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OPTIMIZATION OF HAIRY ROOT TRANSFORMATION SYSTEM FOR GENE FUNCTIONAL STUDIES IN POPLAR (*POPULUS* SPECIES)

MEI HAN[1], JUNHU FENG[1], SHUYUE SUN[1], YUJIA SI[1], XIAONING LIU[1], MINGYUE XU[1] AND TAO SU[1,2*]

[1]*Co-Innovation Center for Sustainable Forestry in Southern China, College of Life Sciences, Nanjing Forestry University, Nanjing 210037, People's Republic of China*
[2]*Key Laboratory of State Forestry Administration on Subtropical Forest Biodiversity Conservation, Nanjing Forestry University, Nanjing 210037, People's Republic of China*
[*]*Corresponding author's email: sutao@njfu.edu.cn*

Abstract

Poplars (Populus spp.) are one of the most widespread ligneous plants in the world, which have a variety of applications ranging from landscape, agriculture, and industry to research. Although *Agrobacterium tumefaciens*-mediated gene transformation method has been widely utilized for poplar genetic manipulation, many defective technique defects such as the time-consuming procedures, and the occurrence of chimeric transformant have emerged. To address these problems, a simple, fast, straightforward and effective gene transformation method by using *Agrobacterium rhizogenes* to induce hairy roots in poplars was developed, and regenerated-transgenic poplar plantlets were obtained within a period of two to three months. The optimal procedure comprised the usage of poplar apex buds as explants, infection of *A. rhizogenes* by directly pricking colonies with the cuttings on the agar plates, and improving hairy-root initiation by application of indole-3-butyric acid (IBA) in combination with hairy-root morphological identification. Moreover, the GFP marker gene was applied to track record the expression level of the introduced genes using a portable fluorescence lamp. Further quantitative real-time PCR (qRT-PCR) and the enzyme activity assay showed that in comparison with the non-transformed control, the transcript abundance and enzyme activity of our target gene (i.e., *aspartate aminotransferase*) were significantly elevated in the transformed plant lines regenerated from the hairy roots, confirming that the current *A. rhizogenes*-mediated poplar transformation method is effective and applicable for poplar genetic manipulation and functional genomic study.

Key words: Poplar, Hairy roots, *Agrobacterium rhizogenes*, Green fluorescent protein (GFP).

Introduction

Poplars (*Populus* spp.) are one of the most widespread ligneous plants across the world (Cseke *et al.*, 2007), whose cultivated area and range of application rank high among all the woody plantations (Bryant *et al.*, 2020; Castro-Rodríguez *et al.*, 2016; Weighill *et al.*, 2019). Apart from their usage in industry for pulpwood, veneer and plywood, poplars play a vital role in carbon sequestration and water and soil conservation (Langeveld *et al.*, 2012; Stanton *et al.*, 2002), and they also function as phytoremediation to remove atmospheric heavy metals and contaminations from municipal biosolids (Assad *et al.*, 2016; Pásztory *et al.*, 2019). Moreover, poplar is the first woody plant, whose entire genome has been sequenced (Jansson & Douglas, 2007; Tuskan *et al.*, 2006), and extensively used as a model tree species for basic research, in particular, for the investigation of the cellular and molecular processes, gene function analysis (Arnaud *et al.*, 2012; He *et al.*, 2021; Luo *et al.*, 2019; Zhao *et al.*, 2021), and stress resistance (Priya *et al.*, 2023; Tong *et al.*, 2022; Wu *et al.*, 2022; Yang *et al.*, 2021).

The technique of gene transformation serves as a fundamental tool for gene functional study and engineering of new traits (Busov *et al.*, 2005), including disease resistance (Wang *et al.*, 2018), as well as drought and salt tolerance in poplars (Yu *et al.*, 2017). So far there have been many methods used for variety-independent genetic transformation for plants, such as biolistic (Vainstein *et al.*, 2011), *Agrobacterium*-mediated transformation (Surya, 2020), electroporation (Miao & Jiang, 2007), microinjection (Yevtushenko & Misra, 2010) and other gene transfer methods. Among these methods, the *Agrobacterium*-mediated transgenic approach has been commonly utilized in poplar, as it is the natural host of *Agrobacterium* (Parsons *et al.*, 1986), and also because this approach is easy to set up at a low cost compared with other approaches (Ashraf *et al.*, 2012).

