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Full Length Research Paper

Ethanol inducible isopentenyl transferase as a high efficiency marker for tobacco transformation

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The isopentenyl transforase gene from *Agrobacterium tumefaciens* is one positive selectable marker genes for plant transformation. In this research, the *ipt* gene was placed under the control of ethanol-inducible system and was introduced into *Nicotiana tabacum* cv. Xanthi via *Agrobacterium*-mediated transformation. On a hormonefree medium (MSI) with 0.1% ethanol vapor, a large number of transgenic calli and shoots were obtained from explants that were infected with *A. tumefaciens* LBA4404 harboring the *AlcA::ipt* gene or *35S::ipt* gene. On the same medium without inducer under identical culture conditions, explants can produce a larger number of calli and shoots transformed using *35S::ipt* gene, but no calli or shoots were induced from explants transformed using *AlcA::ipt* gene. After being transferred to MSII medium, the *AlcA::ipt* transgenic plants displayed normal shoot morphology compared to the *35S::ipt* transgenic plant lines. Our results demonstrated that ethanol-inducible ipt system can be used as a positive selection marker for tobacco plant transformation.

Key words: Selectable marker, *ipt* gene, ethanol inducible system, transformation.

INTRODUCTION

Selectable marker genes, acting as a necessary tool for selecting transformants, play an important role during transgenic plant production (Yoder et al., 1994). The standard for classification of marker genes is various. According to the living condition of untransformed cell on the selective medium, it can be classified as positive and negative selection. Many negative selection genes have been reported, such as the antibiotic resistance gene and anti-herbicide gene. Because of these negative genes conferring resistance to a selection agent, transformed cells survive selection while untransformed cells would be killed or their proliferation would be inhibited (Luo et al., 2006). And these selectable marker genes have potential problems to human health and the ecosystem (Dale et al., 2002). To resolve these problems, several positive selectable marker genes including hormone metabolic genes, carbohydrate metabolic genes and fluorescence genes have been developed for plant transformation (Morten and Okkels, 1996; Christy et al., 1997; Zuo et al., 2002; O'Kennedy et al., 2004). Moreover, it has been reported that the selective efficiency of the hormone metabolic gene is higher than some kinds of the antibiotic resistance genes (Joersbo, 2001). One of this type of marker genes is the isopentenyl transforase (*ipt*) gene, which was an oncogene encoding the enzyme isopentenyl transferase to catalyze one of the early steps in cytokinin biosynthesis, which enables cells to proliferate and differentiate into shoots without exogenous cytokinins in tobacco, tomato, muskmelon, and sweet pepper

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Abbreviations: BA, N^6 Benzyl adenine; **MS,** murashige and skoog; **NAA,** naphthalene acetic acid; PCR, polymerase chain reaction; **IBA,** indole butyric acid; **CTAB,** cetyl trimethyl ammonium bromide.

