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# Development of tobacco plant cells in the presence of kanamycin at various levels for transgenesis

*Perkembangan sel tanaman tembakau pada kanamisin berbagai konsentrasi untuk transgenesis*

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**_Ringkasan_**

*Diferensiasi sel tanaman dalam proses regenerasi tanaman transgenik umumnya dilakukan bersamaan dengan proses seleksi menggunakan bahan penyeleksi. Kanamisin merupakan salah satu antibiotika yang biasa digunakan dalam proses seleksi. Dengan spektrum yang luas, kanamisin menghambat pertumbuhan sel dan mengganggu proses translasi pada saat ekspresi gen. Untuk tujuan regenerasi tanaman transgenik yang mengeks-presikan gen NPTII, konsentrasi kanamisin perlu dioptimasi sehingga cukup untuk membedakan sel yang tertransform dengan yang tidak tertransform. Penelitian ini betujuan untuk mempelajari perkembangan eksplan tembakau pada media regenerasi yang mengandung kanamisin. Kecepatan inisiasi tunas dan jumlah tunas terbentuk merupakan kriteria utama untuk evaluasi. Ekstrak protein dari eksplan yang berregenerasi dianalisa dengan SDS-PAGE untuk melihat kemungkinan keterlibatan protein tertentu dalam proses regenerasi tersebut. Hasil penelitian menunjukkan bahwa konsentrasi optimum kanamisin untuk tujuan tersebut adalah 50 mg/L, dimana tunas tembakau transgenik terinisiasi pada hari ke 25 kultur sedangkan eksplan non-transgenik hingga hari ke 56 kultur tidak mampu menginisiasi tunas. Data analisis protein menunjukkan adanya 3 protein dengan ukuran terdenaturasi relatif kecil, antara 14,5 hingga 21,5 kDa pada eksplan yang berregenerasi, namun tidak dijumpai pada ekstrak eksplan yang tidak berregenerasi. Ketiga protein ini kemungkinan terlibat dalam proses regenerasi.*

**Summary**

Plant cell differentiation toward regeneration of transgenic plants is usually conducted simultaneously with selection process in the presence of selecting agent. Kanamycin is one of antibiotics widely used as selection agent. Having a wide spectrum activity, kanamycin hinders cell growth through inhibition of translation process during gene expression. To regenerate transgenic plant expressing *NPTII* gene, the level of kanamycin has to be optimized so that high enough to differentiate genetically transformed cells from those untransformed. This research is aimed to investigate *in vitro* development of tobacco cells toward plantlet regeneration on the media supplemented with kanamycin. The rate of shoot initiation and number of shoots formed are the major criteria evaluated. Protein extracts of the regenerated explants were analyzed with SDS-PAGE to examine possible involvement of specific proteins in the regeneration process. The experimental result suggested that optimum level of kanamycin for the purpose is 50 mg/L, by which transgenic tobacco shoots were initiated at 25-day culture whereas the untransformed explants did not initiated any shoots even though after 56-day culture. Preliminary data from the protein analysis indicated the presence of relatively small size denatured proteins, between 14.5 and 21.5 kDa, likely to involved in the regeneration process.

[*Key words***:** *Nicotiana tabacum*, shooting, selecting agent, protein marker]

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## Introduction

Developmental process of meristematic plant cells toward differentiation is highly regulated at gene expression level. Studies on *Arabidopsis* indicated the involvement of several regulatory genes such as *LEAFY (LFY), APETALA1 (AP1), AGAMOUS (AG)* genes (Mizukami & Ma, 1997; Levy & Dean, 1998). Those genes interactively control the flowering through promotive and repressive pathways of genetic expression (Koorneef *et al.*, 1998; Nilsson *et al.*, 1998). In plant, regulatory elements involve in this pathway could be from various classes, physical signal from leaf (Tooke & Battey, 2000); chemicals from environment such as gibberellins (Blázquez, *et al.*, 1998), or trans-acting elements such as enhancer (Deyholos & Seiburth, 2000).