Generally, both genetic pathogens: *Agrobacterium tumefaciens* and *A. rhizogenes* possess the ability to horizontally transfer a variety of exogenous genes into the plant cell genome, altering the plant metabolism to introduce the formation of crown galls or hairy roots (Britton *et al.*, 2008; Fernández-Piñán *et al.*, 2019). Even though the *A. tumefaciens* has been routinely used in poplar genetic transformation, many technical drawbacks, such as the tedious work on preparation of the bacterial solution, optimization of optical density (OD) and time period for inoculation have been noticed (Song *et al.*, 2019; Yang *et al.*, 2018; Zheng *et al.*, 2021). In fact, the *A. tumefaciens*-mediated poplar transformation method is not only less certain in efficiency, but also time-consuming. Besides, the transgenic poplars developed from *A. tumefaciens*-mediated transformation are regularly chimeric, ascribed to the asexual reproduction system of the poplar transformant in which many non-identical cells but not one regenerates new plant (Daspute *et al.*, 2019). As a consequence, the stability of gene expression and heredity is less conserved. These problems confine the research in engineering poplars with desired agronomy traits by means of *A. tumefaciens*-mediated transformation in poplars (Movahedi *et al.*, 2014).

A. rhizogenes has been identified as a plant pathogen since the beginning of the 20th century. It is known to be equally competent to *A. tumefaciens* to deliver exotic genes into the plant genome (Cseke *et al.*, 2007). Compared with *A. tumefaciens*, *A. rhizogenes*-mediated transformation showed

some potential merits in terms of genetic stability, morphologically easily distinguishable phenotype and fast growth rate of the transgenic roots (Cseke *et al*., 2007). Specifically, in the *A. rhizogenes*-mediated gene transformation system, the transgenic roots and subsequently regenerated whole plants developed from a single cell, thus they are genetically stable (Britton *et al.*, 2008). Also, *A. rhizogenes* instigates the neoplastic proliferation of plant cells leading to the formation of "hairy roots" characterized by root elongation, multiplication and branching (Cheng *et al.*, 2021). Such hairy-root characteristics could be easily distinguished from the untransformed roots, as a result, the positive transgenic lines can be easily screened out by simple visual detection (Cseke *et al.*, 2007). Hence, *A. rhizogenes* has become an alternative pathogen that has been attempted to introduce exogenous genes into plant in a diverse range of plant species (Van Nguyen *et al.*, 2024).

In some earlier studies, hairy roots have been successfully produced in different variety of poplars, including *Populus davidiana* × *Populus bolleana* (Zheng *et al.*, 2021), *P. tremuloides* (Cseke *et al.*, 2007), *P. tremula* × *P. tremuloides* (Neb *et al.*, 2017) by *A. rhizogenes*-mediated transformation, however, the transformation in these studies is either less efficient, or ended up with composite plants but made no reference to the regenerated whole plants (Table 1). An unmet demand remains for more effective transformation and regeneration of hairy root-derived plants for poplar. To address this issue, in the current study, we developed a fast and simple gene transfer procedure to induce hairy roots in poplars and obtained hairy-root-regenerated poplar transformants within a period of two to three months. Our study provides a straightforward and effective means for poplar stable transformation and gene function analysis.

Material and Methods

Plants and growth conditions: Hybrid poplar 'Nanlin895' (*P. deltoides* × *P. euramericana*) clones were cultivated aseptically on the half-strength Murashige and Skoog medium (1/2 MS) plates supplemented with 20 g L^{-1} sucrose, 8 g L^{-1} agar in a growth chamber at 25°C with 16 h light and 8 h dark photoperiod, and 20 µmol m^{-2} s^{-1} light intensity. *P. tremula* × *P. tremuloides* (clone T89) and *P. alba* × *P. glandulosa* (84K) were cultured following previously established method (Neb *et al.*, 2017; Wen *et al.*, 2022). Unless otherwise indicated, explants for the transformation in this study were sourced from four-week-old plantlets.