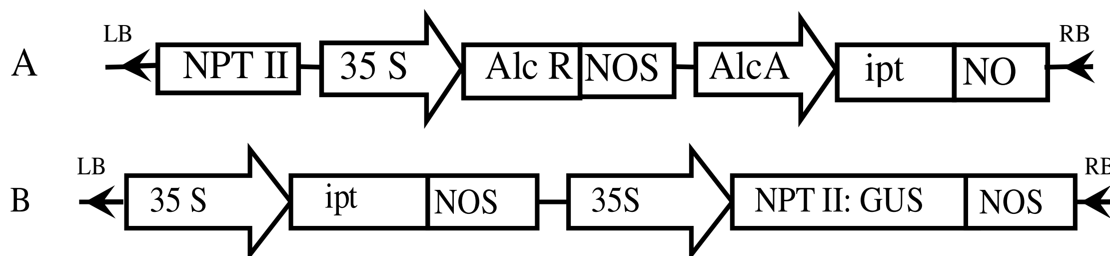

Figure 1. A schematic presentation of the binary vectors used for plant transformation. **A.** Binary vector pBinSRNAIN containing isopentenyl transferase gene (*ipt*) under the control of an *AlcA* promoter; **B.** binary vector pBIG containing the isopentenyl transferase gene (*ipt*) under the control of a CaMV 35S promoter; RB, right border sequence of a T-DNA; LB, left border sequence of a T-DNA; *AlcR,* alcohol sensor protein; *AlcA, AlcA* promoter of alcohol dehydrogenase from *A. nidulans*; NPTII, an antibiotic resistance gene, which is the nopaline synthase gene driven by the nopaline synthase gene promoter. The *nos* terminators of *ipt*, *AlcR*, and *NPTII*, are from the terminator region of the nopaline synthase from *A. Tumefaciens*.

(Ebinuma et al., 1997; Kunkel et al., 1999; Endo et al., 2001, 2002; Mih´alka et al., 2003). To rigorously control the expression of *ipt* gene in plant special development stage or special tissue, a number of promoters including those inducible by heat (Medford et al., 1989), wounding (Smigocki et al., 1993), light (Thomas et al., 1995), copper (McKenzie et al, 1998) have been used to drive *ipt* gene expression. Unfortunately, transgenic plants with over expression of *ipt* gene exhibited morphological abnormalities since overproduction of cytokinins interferes with many developmental processes (Gan and Amasino, 1997). Although the *ipt* gene was successfully used as a positive selectable marker controlled by the dexamethasone-inducible system in plant transformation (Kunkel et al.1999), dexamethasone addition sometimes caused growth defects and induction of specific defenserelated gene expression (Kang et al., 1999; Mori et al., 2001; Ouwerkerk et al., 2001). Recently, ethanol-inducible system, which is one of the most promising inducible systems for both laboratory and field use, is developed and used in many species (Caddick et al., 1998; Roslan et al., 2001). Up to now, the utility of the *ipt* gene as a promising marker controlled by the ethanol-inducible system in genetic transformation of higher plants has not been demonstrated.

In this paper, we report the inducible expression of the *ipt* gene as a positive selection system for plant transformation. Our results demonstrated that inducible expression of the *ipt* gene under the control of the ethanol-inducible system is a positive selection system for plant transformation.

MATERIALS AND METHODS

Vector construction

The *Hin*dIII 0.73-kb fragment of the *ipt* gene coding sequence amplified by polymerase chain reaction (PCR) was ligated into pACN (Syngenta, Bracknell, Berkshire UK) to generate pAIN. The KpnI fragment containing *ipt* gene fused with the *AlcA* promoter and Nos terminator region was excised from pAIN and then inserted into the KpnI site of pBinSRNACatN (Syngenta, Bracknell, Berkshire UK). The vector was designated as pBinSRNAIN (Figure 1A). The 35S-ipt-NOS cassette was also ligated into the *Hin*dIII site of pBI121 to produce the binary vector pBIG (Figure 1B). The two plasmids were introduced into *A. tumefaciens* LBA4404 by the freeze-thaw method (H¨ofgen and Willmizer, 1988), and then used for transforming leaf discs of *Nicotiana tabacum* cv. Xanthi.

Plant transformation

N. tabacum was grown in a greenhouse at 25°C under an 18 h light/6 h dark photoperiod. Expanded leaves were harvested from a wild type tobacco plant of *N. tabbacum* cv "Xanthin" and washed with tap water followed by sterilization with 10% bleach plus 0.1% Tween-20 for 15 min. After being immersed in sterile water 5 times, the leaves were cut into small pieces (approximate 0.5 - 1 cm x 0.5 - 1 cm). Leaf discs were incubated with *A. tumefaciens* strain LBA4404 (OD600 = 0.6) for 10 min then put on a sterile filter paper to remove excess bacteria in suspension for 40 - 50 min. The infected leaf discs were transferred onto a co-culture medium without kanamycin and plant hormones and cultured for 3 days in the dark at 24°C. The leaf discs were then transferred to two different types of media, respectively: (1) MSI, which was an MS medium without hormones and with 100 mg/l kanamycin. (2) MSII, which was an MSI medium with 0.1% ethanol (Justin, et al., 2002; Caddick, et al., 1998) in a sealed glass Petri dish; ethanol was added after autoclaving. After 14 days, all explants were transferred to MSI medium. After one month, the independent shoots wan cut and cultured on the medium 1/2 medium plus 1mg/L indole butyric acid (IBA) for inducing roots. Cultures were maintained at 25°C under light (3000 lux). All three types of media were supplemented with agar (8 g/l), sucrose (20 g/l), and timentin (100 mg/l) and adjusted to pH 5.8. Thirty leaf disks were used for each treatment for the gene constructs and all experiments were repeated three times.

