EFFECTS OF ACETOSYRINGONE, PH AND CONCENTRATION OF *AGROBACTERIUM TUMEFACIENS* ON PUTATIVE TRANSIENT GUS GENE EXPRESSION IN *POPULUS*

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Abstract. -- Efficiency of transient GUS gene transfer mediated by *Agrobacterium tumefaciens* has been studied on *Populus* hybrid NC-5331 leaf explants. Included in this study were three factors which may affect the gene transfer efficiency: concentrations of acetosyringone (0 to 100 µM), dilution of the bacterium (25 and 50 times) and pH (5.5 to 6.4). All three factors were very important to achieve high efficiency gene transformation in the poplar. Interactions among the factors obviously existed. However, several tendencies were evident: 1) exogenous acetosyringone did not always enhance the gene transformation frequency, which was dependent on its concentration and the other factors; 2) acetosyringone preferred higher pH for higher transformation efficiency; 3) the most beneficial range of acetosyringone was between 25 and 75 µM, depending on the other factors; 4) dilution of the bacteria (overnight culture) 50 times, in most cases, resulted in higher transfer rate than the 25 times under the same conditions.

Key words: acetosyringone, *Agrobacterium tumefaciens,* gene transformation efficiency, GUS, pH, *Populus.*

INTRODUCTION

The soil bacterium *Agrobacterium tumefaciens* has the ability to transfer, insert and express a particular segment of DNA in the cell genome on all tested dicotyledonous and some monocotyledonous plants due to a tumor-inducing Ti plasmid. The segment of the Ti plasmid DNA is called transferred or T-DNA. The transfer of T-DNA is dependent upon the *vir* or virulence region of Ti plasmid as well as genes on the bacteria chromosomes (Hille *et al.*, 1984; Douglas *et al.*, 1985). The induction of transcription of the *vir* region is mediated by signal molecules such as acetosyringone (AS, 3',5'-dimethoxy 4'-hydroxyacetophenone), a phenolic compound (Bolton *et a!.*, 1986; Ashby *et al.*, 1988). However, an inhibiting effect by AS on the growth of certain strains of A. *tumefaciens* has been noticed. Further studies indicated that inhibition by AS was accompanied by the accumulation of avirulent mutants (Fortin *et al.*, 1992).

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Researchers have reported on gene transformation in several poplar *(Populus sp.)* species (Parsons *et al.,* 1986; Fillatti *et al.,* 1987; De Block, 1990; Wilde *et al.,* 1992). The highest frequency of *Agrobacterium-mediated* gene transfer approximately 40% has been achieved on a poplar clone (De Block, 1990). In this experiment, three factors, AS, pH and the bacteria concentration were investigated in an attempt to achieve higher gene transfer efficiency in a hybrid poplar.

MATERIALS AND METHODS

Bacteria strain and its preparation

The A. *tumefaciens* strain ASE-9749 with binary vector plasmid (pMON9749, obtained from Monsanto Co., St. Louis, MO) was used in this study. This plasmid carries an intact *vir* region and four chimeric genes, including three antibiotic (kanamycin, spectinomycin, and chloramphenicol) resistant genes and a ß-glucuronidase (GUS) gene. The strain, stored at -20 °C, was streaked on solidified LB medium supplemented with 50 mg/1 kanamycin, 75 mg/1 spectinomycin and 25 mg/1 chloramphenicol for 2 days at 28 °C, and then grown overnight in LB liquid medium on "Roto-torque" at 100 rpm. The bacteria concentration was determined at OD_{600}. Before inoculation, the liquid culture was diluted 25 or 50 times with LB liquid medium supplemented with 0 to 100 µM AS. The pH of LB liquid culture media was adjusted to **5.5, 5.6,** 5.8, 6.1 or not adjusted (pH 6.4) in different experiments. Then the bacteria solutions were incubated for 3 hr at 28 °C before mixed with leaf segments.

Plant materials and putative gene transformation

The *in vitro* grown hybrid poplar line NC5331 *(Populus nigra* L. *var. betulifolia* Ton X *P. trichocarpa* Ton and Gray) was used as plant material. Leaf segments were mixed with bacteria liquid culture for 1 to 2 min and then placed on a co-culture solid medium (MS supplemented with 0.2 mg/1 kinetin, 0.5 mg/1 2,4-D, 20 g/1 sucrose and 6 g/1 agar; pH was 5.5, 5.6, 5.8 or 6.1 depending on experiments and treatments). After 2 days of co-cultivation at 25°C in darkness, the leaf segments were transferred to the selection medium (co-culture medium supplemented with 60 mg/1 kanamycin, 100 mg/I cefotaxime and 200 mg/1 carbenicillin; pH 5.8). Petri dishes were para-film sealed and incubated at 25°C in darkness.