Agrobacterium strains and vector construction: Aspartate aminotransferase (AspAT) is one of the most important transaminases in the cell. It is a pyridoxal-5'-phosphate-dependent enzyme, catalyzing the conversion of aspartate and 2-oxoglutarate to glutamate and oxaloacetate. The full-length complementary DNA (cDNA) sequence (without stop codon) of *P. trichocarpa* AspAT gene isoforms *AspAT1* (Potri.005G079200), *AspAT4* (Potri.006G241600), *AspAT5* (Potri.006G260200), *AspAT10* (Potri.006G260200) (Su *et al.*, 2019) was separately cloned into the modified pRI101 vector (SinoGene Scientific Co., Ltd, Beijing, China), driven by the cauliflower mosaic virus 35S promoter (CaMV 35S), and fused with green fluorescence protein (GFP) C-terminal tagging. Primers used for *AspATs* gene cloning were shown in Table S1. The constructed plasmids *pRI101-AspAT-GFP* were sequenced to ensure the correctness of the constructs. After sequence validation, the positive constructs in parallel with the control vectors pRI101 and pRI101-GFP were transformed into poplars, respectively.

Poplar hairy roots induction and regeneration of whole plantlets: *Agrobacterium rhizogenes* strain K599 cells harboring the above vectors were transformed into poplars. The components of the culture media have been delineated in Table 2. The *A. rhizogenes* K599 cells were grown in the Luria-Bertani (LB) solid plates. The medium is composed of tryptone (10 g), NaCl (10 g), yeast extract (5 g), agar (15 g) in a volume of 1 liter, with the addition of the antibiotic: 50 mg L^{-1} Kana and 25 mg L^{-1} Streptogramin (Strep). An isolated colony was selected and transferred to the co-culture medium, incubated at 28℃ for two to three days. After this preconditioning, poplar apical buds with two to four leaves from the *In vitro* culture were cut off and immediately used to prick the *A. rhizogenes* K599 colonies into the co-culture medium. Approximately two to three days later, the *A. rhizogenes* bacteria completely wrapped around the injured area of the explants. Then the explants were pulled out and transferred to the selection medium to induce hairy root formation. When the initiated hairy roots extended to a length of 3 cm on average, they were moved to the differentiation medium to stimulate buds' emergence. On growing above 1.5 cm, the buds derived from the hairy roots were isolated from the roots and cultivated in the shoot elongation medium until the shoots grew to 1–2 cm. After that, the shoots were relocated to the rooting medium to regenerate complete plantlets.

GFP fluorescence detection and the subcellular localization: The GFP fluorescence was detected under a confocal laser scanning microscope (Carl Zeiss, Jena, Germany). The subcellular localization of the introduced gene was determined by using the 488 nm excitation laser. In addition, the GFP signal for the whole plant was detected by a portable fluorescence lamp (LUYOR-3415, Shanghai, China). The GFP signal (emission at 505-525 nm wavelength), auto-fluorescence (emission at 655–755 nm wavelength) and corresponding bright-field images were photographed at various stages of the transformation procedure.

DNA isolation and PCR analysis: Genomic DNA for PCR analysis was isolated from the fresh roots and leaves of poplar plantlets regenerated from the transformed and non-transformed poplar plants (negative control), respectively, with a modified cetyl trimethylammonium bromide (CTAB) method adapted from a previous report (Porebski *et al.*, 1997). The extraction procedures were carried out as follows: Ground the sample into a fine powder under liquid nitrogen, transfer it to a new centrifuge tube (1.5 mL) containing 200 µL CTAB extraction buffer (preheated at 65℃) with the addition of 2 µL of RNase H and mix well; add equal volume phenol: chloroform, centrifuge 15,000 rpm at ambient temperature for 5 min, and collect the supernatant; add the same volume of chloroform; after centrifugation, dilute the supernatant to an appropriate concentration and use it as the template for further Polymerase Chain Reaction (PCR) analysis.