Verification of stable incorporation of transgenes into tobacco genome

Because all of the shoots used for molecular analysis were primary transformants, it was highly likely that these shoots contained *Agrobacterium* cells and the Ti-plasmid DNA. To circumvent this problem, a representative PCR method was used according to Luo et al. (2006) to verify the stable incorporation of transgenes into tobacco genome. The modified method is as follows: genomic DNA

was isolated from transgenic and wild type *N. tobacum* with a modified cetyl trimethyl ammonium bromide (CTAB)-method (Doyle and Doyle 1990). To avoid contaminations of Ti-plasmid DNA from *Agrobacterium* left in transgenic plant tissues, 1 µg plant genomic DNA samples isolated from all transgenic shoots were fractioned on 1.0% (w/v) agarose gel with the pBinSRNAIN Ti-plasmid DNA loaded on the side as a reference; and then large-sized-genomic DNAs (about 20 - 25 kb that is much larger than the Ti-plasmid DNA) were recovered from the agarose gels and used as templates of PCR reactions to amplify the *ipt* gene within the T-DNA region and the tetracycline resistance (tetA) gene out of the T-DNA region. PCR reactions were done under standard conditions with 3 min predenaturation at 94°C, 1 min denaturation, 1 min annealing, and 1 min extension at 94, 55, and 72°C, respectively, for 35 cycles. The primers used to amplify the *ipt* gene are 5'-ATGGATCTGCG TCTAATTTTCGG-3' and 5'- CTAATACATTCCGAATGGATG ACC-3'. The size of the *ipt* DNA segment amplified is 723 bp. The primers used to amplify the tetA gene are 5'-TGAGTGAGT GAGCTGATACC-3' and 5'-ATGTTGGGTTTCA CGTCTGG-3'. The size of the amplified tetA DNA segment is 1248 bp.

RESULTS

Production of transgenic calli and shoots from tobacco

Tobacco leaf discs were infected with the *A. tumefaciens* LAB4404 harboring binary vectors pBinSRNAIN(AlcA::ipt) and pBIG (35S::ipt), then transferred to MSI and MSII. Within 2 weeks, initiation of calli were observed in leaf discs infected with *Agrobacterium* harboring pBinSRNAIN (AlcA::ipt) on MSII (Figure 2D) and pBIG (35S::ipt) on MSI (Figure 2A) or MSII (Figure 2C). About 1 month after culture, a number of shoots were produced from these leaf discs on the medium (Figure 2E and F). These shoots produced from explant transferred with *AlcA::ipt* gene could be distinguished easily from shoots produced from explant transferred with *35S::ipt* gene. These morphological alternations were possibly caused by over-expression of the *ipt* gene in tobacco plants. In contrast, 6 weeks after infection, there were no visible calli produced from leaf discs infected with *Agrobacterium* harboring pBinSRNAIN(*AlcA::ipt*) cultured on MSI (Figure 2B).