The histochemical procedure of GUS activity was performed according to Jefferson *et al.* (1987). The calli or survived leaf segments were assayed with X-gluc (5-bromo-4-chloro-3-indolyl-ß-glucuronide) in 6 weeks. The small pieces of calli about 50 mg or whole leaf segments were mixed with 100 µl X-gluc at 37°C for overnight. The blue color indicated the putative GUS gene expression. The number of transformed explants was determined based upon at least one visible blue spot on each leaf segment or callus under a dissecting microscope. The percentage of transformed from total explants was calculated as the putative gene transformation efficiency.

Experimental Design

Three experiments were conducted in this study. In the first experiment, the influences of AS concentrations and dilution times of the bacteria solution were investigated. The pH of the

bacteria solution and the co-culture medium were 6.4 and 5.8, respectively (Table 1). In the second experiment, only the dilution at 50 times was used. The effects of AS concentrations and pH of the bacteria solution on putative gene transfer efficiency were determined. In the last experiment, possible interactions among AS concentrations, bacteria concentration and the pH of bacteria solution and co-culture medium were investigated. The pH of the bacteria solution and co-culture medium were adjusted to the same level in each treatment (Table 1).

In each treatment, three petri dishes were used; each contained 15 to 21 leaf segments (Table 1). The pH of the bacteria solution containing AS and that of the co-culture media were adjusted as designed. After the solution was cultured overnight, its OD_{600} value was about 0.45. It was diluted 25 or 50 times dependent on the experiments. The AS was filter-sterilized to the bacteria solution.

RESULTS

Experiment 1

Addition of AS promoted the putative GUS gene transformation with bacteria solution diluted 50 times but inhibited transformation with the bacteria dilution of 25 times (Table 1). However, the putative gene transformation efficiency gradually decreased with the increasing of AS concentration regardless of the bacteria concentration. Without the supplement with AS, the dilution of 25 times achieved 22.2% gene transfer frequency while the dilution of 50 times did not achieve transformation (Table 1). The enhanced putative gene transformation frequency with the assistance of AS at the dilution of 50 times was achieved without reducing pH of the bacteria solution or co-culture media.

Experiment 2

Only the bacteria dilution of 50 times was used in this study, and the pH of that solution was adjusted while the pH of co-culture medium was maintained at 5.8. Under these conditions, the impact of pH was clear in that the addition of AS only slightly increased the putative transformation efficiency when the pH of bacteria solution was adjusted to 5.6 while it significantly improved the putative transformation at pH 6.4 (Table 1). Higher AS concentration did not enhance or decreased the putative transformation efficiency at pH 5.6 or 6.4, respectively. Addition of AS combined with higher pH could promote putative *Agrobacterium-mediated* gene transfer compared with no supplement with AS. Also this experiment confirmed that putative gene transfer efficiency could be increased with the assistance of AS when the bacteria solution was diluted 50 times and its pH was at 6.4.

Experiment 3

Surprisingly, the highest putative transformation frequency of 82% was achieved at pH 5.8 with the bacteria dilution of 50 times and without AS addition (Table 1). Regardless of the bacteria concentration, higher concentration of AS slightly increased the putative gene transformation efficiency at pH 5.5. At pH 5.8, the different bacteria dilutions produced completely different outcomes in that the 50 times dilution resulted in significantly higher transfer

rate at AS 0 and 25 μM than the solution diluted 25 times. At pH 6.1, addition of AS decreased putative transformation rate under the dilution of 25 times compared with no AS treatment, but putative transformation increased with increasing the AS concentration. The addition of AS at 25 to 75 μM significantly enhanced transfer efficiency with the bacteria dilution of 50 times at pH 6.1 compared with no addition of AS. This confirmed the results in experiment 1 that addition of AS could increase the putative transformation efficiency at the dilution of 50 times at higher pH. The low transformation efficiency achieved at pH 5.5 was similar to the results at pH 5.6 in the experiment 2.

DISCUSSION

AS is an inducer of the virulence region, which can mediate the T-DNA transfer. In nature, wounded plant cells contain defined signal molecules, such as AS (Stachel *et al.,* 1986). Thus, addition of AS should enhance gene transfer efficiency. This has been demonstrated in some species (Sheikholeslam and Weeks, 1987; Owens and Smigocki, 1988; Godwin *et a!.,* 1991). Our results showed that AS, in most cases, did enhanced the putative gene transformation efficiency but the extent of influence was dependent upon its concentration, the pH and the bacteria concentration. An AS level up to 200 μM is not considered to be significantly toxic to *Agrobacterium* cells (Stachel *et al.,* 1985). However, in our investigation, more than 75 μM AS rarely promoted the gene transfer. Similar results have been reported on carrot (Guivarc'h *et al.,* 1993). It was not surprising that the addition of AS did not always raise the transformation frequency because wounding of tobacco cells is known to induce more than a 10-fold increase of AS in cell exudate (Stachel *et al.,* 1985). We do not know exactly how high the concentration of AS was that explants were exposed to. The non-effect with AS addition has also been demonstrated on other species (Godwin *et al.,* 1991). In addition, decreased transformation efficiency by AS may be associated with the accumulation of avirulent mutants as indicated by Fortin *et al.* (1992).