Table 1. Comparison of *A. tumefaciens* and *A. rhizogenes*-mediated transformation in poplars.

Populus species	Agrobacteria strain	Explants	Pre-culture duration	Infection methods	Co-culture duration	Transformation efficiency and minimal regeneration time	Reference
P. nigra L. × *P. maximowiczii*	*A. tumefaciens* MP90	Full-length petioles, internodal stem segments, and 2–3 cm^2 leaf pieces	2 d	The explants were put into *Agrobacterium* solution for 1 h	3–4 d	90–93% > 60 d	(Yevtushenko & Misra, 2010)
P. simonii × *P. nigra*	*A. tumefaciens* EHA105	Leaves	No need	Cut the midrib in the base, and then inoculated with *Agrobacterium* for 30 min	2 d	86% > 60 d	(Yang *et al.*, 2018)
P. davidiana × *P. bolleana*	*A. tumefaciens* GV3101	Transient expression leaves for 9 days	No need	Transient expression by agroinfiltration under aseptic conditions	No need	66.6% 90 d	(Zheng *et al.*, 2021)
P. nigra L. var. *italica* Koehne	*A. tumefaciens LBA4404*	Leaf, stem, petiole and root segments	No need	Explants were incubated in the diluted *A. tumefaciens* culture for 30 min	2 d	50% > 60 d	(Mohri *et al.*, 1996)
P. tremuloides Michx. clone 271	*A. tumefaciens* C58	Young leaves from cuttings	2 d	Explants were inoculated with an overnight-grown agrobacterial suspension for 2 hours on a shaker	2 d	Not applicable 90 d	(Tsai *et al.*, 1994)
P. alba ×*P. glandulosa* cv.(84K)	*A. tumefaciens* GV3101	Callus (1 cm^3)	6 d	Callus (1 cm^3) were submerged in the *Agrobacterium* suspension	2–3 d	> 50% > 60 d	(Wen *et al.*, 2022)
P. tremuloides Michx	*A. rhizogenes* ARqual	Seedlings	4–5 d	Collected *Agrobacterium* with a needle, then stabbed in the hypocotyls	7–9 d	< 5% 56 d	(Cseke *et al.*, 2007)
P. berolinensis Dippel ×*P. alba* L	*A. rhizogenes* A4	Stem segments 1–2 cm	3 d	The stem segments were put into *Agrobacterium* solution for 1h	7 d	40–60% Not applicable	(Ding, 2017)
P. tremula × *P. tremuloides* (clone T89), *P. tremula* × *alba* (INRA clone no. 717.1B4), and *P. trichocarpa* (Nisqually 1)	*A. rhizogenes* 1724, K599, 8196 and 15834	Shoot cuttings	No need	The cut surface was dipped into destinated colony and then grown on MS agar	3 d	60% Not applicable	(Neb *et al.*, 2017)

Table S1. Primers used in this study.

Name	Gene ID	Forward Primer	Reverse Primer
Primers for gene clone			
AspAT1	Potri.005G079200	GGAATTCCATATGGCTGCTTCAACTTCAA	CGGGATCCCACAGGGACGGCAGACTTGA
AspAT4	Potri.006G241600	GGAATTCCATATGAACCCAGAATTGACATCA	CGGGATCCCACAACTCGCTTAACAGCTG
AspAT5	Potri.006G260200	TCTTCACTGTTGATACATATGGCTTCAACAATGCTTTC	GCCCTTGCTCACCATGGATCCACTGACATTATGGTAAGAGTC
AspAT10	Potri.018G082500	GGAATTCATATGGAGTCTTCTTCTGTG	CGGGATCCGCCAACACGGGTAACAG
Primers for sequencing and selection			
P35	-	ACTGACGTAAGGGATGACGCAC	CGTCGCCGTCCAGCTCGACCAG
EGFP	-	ATGGTGAGCAAGGGCGAG	CTTGTACAGCTCGTCCATGC
rolB	-	ACGAAGAAGGTGCAGGCTAC	TCAACGCTTTCAACCACGAG
Kana	-	TGATATTCGGCAAGCAGGCA	CAAGATGGATTGCACGCAGG
Primers for qPCR			
AspAT5	Potri.006G260200	TGCACGTGGTATGGAGCTTT	TCGGGCAATCCTTTTCAGCT
AspAT10	Potri.018G082500	CACTGGCTTGAACACTGAACA	AAAAGATCCCCAGAAGCGGA
UBIC	Potri.006G205700	GATTACCCGGAGAAGCCACC	GTTGTGTGGTGCTGTCATCT