*In vitro* differentiation of plant cells for transgenesis is expressed in the presence of a selecting agent such as antibiotics. Kanamycin is a wide-spectrum antibiotic commonly used in selective regeneration of transgenic plants (Angenon *et al.*, 1994). Like mostly antibiotic, kanamycin hinders growth of living cells by inhibiting gene expression process particularly at translation step. Mimicking amino acid-carrying tRNA, antibiotic interacts with ribosome-mRNA complex binding to the A site of the complex and blocking the synthesis of the polypeptide (Lehninger, 1993).

Inhibitory capacity of kanamycin to the growth of some plant species is varied. To select plant cells expressing *NPTII* transgene, kanamycin is added to selection media at optimum concentration, which is ranging from 50 to 500 mg/L depended on the species tested. Therefore in plant genetic transformation, the optimum concentration of selecting agent added to the media is determined before hand.

This research is aimed to investigate the effect of kanamycin level on the development of tobacco plant cells cultured on regenerati-on media. The media were prepared for induction of organogenesis. Along with morphological characteristics, molecular characterization related to the development is also determined on the differentiating explants. Possibility of the presence of a specific protein induced in response to a signal of cell differentiation was examined with SDS-PGE. In addition to resistance to kanamycin, transgenesis was confirmed by detecting the transgene using PCR methods.

## Materials and Methods

### In vitro *inhibition of kanamycin on the development tobacco leaf explant*

Tobacco explants were harvested from fully expanded young leaves of tobacco plants grown *in vitro*. After sliced into pieces of 1 cm x 1 cm, the explants were inoculated onto regeneration media of MS (Murashige & Skoog, 1962) supplemented with 3% sucrose, 0.5 mg/L BAP, without or with kanamysin varied from 25 to 200 mg/L, and solidified with 0.2% phytagel. After the pH is adjusted to 5.6, the media were autoclaved at 121$^{o}$C at 1.0 atm for 20 minutes. Regeneration to induces shoots was conducted *via* organo-genesis by incubation under 12-hr light regime at 25-26$^{o}$C.

### *Protein analysis*

Total proteins were extracted from tobacco plant tissues of several stages of development. The protein extracts were prepared in buffer solution as described in Santoso & Thornburg (1995). Extraction buffer containing 100 mM Tris.HCl pH 7.5, 10 mM 2-mercaptoethanol, and 4 mM EDTA was used for this extraction. The extracts were stored in –20$^{o}$C until needed for the analysis. Protein content of the extract is assayed using coommasie blue (Bradford, 1977). To identify the presence of specific protein regulated at this culture stage, extracts containing each 100 µg total protein were fractionated on SDS-PAGE containing 12% polyacrylamide. Protein bands were visualized by coomassie blue staining.

*Genetic transformation of explant*

Transformation of recombinant DNA carrying *NPTII* gene into tobacco leaf explant was carried out according to procedure slightly modified from Sain *et al.* (1994). After 15 minute incubation in 1/10 volume of *Agrobacterium* of a 3-hr on culture, the leaf slices were co-cultivated on MS solid media containing 100 mg/L acetosyringone for 2 days. Afterward, the explants were rinsed with MS liquid media added with 500 mg/L cefotaxime then cultivated on regeneration media containing 50 – 100 mg/L kanamycin and 500 mg/L cefotaxime. Regeneration for transgenic plantlets of tobacco was performed with MS selection media containing 0.5 mg/l BAP, 3 % sucrose and 50 mg/L kanamycin unless indicated different. Plantlets having two pairs of expanded leaves were cut at the lower part of the stem and transferred onto MS rooting medium containing 50 mg/L kanamycin without hormone

*DNA analysis*

Examination for transgenesis was performed at DNA level as well as expression product of the transgene. To confirm the presence of transgene in the regenerated tobacco plantlets, the genomic DNA of the plantlets was first purified using CTAB-containing extraction buffer as described by Rogers & Bendich (1994). Amplification a fragment of the transgene with PCR using a pair of NPT II primer to amplify a fragment of the transgene was used to detect the presence of the kanamycin resistance encoding gene. The detail procedure of PCR is described in Davis *at al.* (1994) with the total volume of the reaction was reduced to 25 $\mu$ L . For optimization the annealing temperature was varied from 45 – 58$^o$C. The amplification products were examined on agarose gel 1% containing 0.5 µg/mL ethidium bromide and visualized using UV illumination.