Molecular analysis of transgenic shoots

We used PCR technique to confirm the stable incorporation of transgenes into the tobacco genomes. Genomic DNA was extracted from each of kanamycin resistance plant lines and fractionized on agarose gels with pBinSRNAIN plasmid DNA as references. As shown in Figure 3A, high molecular weight tobacco genomic DNA can be easily separated from the Ti-plasmid DNA. Then high molecular weight tobacco genomic DNA were recovered from the gels and used as PCR templates. Using this method, the contamination of the Ti-plasmid DNA from residual *Agrobacterium* cells would be eliminated. To confirm that there was no Ti-plasmid DNA contamination in the recovered plant genomic DNA samples, an internal control PCR reaction was done in the same reaction tube. For the internal control PCR reaction, 5'-TGAGTGAGTGAGCTGATACC-3' and 5'-ATGTTG GGTTTCACGTCTGG-3' were used as primers to amplify 1248 bp of the tetA gene outside of the T-DNA region. The presence of the 1248 bp tetA DNA segment would indicate that the genomic DNA samples were contaminated with the Ti-plasmid DNA from residual *Agrobacterium* cells in the plant tissues. The representative PCR analyses of transgenic plants are shown in Figure 3B. All of the PCR reactions (Lanes 1 - 4 and 6 - 9) with the recovered genomic DNA samples as templates did not produce the tetA DNA segment. With two primers specific for the *ipt* gene within the T-DNA region that were incorporated into the plant genome, we amplified a 723 bp segment from all kanamycin resistance transgenic lines, as shown in Figure 3B. The *ipt* DNA fragment was amplified from two types of transgenic shoots (Lanes 1 - 4 and 6 - 9). PCR using genomic DNA isolated from wild type plants did not produce *ipt* DNA fragment (Lane 5).

Comparison of transformation efficiencies

To determine inducible expression of the *ipt* gene as a positive selection system for plant transformation under the control of ethanol-inducible system, transformation efficiencies was calculated based on the number of transgenic shoots produced per 10 explants. The PCR method (genomic DNAs with Ti-plasmid DNA removed as templates) was used for confirmation of stable incurporation of transgenes into the tobacco genome. As shown in Table 1, without exogenous plant hormones (that is, cytokinin and auxin) added to a kanamycin medium (MSII), leaf discs infected with *Agrobacterium* harboring *35S::ipt* gene produced 36 calli and 29 transgenic shoots per 10 explants, similar to that obtained with selection on MSI, 42 calli and 27 shoots. Without ethanol inducer in the culture medium MSI, there was no callus and shoot production from the explants infected with *Agrobacterium* harboring *AlcA::ipt* gene. However, 32 calli and 22 transgenic shoots per 10 explants were produced in the same culture conditions when they were grown on MSII, a medium containing ehanol inducer. The transformation efficiency of *35S::ipt* gene is higher than that of *AlcA::ipt* gene cultured on MSII medium. After two weeks culture on MSII medium, callus and shoots were produced from explants transformed with *AlcA::ipt* gene and *35S::ipt* gene. One month later, shoots produced from explants transformed with *35S::ipt* gene expressed shoot characteristics typical of constitutive cytokinin expression and could be distinguished easily from normal shoots produced through tissue culture, but more than 17% shoots transformed with *AlcA::ipt* gene displayed a range of growth habits from plants that appeared similar to the wild-type. Within 20 days of transfer to root-inducing

Table 1. Efficiencies of callus and shoot formation from tobacco leaf discs infected with *Agrobacterium tumefaciens* LAB4404 containing pBIG (35S::ipt), pBinSRNAIN (AlcA:ipt) vectors.

Vector medium		Average number of calli per 10 explants	Average number of shoots per 10 explants	Transformation efficiency (%)
pBIG(35S:ipt)	MSI	36.3	29.0	290
pBinSRNAIN(AlcA:ipt)	MSI	0	0	0
pBIG(35S:ipt)	MSII	42.0	27.0	270
pBinSRNAIN(AlcA:ipt)	MSII	32.3	22.0	220

MSI: No hormones, but kanamycin added to MS medium; MSII: MSI with 0.1% ethanol inducer. Shoots were verified for stable incorporation of transgenes into the tobacco genome according to the methods.

Transformation efficiency (%) = transgenic shoots positively /the total number of explants infected with *Agrobacterium* ·100.

