Plant regeneration and *Agrobacterium*-mediated transformation of Vacuolar H^+-ATPase c Subunit Gene in hybrid poplar *populus davidiana* Dode × *P. bollena* Lauche

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Abstract. An efficient regeneration and transformation system was developed for hybrid poplar *populus davidiana* Dode × *P. bollena* Lauche. Several factors, such as a competent regeneration protocol, antibiotics, bacterial concentration, infection time have been shown to affect transformation efficiency. It is the first report describing an efficient protocol for Vacuolar H^+-ATPase c Subunit Gene from salt tolerant plant *Puccinellia chinampoensis*, which transformed into *populus*. Stable transgene integration was confirmed by the expression of GFP fusion proteins in leaves, stem and root for populus.

1 Introduction

Development of an efficient, competent plant regeneration system is essential for transformation. Poplar regeneration has been reported in the past decades. Different genotype of poplar have been used to perform the regeneration experiment [1-3]. The leaf disc transformation method established by Horsch et al. [4]. The technology has been applied to various *populus* species for transformation effeiency [5-7], The systematic analysis of several factors to improve the transformation efficiency, including poplar genotype, *Agrobacterium tumefaciens* strain, bacterial concentration and so on [8-10].

Populus davidiana Dode × *P. bollena* Lauche is the hybrid cultivar of poplar that using *Populus bolleana* as male parent and *Populus davidiana* Dode as female parent, which is planted on a significant scale in northeast of China due to its characteristics as graceful shape, cold resistance, fast growth, and suitability to develop transgenic.

Puccinellia chinampoensis is a special gramineous plant, which can survive well in the saline and alkali environment with severe drought and salt stress. *Puccinellia chinampoensis* has resistance mechanism of adaptation in the saline and alkali soil. Vacuolar H^+-ATPase c Subunit Gene (VHA-c) was isolated from *Puccinellia chinampoensis*, which was used to transformation in the study. Several researchs and informations about VHA have been published including structure, function and regulation mechanism[11-14]. Vacuolar H^+-ATPase c Subunit Gene has been studied in *Mesembryanthemum crystallinum* [15], sugar beet [16], *Arabidosis thaliana*[17,18] and tobacco [19].

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In the present study, we developed a high efficient regeneration system for *Populus davidiana* Dode × *P. bollena* Lauche. In the basis of the regeneration system, a simple and efficient protocol for transformation, selection and regeneration of *popolus* was developed. Although VHA-c has been successfully used to transfer into a wide range of gramineae plant species, it has received little attention for transformation of woody plant. To our knowledge, this is the first report describing an efficient protocol for Vacuolar H^+-ATPase c Subunit Gene from salt tolerant plant *Puccinellia chinampoensis*, which transformed into *populus*. Stable transgene integration was confirmed by the PCR analysis and the expression of GFP fusion proteins.

2 Materials and methods

2.1 Plant material, *Agrobacterium* strain and plasmid

Populus davidiana Dode × *P. bollena* Lauche leaf explants were obtained from shoot cultures. The *Agrobacterium tumefaciens* EHA105, containing the plasmid pBI121-VHA-c-GFP. This plasmid driven by the CaMV 35S promoter.

2.2 Regeneration system

MS medium was supplemented with 6-BA and NAA such as Table 1. The leaves were cut into 0.5*0.5 cm^2 blade and were cultured in flasks. They were transferred to fresh regeneration medium at 10-day intervals.

Regenerated shoots, 3–5 cm in length were excised and rooted on MS medium containing various concentrations NAA (0.1, 0.2, 0.3 or 0.4 mg/L). All the media included 3% (w/v) sucrose and 0.8% (w/v) agar as gelling agent. The pH value of the media was adjusted to 5.8 after adding the hormone and autoclaved at 121°C for 20 min. AS and all antibiotics such as Kan and Cef were filter-sterilized and added to media cooled to 50–60°C after autoclaving.

After 1-2 months the root was lignified, and the well-developed plantlets were taken out from media. They were washed under gentle stream of running tap water to remove the adhering media, and then transferred to plastic cups containing a mixture of sterile compost. The plastic cups were covered with transparent polyethylene bags to maintain the internal humidity and placed in growth room. Plantlets in plastic cups were watered every 2 days with regular water and acclimatized. After 2 weeks in the culture room, opening the plastic bags progressively until the plants were ready for transfer to the greenhouse.