**Results and Discussion**

Selecting agent widely utilized in selection and processes for transgenic plants has been kanamycin. (Topfer *et al.*, 1980; Chung *et al.*, 2000). Interfering with the process of gene expression particularly translation, kanamycin is capable of inhibiting the growth and development of living cells. Expressing a bacterial transgene of *NPTII*, transgenic plant cells were distinguishable from non-transgenic cells as they are tolerant to kanamycin. *NPTII* detoxifies kanamycin through its neophos-photranferase activity. In a culture selection the process of kanamycin detoxification by the *NPTII* and inhibition of cellular gene expression by kanamycin occurs simultaneously. Therefore in practice, kanamycin level in selection media needs to be optimized. The concentration should not be too high, but it should just enough to inhibit the growth of development of non-transgenic cells.

Table 1 shows the effect of kanamycin level added to regeneration media on the development of tobacco leaf explants. These data demonstrate that the more kanamycin added into selection media, the stronger the inhibition effect of kanamycin on the development of the plant cells. In the absence of kanamycin, shoots were initiated at the 2$^{nd}$ week of the culture and could clearly be seen at 3$^{rd}$ week of the culture or after. Kanamycin level of 25 mg/L inhibited slightly the cell development. Shoots emerging from the explant could clearly be seen only after 5 weeks of the culture.

On the regeneration media with 50 mg/L kanamycin, the plant regeneration was completely inhibited. Shoot initiation was not detected even though the explants had already been cultured for 8 weeks. At the other higher concentration tested, the inhibitions were stronger. After cultured for 8 weeks, the edges of the leaf explants were browning that indicates the cells on those parts were likely died. These data

Table 1. The effect of kanamycin added in the regeneration media to the development of untransformed tobacco leaf tissue

| Kanamycin (mg/L) | Parameters on explant | Development at various culture ages (weeks) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 8 |
| 0 | Viability | +++ | +++ | +++ | +++ | +++ | +++ |
| | Shoot | - | + | ++ | +++ | ++++ | +++++ |
| 25 | Viability | +++ | +++ | ++ | ++ | ++ | ++ |
| | Shoot | - | - | - | + | ++ | +++ |
| 50 | Viability | +++ | +++ | ++ | ++ | ++ | ++ |
| | Shoot | - | - | - | - | - | - |
| 75 | Viability | +++ | +++ | ++ | ++ | ++ | + |
| | Shoot | - | - | - | - | - | - |
| 100 | Viability | +++ | +++ | ++ | ++ | ++ | + |
| | Shoot | - | - | - | - | - | - |
| 150 | Viability | +++ | +++ | ++ | ++ | ++ | + |
| | Shoot | - | - | - | - | - | - |

Note: Stages of shoot development & viability: - = no shoot, + = small shoot initiation & bit browning, ++ = clearly seen shoot & pale-green, +++ = leaf initiated & fresh-green, ++++ = leaf clearly formed, ++++ = transferable plantlet for rooting

suggested that the optimum concentration of kanamycin for regeneration of transgenic tobacco expressing *NPTII* gene is 50 mg/L. A lower concentration may result in also regenerated non-transgenic plantlets. On the other hand, at higher than 50 mg/L, some added kanamycin might be out of the detoxification, and consequently inhibited gene expression process in the cells and therefore decrease the frequency of regeneration. The level of kanamycin required for selection and regeneration of transgenic plants is depended on plant species. From previous report on selectable marker of transgenesis, kanamycin level of 50 mg/L was effective in selection for transgenic plant of *Arabidopsis thaliana* (Becker *et al.*, 1992; Chung *et al.*, 2000). Whereas selection for transgenic *Cucurmis sativa L.*, 75 mg/L kanamycin was considerably effective (Sarmento *et al.*, 1992). Our experiments on calli of *Saccharum officinarum* L., developmental differences were distinctively occurred only at kanamycin levels more than 100 mg/L (Minarsih, 2001).