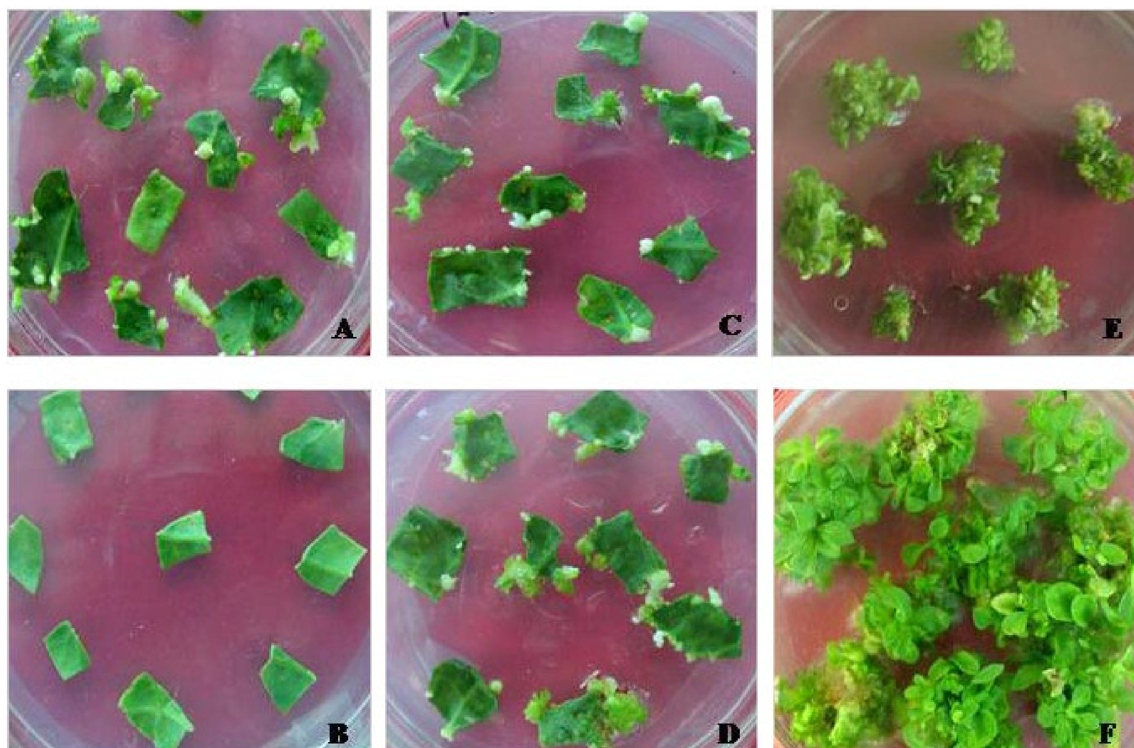

Figure 2. Regeneration of shoots from tobacco leaves transferred with *AlcA::ipt* gene, or *35S::ipt* gene. A. Two weeks after inoculation on MSI medium, calli and shoots were initiated in the explants transferred with *35S::ipt* gene; B and no calli and shoots were initiated in the explants transferred with *AlcA::ipt* gene; C. two weeks after inoculation on MSII medium, calli and shoots were initiated in the explants transferred with *AlcA::ipt* gene; or D. *35S::ipt* gene; F. one month later, shoots were regenerated from the explants transferred with *AlcA::ipt* gene, or E. *35S::ipt* gene.

media, more than 90% of the transgenic *AlcA::ipt* tobacco shoots developed a strong root system but over 50% of the transgenic *35S::ipt* tobacco shoots developed a weak root system. The plants transformed with *AlcA::ipt* could be transferred easily to soil and they developed normal leaves, flowers, and seeds. These data demonstrate that *ipt* gene can be used as a selection marker gene under control of the ethanol-inducible system for plant transformation in hormone free media with suitable contraction inducer.