Regeneration experiments were designed with 10 Petri dishes per treatment, each dish contained at least 6 leaves for shoot regeneration and 3 shoots for the rooting of shoots. Experiments were replicated three times. These cultures were incubated at 25±2°C temperature under 16 h photoperiod and were observed for regeneration.

2.3 Assessing antibiotics for transgenic shoot selection and bacterial elimination

For the experiments testing Kan as a selective agent, untransformed leaf explants were cultured on induction media containing 0, 5, 10, 20, 30 or 40 mg /L Kan, aiming to identify the most suitable concentration for selection of transgenic plants. The experiment was carried out with 30 explants per treatment and replicated three times. Growth and differentiation were observed for 1 month.

Sensitivity of rooting of shoots to Kan was determined by culturing the elongated adventitious shoots (2–3 cm) on the rooting medium with 0, 5, 10, 20, 30, and 40 mg/L Kan for 1 months. The experiment was carried out with 30 shoots per treatment and replicated three times. Rooting was observed over 1 month.

For the experiments testing Cef as a bacterial elimination, untransformed leaf explants were cultured on induction media containing 0, 100, 200, 300, 400, 500, 600, 700, 800 mg /L Cef, aiming

to identify the most suitable concentration for control bacterial growth and the plant growth well. The experiment was carried out with 30 shoots per treatment and replicated three times. Growth and differentiation were observed over two-month period.

2.4 *Agrobacterium* concentration and infection time

A single colony was inoculated into 3 mL of liquid YEP medium with 100 mg/L Rif and 50mg/L Kan, incubated at 28°C for 24 h with constant agitation (175 rpm). An additional 30 mL YEP was then added and the culture was grown overnight at the same culture conditions upto OD_{600} =1.2. The cells were then centrifuged at 5,000 rpm for 5 min and the pellet was diluted to OD_{600} = 0.5, 0.8, 1.0, 1.2 for transformation. The leaf explants were incubated in the bacterial suspension with different times such as 10min, 20min, 30min, 40min.

2.5 Transformation and plant regeneration

The bacterial suspension containing 100μmol/L AS and 0.1% TritonX-100 was poured into petri dish. Leaves were cut into 0.5 cm*0.5 cm blade in the bacterial suspension of petri dish, and inoculated for different time in the bacterial suspension . Leaf explants were then dry on sterile filter paper and cultured in the dark on the appropriate tissue culture medium without Kan and Cef. After 48h co-cultivation, leaf explants were washed 3 times (20-30 min each) in liquid co-cultivation medium plus 200 mg/L Cef, and then blotted dry on sterile filter paper. The explants were transferred to regeneration medium containing Kan and Cef for selection and cultured in the light at 25±2°C for 4-6 weeks. To inhibit *Agrobacterium* overgrowth, cultures were transferred to fresh selective medium at 15-day intervals until the leaves differentiation. Shoots were transferred to rooting medium containing Kan and Cef after the transformation leaves differentiation shoots . Approximately 100 leaf explants were used for each transformation experiment and replicated three times. Transformation efficiency were calculated from the number of genetically independent transgenic polar plants divided by the number of explants that were infected as to optimize parameters affecting poplar transformation.

2.6 DNA extraction and PCR analysis

After selection, leaves from regenerated transgenic shoots were harvested for PCR analysis. Genomic DNA was extracted from transformed and non-transformed leaves according to the method of CTAB. The primers 5 - ATGTCGTCGGTGTTCAGCG -3 and 5 - ATCTGCGCGGGATTGGCCG -3 that amplifies a 498-bp fragment corresponding to the coding region of VHA-c gene were used. Reactions were performed in a 20 μL volume containing 1 μL (100–500 ng) of genomic DNA, 1μL (20 μmol/L) of each primer, 10 units GoTaq DNA polymerase. Reactions were first heated at 94°C for 5 min followed by 30 cycles of 94°C for 30 s , 58°C for 30s , 72°C for 1 min, and followed by a final extension at 72°C for 10 min. Amplified DNA was detected under UV light, after electrophoresis of the amplification reaction mixture for each sample in 1% (w/v) agarose gels and staining with ethidium bromide.

2.7 Expression of GFP fusion proteins in populus of tissue culture

The leaf, shoot and root were taken away from *populus* of tissue culture and observed in confocal microscopy. Confocal microscopy was performed with a TCS SP2 laser-scanning confocal imaging system (Leica). GFP fluorescent signals were detected with excitations at 488 nm and 543 nm, respectively.