Quantitative data on the *in vitro* plant cell development in the presence of selecting antibiotic are presented in Table 2. These data shows that, in addition to by the selecting agent, the regeneration capacity was also affected by the event of genetic transformation. The transformation decreased the number of regenerated shoots from 5 to 4 shoots on each explant. The transformation slowed down the shoot initiation from 18 days to 20 days. The two stressing treatments transformation coupled with addition of the selecting agent reduced the rate of shoot formation even higher. The shoot initiation of the transformed explants on the selection media was significantly slower, which was 7 days longer than that of control without,

Table 2. Shoot initiation and number developed from the leaf explants cultured on regeneration media containing kanamycin at sub- and lethal concentration

| Treatment of explant | Kanamysin (mg/L) | Time of shoot initiation (days) | Shoot number at 37 days | |
|---|---|---|---|---|
| | | | per explant | Total |
| Un-transformed | 0 | 18 | 5 | 60 |
| Un-transformed | 50 | >55 | - | - |
| Transformed | 0 | 20 | 4 | 48 |
| Transformed | 50 | 25 | 0,4 | 6 |
| Transformed | 200 | > 55 | - | - |

kanamycin. More over the frequency of regeneration in the transgenesis was 92% lower than that in the regeneration of positive control untransformed explants on media with no selecting agent.

Those arguments suggest that the rate of regeneration for transgenic plant in recalcitrant trees such as conventional estate crops will consequently much lower. Therefore, development of transgenic estate crops requires better techniques particularly for tissue culture and genetic transformation. Technology of tissue culture capable of regenerating plantlets at high frequency is indispensable. Availability of genetic transformation and selection protocols less stressful to the plant cells is required.

In addition to the treatments above, visual appearance and development of the cultured leaf explants was clearly affected bythe concentration of kanamycin. On media with no or low level of kanamycin (25 mg/L), the explants were freshly green in color and able to initiate and develop viable shoots. Whereas on the media with kanamycin at concentration of 50 mg/L or more (examination was conducted at kanamycin level up to 200 mg/L), the color of explants was relatively unchanged but regeneration ability was drastically low (Figure 1). They did not initiate any observable shoot even though the induction had already been 8 weeks. Unchanged green color of the explants suggested that the kanamycin does not inhibit expression of genes for synthesis of chlorophyl. Alternatively, the amount of chlorophyl in the explants had been already saturated and relatively stable in the cultured. The antibiotics taken up by the cells from low kanamycin containing media, 25 mg/L, was not enough to significantly inhibit expression of genes including those involved in differentiation process. Therefore the cells in the explants on the low kanamycin media could undergo organogenesis to finally regenerate plantlet as those on media with no kanamycin.

In addition to resistance to kanamycin, transgenesis was confirmed by detecting the presence of the transgene using PCR methods. PCR reaction using *NPT II* primers that amplify fragment of *NPTII* gene on several tobacco transgenes is depicted on Figure 2. The results showed that there were amplification of the transgene on four selected tobacco planlets with a size slightly lower than 500 bp (lane 3 to 6), and no bands on un-transformed plant (lane 7). This indicated that the pBI-P5CS construct containing the *NPTII* gene is successfully transformed into the tobacco explants.

Figure 1. Development of untransformed tobacco leaf explants on regeneration media with and without kanamycin. From left to right are from explants on the media with 0, 25 and 50 mg/L kanamycin respectively.

Varying intensity of DNA bands amplified using *NPTII* primers, indicated likely aselectable marker gene stable inserted into the plant genome. Genetic transformation of correspondence to the copy number of the cucumber through *Agrobacterium* followed by selection in the presence of 75 mg/L kanamycin resulted in insertion of 1 to 20 copies of *NPTII* gene into the regenerated plant genomes (Sarmento *et al*., 1992)

Most of biological processes are regulated synchronically via enzymatic control. To determine the existence of protein involvement in the processes of cell differentiation and transgenesis, protein extracts of various stages of explants were prepared and analyzed simply using denaturing gel SDS-PAGE. The protein banding patterns of each samples are presented in Figure 3. This preliminary data show that there are typical differences on the pattern of tested samples.