DISCUSSION

To address the concerns regarding biosafety of using antibiotic herbicide selectable marker genes in genetically modified crops, a number of alternative selection systems have been developed (Penna et al., 2002). Previously, the *ipt* gene was used successfully as a positive selection marker gene (Zuo et al., 2002; Ebinuma et al. 2005). They have also demonstrated that using the ipt gene as a selection marker gene dramatically improves transformation

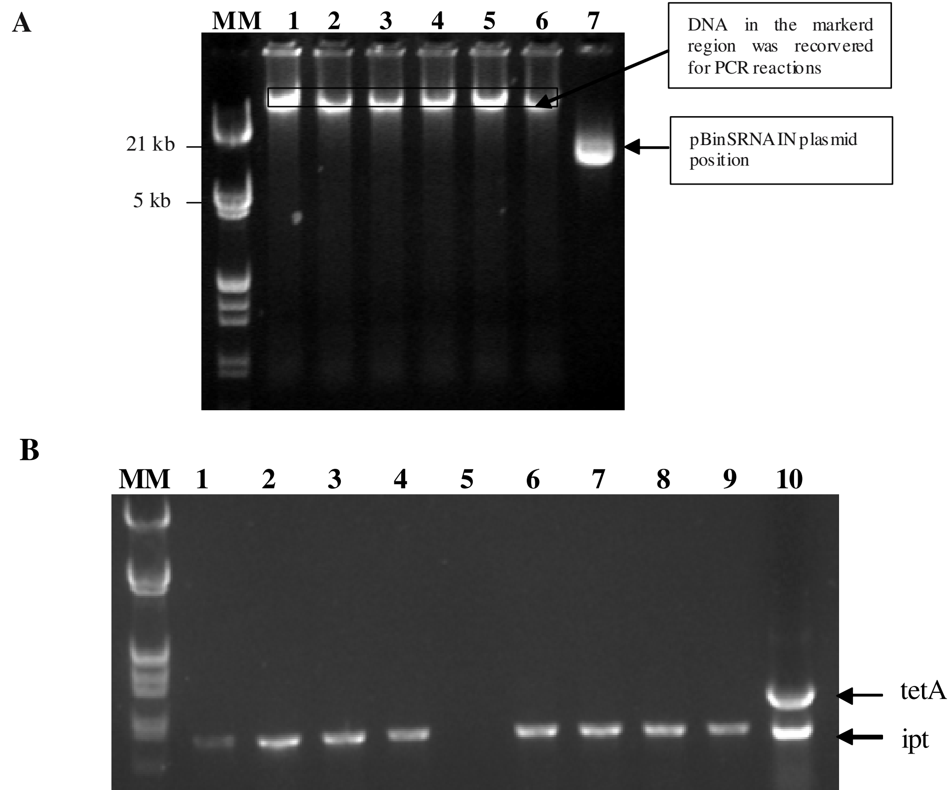

Figure 3. Confirmation of stable incorporation of transgenes into the tobacco genome with PCR analysis. A, *Lane MM*: DNA molecular size marker (Lambda DNA was digested by *Hin*dIII and EcoRI). *Lane 1*: Ti-plasmid pBinSRNAIN DNA. *Lanes 1–6*: genomic DNA extracted from transgenic plants. The plant genomic DNA in the rectangle region of each lane as marked was recovered individually from the agarose gel and used as templates for PCR reactions. The PCR results are shown in Figure 3B. B, PCR reactions were performed as described in materials and methods with primer sequences for both the *ipt* gene (within the T-DNA region and should be incorporated into the plant genome) and for the tetA gene (outside the T-DNA region, should not be incorporated into the plant genome). *Lanes 1, 2, 3 and 4*: Representative transgenic AlcA::ipt shoot lines; *Lanes 6–9*: representative transgenic 35S::ipt shoot lines; *Lane 10*: positive control (pBinSRNAIN Ti-plasmid, DNA used as the template); *Lane 3*: wild-type plant control (Wild-type plant DNA as template). *Lane MM*: DNA molecular size marker (Lambda DNA was digested by *Hin*dIII and EcoRI).