3 Results and Discussion

3.1 Effects of media composition on plant regeneration

The adventitious shoot induction is influenced by the growth regulators concentration and combination. After 10 days of culture, the leaves were curled and expanded. Shoot buds differentiated directly from both the cut blades of leaves explants on some growth regulators concentrations and it took 15–20 days for bud differentiation. All combinations of different concentrations of cytokinins (BA) and auxins (NAA) induced direct shoot buds from leaves explants, especially in the main vein of leaves blades. A combination of BA (0.1 mg/L) and NAA (0.01mg/L) regenerated minimum leaf explants differention frequency (0%) (Table 1). The combination of BA (0.1 mg/L) , NAA (0.05, 0.08, 0.1 mg/L) and BA (0.5 mg/L) , NAA (0.01, 0.05, 0.08, 0.1 mg/L) also regenerated lower shoots than others. BA has vital effect on shoots differentiation, lower or higher concentration lead to the lower percent explant regeneration directly. A combination of BA (0.3 mg/L) and NAA (0.08 mg/L) regenerated 91% shoots. (Table1, Figure 2ABC).

Table1. Effect of different combinations and concentrations of cytokinins (BA) and auxins (NAA) in MS medium on shoot regeneration from leaf explants of *populus davidiana* Dode × *P. bollena* Lauche

Medium	BA(mg/L)	NAA(mg/L)	Per cent explant regeneration(%)
1	0.1	0.01	0
2	0.1	0.05	15.8±1.37c
3	0.1	0.08	6.56±1.37c
4	0.1	0.1	3.12±1.62c
5	0.3	0.01	78.7±6.47ab
6	0.3	0.05	18.6±4.17c
7	0.3	0.08	91.0±7.98a
8	0.3	0.1	58.6±3.09b
9	0.5	0.01	17.4±2.85c
10	0.5	0.05	41.0±10.8bc
11	0.5	0.08	3.83±3.54c
12	0.5	0.1	6.56±2.85c

a Denotes highly significant value

Rooting experiment results showed that formation and growth of the new roots were promoted by NAA at any concentrations that have been setting, the percents of root regeneration were 100%. All the tissue culture derived shoots (2–4 cm in length) developed roots within 10–15 days of MS medium supplemented with various concentrations of NAA. The growth of roots was rapid within 30 days. Of the two concentrations of NAA (0.1 mg/L , 0.2 mg/L) evaluated in the present study, the root of growth and development status were fine root , the number of the roots reduces and the root of length shorter. The status of the rooting were the length was smaller, the number of the roots reduces and thick root at the concentrations of NAA (0.3 mg/L, 0.4mg/L). NAA at 0.3 mg/L was the best for rooting as the growth status of roots were length and polyrhizal (Figure 2D).

Plantlets were taken out of the culture tubes and hardened after two months when the root induction. 90% of plants survived after the hardening process(Figure2 EFGK).

3.2 Assessing antibiotics for transgenic explants selection and bacterial elimination

At 20 mg/L Kan, leaves chlorosis were observed in all explants by the end of the 2 months. Concentrations above 30 mg/L Kan completely inhibited regeneration. At 30 mg/L Kan, non-rooting were observed in all explants by the end of the 2 months. Concentrations above 40 mg/L Kan completely inhibited the roots regeneration. Based on these datas, 30 mg/L Kan is the most suitable concentration for use to shoots regeneration and 40 mg /L Kan is the most suitable concentration for use to roots regeneration in genetic transformation experiments of *populus davidiana* Dode × P.

bollena Lauche(data not shown).

The percentage of shoot regeneration on culture medium containing 200 mg/L Cef did not significantly differ from that obtained on the control treatment (culture medium devoid of antibiotics). The percentage of shoot regeneration on culture medium containing 200–500 mg /L Cef reduced. Cef at 500 mg /L or more gave the worst responses, the status of having little shoots regeneration. (data not shown)

3.3 Effects of Agrobacterium concentration and infection time on plant transformation

The higher transformation frequency were observed at OD_{600} = 0.8 with little contamination(Figure1). Lower transformation frequency were obtained in lower *Agrobacterium* concentrations(OD_{600}=0.5). The same with infection time, the frequency of transformation and contamination increased by the time longer, the higher contamination frequency can lead lower frequency of transformation. The explant inoculation time were reasonable at 20-30min (Figure 1).