Quantitative real-time PCR (qRT-PCR): Total RNA for the real-time quantitative PCR (qRT-PCR) analysis was isolated by using the RNAprep pure Plant Kit (TIANGEN, China) following the instruction of the user manual. The extracted RNA (1 μg) was then reverse transcribed into cDNA by the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Japan). RT-PCR was conducted using SYBR Green Pro Taq HS premixed qPCR kit (Accurate Biology, Changsha, China) based on the user manual. The real-time quantitative PCR (qRT-PCR) analysis was implemented on ABI 7500 Real-Time PCR System (Applied Biosystems, USA). The amplification was conducted under the program as follows: initial denaturation at 95°C for 30 s, 40 cycling procedure of 5 s denaturation at 95°C, 30 s of annealing at 60°C, and 15 s of extension at 72°C (Mohammadi *et al.*, 2018). Primers used for the present study were listed in Table S1. Relative mRNA expression levels of the candidate genes were estimated by the formula $2^{-\Delta\Delta Ct}$ described previously (Livak & Schmittgen, 2001). The qRT-PCR analysis was implemented with three biological replicates.

Enzyme activity assay of AspAT: AspAT enzymatic assays were carried out according to the method described previously (Zhou *et al.*, 2009). In brief, the crude enzymes were prepared by extracting the tissues with solutions containing K^+-phosphate buffer (20 mM, pH 7.4), dithiothreitol (5 mM), antipain (10 mM), and phenylmethylsulfonyl fluorid (1 mM). The homogenate was centrifuged at 12,000 g at 4°C for 20 min. After that, the supernatant was mixed with K^+-phosphate buffer (20 mM, pH 7.4), ketoglutarate (2 mM) and aspartate (0.2 mM) and incubated at 37°C for 1 h. Then 2,4-dinitrophenylhydrazine (100 mL) and aniline-citric acid (20 μL) were added to the samples, incubated at 37°C for another 20 min. The absorbance was monitored at 520 nm by using a spectrophotometer (752Pro, Lengguang, China). One enzyme unit was arbitrarily defined as that causing an increase of 1 μmol pyruvate in 30 min at 37°C.

Results and Discussion

Establishment of an effective hairy roots induction and regeneration system: The *A. rhizogenes* K599 colonies carrying constructs of our target gene, *Aspartate aminotransferase* (*AspAT*) fused with the GFP reporter gene which is driven by the CaMV 35S promoter (Fig. 1), and the control vector with or without GFP were separately preincubated on the co-culture medium consists of the antibiotic kanamycin for two to three days.

For infection, the poplar apical buds with two to four leaves from the *In vitro* culture were cut off and immediately inoculated with the preconditioned *A. rhizogenes* K599 strains by pricking the colonies into the co-culture medium using the tip of the poplar cutting. After two to three-days incubation at 25°C with a 16 h light and 8 h dark photoperiod, the bacteria completely wrapped around the wounded sites of the cutting. The explants were then relocated to the selection medium consists of appropriate antibiotics (Table 2). Approximately ten days later, the newborn hairy roots appeared; with additional five to ten days the transformed roots over 1 cm in length were developed. The entire procedure of *A. rhizogenes*-mediated poplar gene transformation was illustrated in Fig. 2. Overall, in our study the agrobacteria colonies acclimatized to

the co-culture medium were directly used for the subsequent infection, no liquid culture was required. This not only saved time but also eliminated troublesome in the preparation of agrobacterial liquid culture. In replicate experiments, it showed that the inoculation implementation with agrobacteria on poplar bud cutting took less than one minute. The hairy roots initiated roughly ten-days post-infection (dpi).