efficiency (Estruch et al., 1991; Li et al., 1992; Smith et al., 1992; Sinha et al., 1993). In addition, the increase on the content of cytokinin of transformed plant leads to morphological changes if the *ipt* gene is continuously expressed. To address this problem, two approaches have been developed. One is to segregate or excise the *ipt* gene from transgenic plants (Dale and Ow, 1991; Russell et al., 1992). Recently, it has been demonstrated that marker-free transgenic plants can be obtained using the MAT vector system with the *ipt* gene deleted once shoots are produced (Ebinuma et al. 1997, 2005; Endo et al., 2001, 2002). The application of this system is strictly restricted due to its low frequency and long cultivation period. Moreover, it is difficult to distinguish transgenic plants that have lost the 35S-ipt gene from plants that are chimeric or that express very low levels of *ipt* (Kunkel et al., 1999). Another approach is to control the expression of the *ipt* gene under an inducible promoter. Kunkel et al. (1999) have shown that in the presence of dexamethasone media, transgenic calli and shoots can be produced from leaf discs. Once the inducer is removed, the transgenic plants will be morphologically or developmentally normal. However, dexamethasone addition sometimes caused growth defects and induction of specific defenserelated gene expression (Kang et al., 1999; Mori et al., 2001; Ouwerkerk et al., 2001), and the inducers (steroidal compounds) are expansive, unsuitable for field applications. The ethanol-inducible system is one of the most promising systems for both laboratory and field use because its inducer, ethanol, is a simple biodegradable organic molecule and is also safe for the ecosystem (Caddick et al., 1998). The ethanol-inducible system has been demonstrated to be successfully effective in a wide range of model and crop plants (Caddick et al., 1998;

Roslan et al., 2001; Deveaux et al., 2003; Schaarschmidt et al., 2004; Garoosi et al., 2005). Michael et al. (1998) reported that the concentrations of ethanol required is 0.1% for seedlings in hydroponics and 5% for leaf spraying of mature plants to achieve maximal induction and there were no visible signs of damage after any ethanol treatment. In this research, we used 0.1% ethanol to induce the *ipt* gene expression for 14 days. After two weeks culture on MSII medium, calli and shoots produced from explants transformed with *AlcA::ipt* gene and *35S::ipt* gene. One month later, shoots produced from explants transformed with *35S::ipt* gene expressed shoot characteristics typical of constitutive cytokinin expression and could be distinguished easily from normal shoots produced through tissue culture. Kunkel et al. (1999) reports only very few exceptions (<2%); the morphology of the transgenic tobacco plants appeared normal, but in our experiment, more than 17% shoots transformed with *AlcA::ipt* gene displayed a range of growth habits from plants that appeared similar to the wild-type. The transformation efficiency of explants infected with *Agrobacterium* harboring *35S::ipt* gene is higher than the explants infected with *Agrobacterium* harboring *AlcA::ipt* gene. The transformation decrease might probably be caused by the unsuitable inducer concentration or inducing time. But for using *ipt* gene as selectable marker genes, 220% transformation efficiency is high enough for plant transformation. All of the transgenic *AlcA::ipt* tobacco shoots can produce a strong root system within 20 days of transfer to root-inducing media, but in Kunkel's research, over 40% of the transgenic tobacco shoots developed a strong root system within 20 days of transfer to root-inducing media without Dex. This might be that the plant materials grown for 40 days under inductive (10 mM Dex) are too long. The transgenic *AlcA::ipt* tobacco plants were transferred easily to soil and they developed normal leaves, flowers, and seeds. For some wood plant which is difficult to regenerate, this inducible system is a compatible transformation to induce shoots. Thus, this approach can be used to address the problems associated with the continuous expression of the *ipt* gene when is used for plant transformation and eliminated potential risks associated with the use of antibiotic-resistance or herbicide-resistance selection marker genes.

Our results demonstrated that if the explants transformed with *AlcA:ipt* gene were cultured on the MSII medium for 14 days continuously, calli and shoots can be produced from the leaf explants; and the undesirable ipt effect can be eliminated after the initiation of shoots. Therefore, the inducible *ipt* system can be used as a very powerful tool to enhance transformation efficiencies in many plant species to take the place of antibiotic or herbicide marker genes in plant transformation. Using *ipt* gene as a selection marker gene under control by ethanol-inducible promoter, no antibiotic-resistance or herbicide-resistance genes are needed so that potential risks associated with the use of these traditional selection marker genes can be eliminated.

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