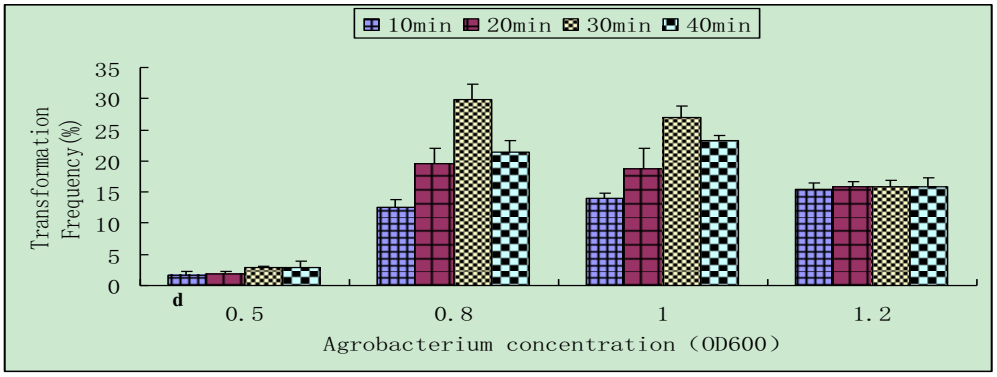

Figure1. Frequency of the transformation of *populus davidiana* Dode × *P. bollena* Lauche with different *Agrobacterium* concentration and the infection time.

Figure 2. High frequency plant regeneration, shoot induction, rooting, micropropagation and high frequency transformation of *populus davidiana* Dode × P. *bollena* Lauche. AB. Regenerated shoots from leaf explants; C. Elongation of regenerated shoots; D. Roots regenerated; E. Regenerated plantlets with medium from flask; FG. Growth of regenerated plantlets in pots after 20 d of transfer. HIJ. Transgenic shoots differentiation. K. Growth of regenerated plantlets in pots after six months of transfer.

3.4 DNA extraction PCR analysis

The successful incorporation of the transgene was proven by the genomic PCR. No positive result occurred with DNA isolated from control plants (non-transformed). Sixteen transformed plants were produced. The data of Figure 3 shown that Vacuolar H^+-ATPase c Subunit Gene has been transferred into hybrid poplar *populus davidiana* Dode × *P. bollena* Lauche.

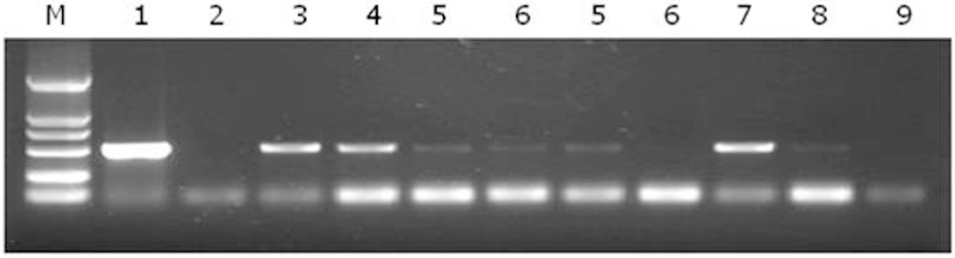

Figure 3. PCR analysis of total genomic DNA isolated from transgenic and untransformed plants by amplification of the VHA-c genes. Lane M, DL2000; Lane1, plasmid pBI121; Lane2, untransformed plant; Lanes 3–8, transformed plants; Lane 9, H_2O.

3.5 Expression of GFP fusion proteins in *populus* of tissue culture

A plant transformation vector designed to express the GFP gene was used to detect expression of GFP fusion proteins in *populus* of tissue culture. From the Figure 4, GFP fluorescence was detected in leaves, stem and root, which proved that the Vacuolar H^+-ATPase c Subunit Gene has been transferred into hybrid poplar *populus davidiana Dode* × *P. bollena* Lauche and expression exactly.