Once emerging, the hairy roots rapidly elongated, in parallel, several lateral branches developed (Fig. 3a). At about 15 dpi, the hairy roots grew to a length of above 1 cm (Fig. 3b), and approximately three-weeks post-infection, a considerable amount of hairy roots were obtained. Compared with the wild roots (i.e., roots of the non-transformed plants), the growth rate and number of the lateral branches of the hairy roots were significantly increased (Fig. 3c). Since the hairy-root morphology (Cheng *et al.*, 2021) can be easily distinguished from the wild roots by the naked eye (Fig. 3c), this distinctive phenotypic feature was employed as a pre-selection method for the following screen of the positive transformants.

To improve hairy-root development, we applied indole-3-butyric acid (IBA) in the growth medium for poplars. The impact of IBA on hairy-root induction at different concentrations in the selection medium was estimated. Our results (Table 3) showed that without IBA supplement, the ratio of *A. rhizogenes* K599-mediated roots formation was much less (13.33%) than that of the wild roots (i.e., the untransformed control roots; set as 100%) cultivated on 1/2 MS medium. By contrast, with the supplement of 0.1-0.5 mg L^{-1} IBA, the formation rate of *A. rhizogenes* induced hairy roots was significantly increased, while negligible changes were found in the wild roots compared with the mock (0 mg L^{-1} IBA). As a whole, the induction efficiency of hairy roots increased along with the IBA concentration and peaked at 0.175 mg L^{-1} IBA, after which it fell down (Table 3). Because higher IBA concentration (≥ 0.175 mg L^{-1}) depressed subsequent root elongation, hereafter 0.1 mg L^{-1} was taken as the optimal dosage for IBA application.

Further temporal comparison of root growth revealed that the average time required for roots appearing upon *A. rhizogenes* infection was approximately 10 days, while for the wild type (WT) control was about 5 - 6 days (Table 4). Nevertheless, it took about 2 - 3 weeks for the *A. rhizogenes*-induced hairy roots to develop maximum number of primary roots, while one week for that of the WT. On average, every explant generated 3 - 4 hairy roots. Insignificant difference in the amount of hairy roots was detected among various transgenic explants.

To estimate the efficacy of the current *A. rhizogenes*-mediated poplar gene transformation method, we took the advantage of the GFP fluorescence detection system to track record the transgenic hairy roots by applying a dual-wavelength excitation light source for fluorescent protein (LUYOR-3415RG). The GFP signal (emission at 505–525 nm), and the autofluorescence (emission at 655-755 nm) of the hairy roots, the hairy-root-regenerated shoots as well as the hairy-root-regenerated complete plants were captured at different dpi (Fig. 4). By LUYOR-3415RG detection, the composite plants which have wild-type buds and transgenic hairy roots grown in the selection medium showed strong green fluorescence in the transformed root part but auto-fluorescence red color in the shoot part (Fig. 4a). The positive hairy roots (confirmed by GFP signal detection) (Fig. 4b) were then transferred to the differentiation medium to induce root suckers/adventitious buds (Fig. 4c), followed by the shoot elongation medium to increase their sizes and numbers (Fig. 4d). After that, the root suckers/adventitious buds were transferred to the rooting medium (Fig. 4e) to regenerate independent transgenic plants (Fig. 4f).

Table 2. Constituent of the culture media used for *A. rhizogenes* K599-mediated transformation of poplar 'Nanlin895'.