The AS-mediated *vir* gene induction increases with the decreasing of pH from 6.2 to 5.1 (Stachel *et al.,* 1986). The optimal induction of *vir* gene is attained when pH is lower (Stachel *et al.,* 1986) than those commonly used in plant tissue culture medium (pH 5.8 to 6.0). However, Godwin *et al.* (1991) indicated that AS-assisted gene transfer frequency was higher at pH 5.5 to 5.8 than at pH 5.2. They suggested that actual pH around explants was depressed by leakage of cell contents into the medium, and hence the optimal pH was reached on the less acidic media. In our work, supplement with AS at lower pH only slightly increased the gene transfer efficiency. Higher pH from 5.8 to 6.4 was preferred by AS to promote the gene transformation. The gene transfer with AS at pH higher than 6.1 was rarely reported. Based on our study including three experiments, AS could enhance the gene transfer at higher pH from 5.8 to 6.1 or even 6.4. This may be due to the interaction between plant and bacteria which optimized the microenvironment. Without addition of AS, pH was also important to gain a higher gene transfer frequency. Over 20% putative transformation efficiency was achieved only at pH higher than 5.8 depending on the concentration of bacteria solution.

The impact of the bacteria concentration on gene transfer has rarely been reported. Based on the experiments 1 and 3, the bacteria concentration was critical to attain a higher efficiency of putative GUS gene transformation. This may be attributed to the negative effects of higher

bacteria concentration on the growth of explants and the pH change induced by the growth of bacteria.

Overall, from the three experiments, several common tendencies were evident. First, exogenous AS did not always enhance the gene transfer, e.g. with the dilution of 25 times in experiment 1, with the dilution of 25 times at pH 6.1 in the experiment 3, and with the dilution of 50 times at pH 5.6 in experiment 3. The bacteria concentration and pH both were important to affect the *Agrobacterium-mediated* transformation by addition of AS. Second, AS, in most cases, preferred medium to high pH (5.8 to 6.4) to raise the putative gene transfer efficiency. With reducing pH even only in the bacterium solution, the gene transfer efficiency decreased under the same conditions, e.g. in the experiment 2 and experiment 3. Third, the bacteria solution diluted 50 times usually produced higher putative gene transfer frequency than that diluted 25 times under the same conditions, especially with the addition of AS. Fourth, the most beneficial range of AS was between 25 and 75 μM, but was dependent on the other factors, especially the bacteria concentrations.

Southern blotting will be tested in the future to provide further evidence that these putative GUS gene expressions are true. The putatively transformed calli had potential to be regenerated. The stable gene transformation efficiency will be determined.

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Table 1. Effects of acetosyringone, pH and concentration of A. *tumefaciens* on the putative GUS gene transformation efficiency in hybrid poplar NC-5331.

AS (P^M)	pH of BS'	pH of CM2	*A. tumefaciens* dilution times	Transformed' explants/total	Transformation efficiency %
Experiment 1					
0	6.4	5.8	25	10/45	22.2
25	6.4	5.8	25	2/45	4.4
50	6.4	5.8	25	2/45	4.4
75	6.4	5.8	25	0/45	0
100	6.4	5.8	25	0/45	0
0	6.4	5.8	50	0/45	0
25	6.4	5.8	50	23/45	51.1
50	6.4	5.8	50	13/45	28.9
75	6.4	5.8	50	6/45	13.3
100	6.4	5.8	50	2/45	4.4
Experiment 2					
0	5.6	5.8	50	3/45	6.7
30	5.6	5.8	50	5/45	11.1
60	5.6	5.8	50	5/45	11.1
0	6.4	5.8	50	1/45	2.2
30	6.4	5.8	50	35/45	77.8
60	6.4	5.8	50	15/45	33.3
Experiment 3					
0	6.1	6.1	25	12/52	23.1
25	6.1	6.1	25	2/57	3.5
50	6.1	6.1	25	7/52	13.5
75	6.1	6.1	25	8/50	16.0
0	6.1	6.1	50	0/52	0
25	6.1	6.1	50	32/49	65.3
50	6.1	6.1	50	23/54	42.6
75	6.1	6.1	50	31/55	56.4
0	5.8	5.8	25	0/58	0
25	5.8	5.8	25	3/54	5.6
50	5.8	5.8	25	4/63	6.3
75	5.8	5.8	25	0/57	0
0	5.8	5.8	50	41/50	82.0
25	5.8	5.8	50	19/47	40.4
50	5.8	5.8	50	3/57	5.3
75	5.8	5.8	50	3/47	6.4
0	5.5	5.5	25	0/59	0
25	5.5	5.5	25	1/54	1.9
50	5.5	5.5	25	4/52	7.7
75	5.5	5.5	25	4/45	8.9
0	5.5	5.5	50	0/50	0
25	5.5	5.5	50	2/52	3.8
50	5.5	5.5	50	1/54	1.9
75	5.5	5.5	50	3/45	6.7

[1] represents bacteria solution.
[2] represents co-culture medium.
[3] at least one blue spot showed in each survived leaf segment or callus.