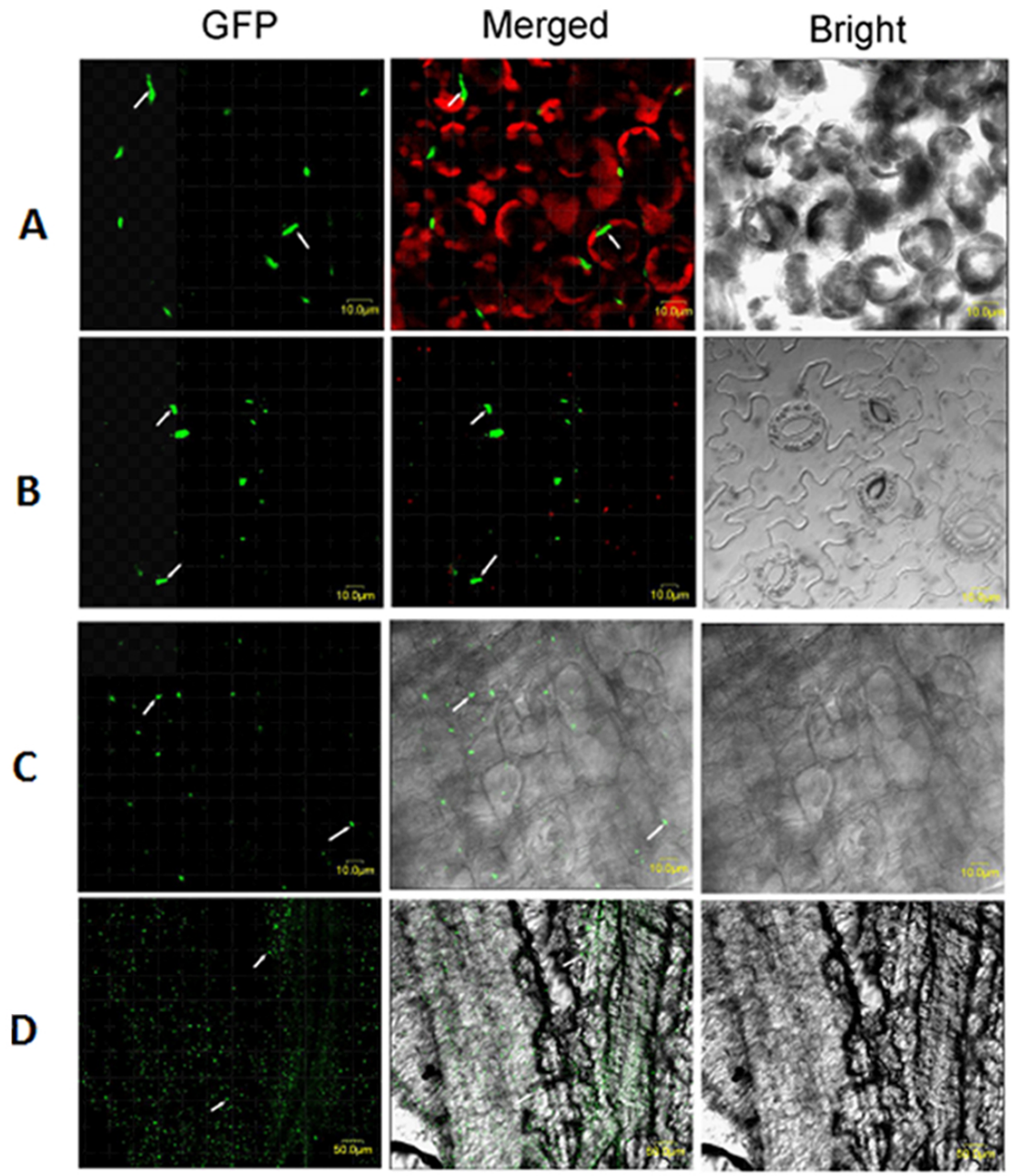

Figure 4. Expression of MD-GFP fusion protein in populous
A Expression of MD-GFP in mesophylls (white arrows). **B** Expression of MD-GFP in epidermal of leaf (white arrows). **C** Expression of MD-GFP in stem (white arrows). **D** Expression of MD-GFP in root (white arrows). Scale bar (A, B, C) 10 μm; (D) 50 μm.

4 Discussion

The regenerated adventitious shoots along with petiole explants of popular oftenly turned brown, woody plants and especially angiosperms, oftenly secrete polyphenols and tannins, which can inhibit

development of explants/organogenesis and cause necrosis, this problem can be partially overcome by frequent sub-culturing or variation in medium nutrients and hormone composition[20]. In this study, the higher concentrations of 6-BA(0.8,1.0) combination with any concentrations of NAA(0.01-0.1) lead the leaf explants turning brown(data not shown). Our data show that the appropriate hormone concentrations and ratio can overcome this problem.

Higher contamination rates can be obtained by increasing the *Agrobacterium* concentration, although transformation frequency increased with increased *Agrobacterium* concentration. It is universally acknowledged that leaf explants were washed five to six times (20-30 min each) in liquid co-cultivation medium plus 200 mg/L Cef may induce leaf explants albinic or low transformation frequency. But our date show that the method was used to remove the excess *Agrobacterium* ahering to leaf explants, which was valuable to inhibit the number of *Agrobacterium*. The result showed that lower *Agrobacterium* contamination rates had a rise in transformation.

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