Culture medium	Basal Medium	Sucrose (mg L^{-1})	IBA[a] (mg L^{-1})	6-BA[b] (mg L^{-1})	TDZ[c] (mg L^{-1})	Kanamycin (mg L^{-1})	Timentin (mg L^{-1})
Co-culture Medium	1/2 MS	-	-	-	-	-	-
Selection Medium	1/2 MS	-	0.1	-	-	10	200
Differentiation Medium	MS	20	-	0.2	0.01	10	200
Shoot Elongation Medium	MS	20	-	0.2	0.001	10	100
Rooting Medium	1/4 MS	20	-	-	-	10	50

[a] IBA, Indole-3-butyric acid; [b] 6-BA, 6-Benzylaminopurine; [c] TDZ, Thidiazuron

Table 3. Effects of IBA concentrations on the rooting efficiency of poplar explants in the selection medium.

IBA Concentration (mg L^{-1})	Frequency of explants producing roots (%)	
	WT untransformed root	K599 infection root
0	100	13.33 ± 5.77 e
0.1	100	83.33 ± 5.77 b
0.175	100	96.67 ± 5.77 a
0.25	100	70 ± 10 c
0.5	100	46.67 ± 5.77 d

Each treatment contains ten poplar explants. Three biological replicates were performed. Different letters within the same column denote significance of differences based on the Duncan test at $p<0.05$

Table 4. Frequency of explants producing transformed roots and untransformed roots.

Days after infection (dpi)	Frequency of explants producing roots (%)	
	WT untransformed root	K599 infection root
0	0 d	0 d
5	0 d	0 d
6	80 ± 10 c	0 d
7	87 ± 5.77 b	0 d
8	97 ± 5.77 a	0 d
9	100 a	33.33 ± 5.77 c
10	100 a	90 ± 10 b
15	100 a	100 a

Time required for root formation from the explants with (n = 40) or without (n = 20) *A. rhizogenes* K599 infection in the selection medium with addition of 0.1 mg L^{-1} IBA. The percentage of the cuttings with at least one root explant^{-1} at a designated time point was shown. Different letters within the same column denote significance of differences based on the Duncan test at $p<0.05$

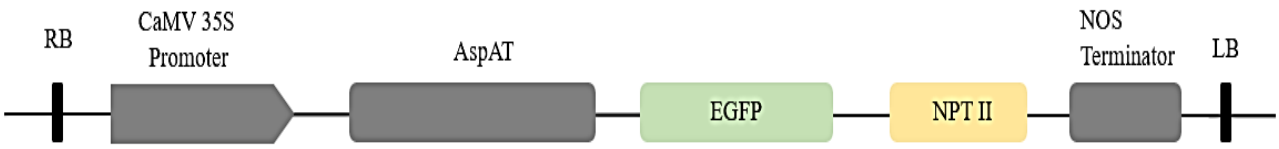

Fig. 1. Schematic illustration of the transgenic gene cassettes cloned into the plant binary vector pRI101. RB, right border; CaMV 35S Promoter, 35S promoter from cauliflower mosaic virus (CaMV); EGFP, the enhanced green fluorescent protein. NPT II, the neomycin phosphotransferase gene for kanamycin resistance; NOS Terminator, NOS terminator from *Rhizobium radiobacter*; LB, left border.

