeze protein gene and all three strawberry plants, transformed with pBB, also produced a band corresponding to the

Table 3. Transformation efficiency of plasmid pSW1 and pBB in the strawberry cultivar Tioga.

Plasmid	Number of explants	Total number of rooted shoot in MS	Number of plantlets examined by PCR with 50 mg.l^{-1} Km	Number of plantlets with PCR positive	Transformation efficiency base on shoot rooted in MS with 50 mg.l^{-1} Km (%)
pSW1	125	15	7	7	12
pBB	138	6	3	3	1.43

antifreeze protein gene. There were no bands of the AFP gene size appearing in control, non-transformed strawberry plants (Figure 5).

Notably, the PCR result of strawberry plants transformed with pSW1 produced more than one band. Since the base sequences of primers used were completely complement with the strawberry optimized condon of the AFP gene in pBB but partially pair with the original AFP gene in pSW1. Thus, nonspecific binding might have occurred. Based on strawberry shoots rooting in MS with 50 mg.l^{-1} Km, the transformation efficiencies for pSW1 and pBB were 12% and 1.43%, respectively (Table 3).

Acknowledgments

The authors thanks Assoc. Prof. Dr. Prasartporn Smitamana, Faculty of Agriculture, Chiang Mai University, for providing *in vitro* strawberry plants. This work was supported by a Thailand Ministry of University Affairs Scholarship and Chiang Mai University's Graduate Research Fund.

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