Three small dominant bands between 14.5 and 21.5 kDa are present in shoot-regenerating cells regardless genetic transformation (lanes 1 and 2). This can be speculated that those proteins may involve in the *in vitro* regeneration process of tobacco explants. Involvement of gene expression in the process of plant cell development has been demonstrated in several species. Some specific mRNAs were differentially expressed during somatic embryogenesis of *Solanum melongena* L. (Afele *et al.*, 1996). Constitutive expression of *Psy*1 transgene in tomato caused dwarfism (Fray *et al.*, 1995). Current reports on *Arabidopsis* indicated that a novel protein, embryonic flower2, mediated shoot development and flowering (Yoshida *et al.*, 2001) and EMF1 involved in the control of shoot architecture and flowering in the species (Aubert *et al.,* 2001).

There other distinctive big sizes protein bands, which are bigger than 97.4 kDa. The biggest size band, which close to the stacking gel, was identified only in the sample from the regenerating transgenic tobacco plantlet

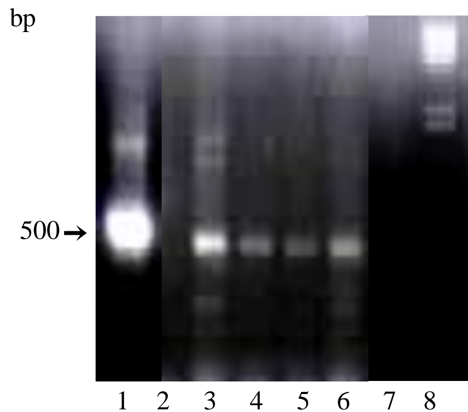


Figure 2. Agarose gel profile of the PCR products of tobacco genomic DNA using the *NPTII* primer forward and reverse. Lane 1) plasmid pBI-P5CS; 2) control $H_20$; 3) to 6) transgenic tobacco planlets; 7) tobacco non-transformed; 8) λ *Hind*III DNA marker

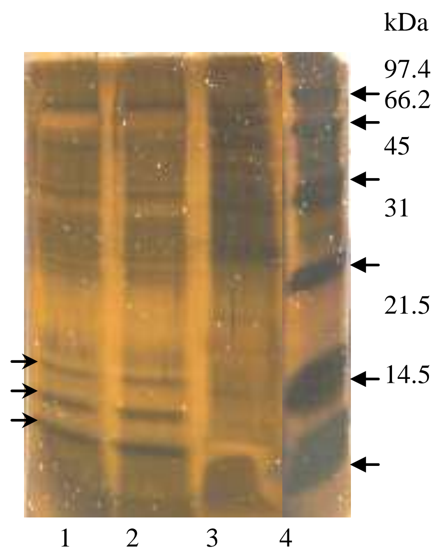


Figure 3. .SDS-PAGE profile of the cultured-explant extracts. Lanes 1-4 are extracts from transgenic shoots, non-transgenic n shoots, untransformed explants on kanamycin, and molecular weight standard respectively.

This protein maybe corresponding to the expressed transgene particularly p5cs. Deducting protein size from the full length of the *P5CS* cDNA, which is 2.4 kb, and assuming the average molecular weight of amino acids is 300 Dalton, therefore the size of the P5CS protein should be about 240 kDa.

## Conclusions

- The experimental result suggested that optimum level of kanamycin for the purpose is 50 mg/L, by which transgenic tobacco shoots were initiated at 25-day culture whereas the untransformed explants did not initiated any shoot even though after 56 days of culture.
- Preliminary data from protein analysis indicated the presence of relatively small size denatured proteins, between 14.5 and 21.5 kDa, likely to involved in the tobacco regeneration process.

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