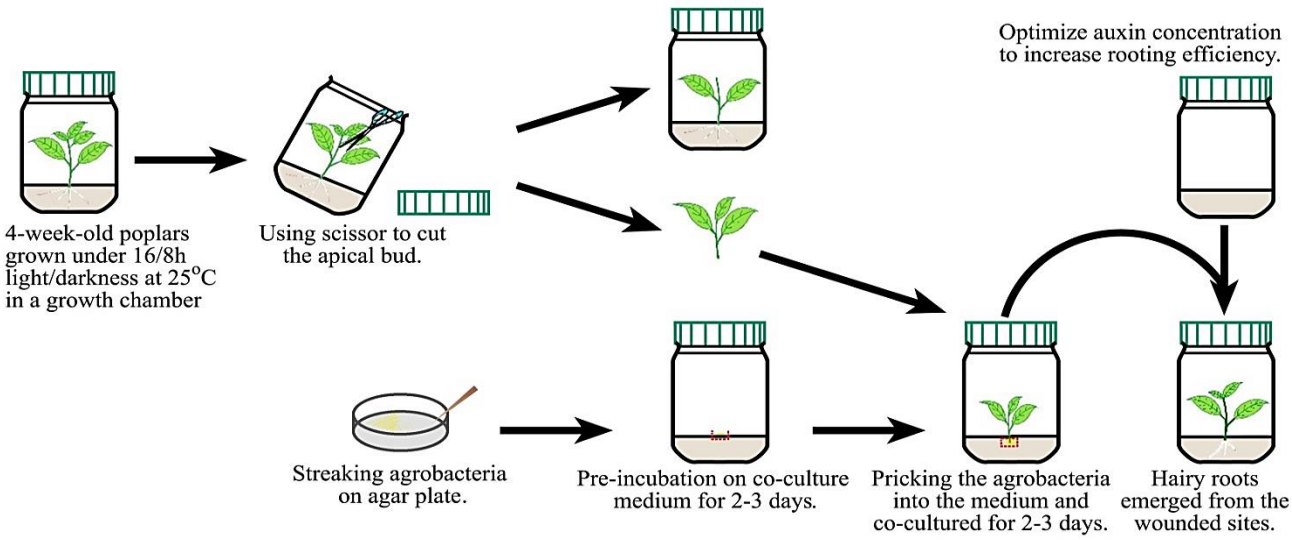

Fig. 2. Workflow of the hairy root gene transformation method by means of *A. rhizogenes* for poplars using apical buds as explants. Dashed boxes indicate the location of the colonies. The four-week-old poplar apical buds were excised using scissors and subsequently placed onto the co-culture medium, followed by two to three-days incubation, the apical buds were relocated to the selection medium containing auxin and kanamycin to develop hairy roots.

Further pairwise comparisons between the transgenic lines and the untransformed control under both bright filed and GFP channels were also performed to check the stability and homogeneity of the overexpressors (Fig. 5). Upon closer inspection, the root suckers/adventitious buds derived from the transformed hairy roots showed strong green fluorescence under the portable fluorescence lamp, whereas their untransformed counterpart control plants were red (Fig. 5a, GFP channel). In the meantime, no significant differences in images between the hairy-root-regenerated lines and the control plants were found under the corresponding bright field (Fig. 5a; BF). Moreover, the GFP fluorescence of the transgenic plant lines was universally present in all the parts of the plant, indicating that transgenic chimera events haven't occurred under the current transformation conditions. To further investigate the stability of the genes in the transformed lines, we continued to track-recording the GFP signals in the rooted plantlets (Fig. 5b) and the regenerated transgenic plants (Fig. 5c). Once again, the GFP fluorescence signals and the auto-fluorescence red colors were observed for the transgenic plant lines and the untransformed control, respectively. Although the GFP signals in the individually rooted plantlet and whole seedling were not as strong as their progenitor due to the chlorophyll interference (Chin *et al.*, 2018), our results demonstrated that the foreign genes introduced by the *A. rhizogenes* were stably expressed in all parts of the transgenic lines examined.

Table 5. Evaluation of the generation efficiency of stably transformed plants.

Target Gene	Number of explants	Hairy root induction rate (%)[a]	Transgenic plants regeneration rate (%)[b]	Co-integration rate (%)[c]
AspAT1	10	100 a	90 b	90 b
AspAT4	10	100 a	90 b	90 b
AspAT5	10	90 b	80 c	88.89 c
AspAT10	10	100 a	100 a	100 a
Average	10	97.5 ± 5	90	92.22 ± 5.21

[a] Induction rate of hairy root (%) = the number of explants developing hairy roots (as revealed by the presence of *rob B* gene via PCR analysis)/the total number of explants examined.
[b] Transgenic plant regeneration rate = the number of lines displaying GFP fluorescence signal/the total number of explants tested.
[c] Co-integration rate = transgenic plants regeneration rate/hairy root induction rate. Different letters within the same column denote significance of differences based on the Duncan test at $p<0.05$. Three biological replicates were performed.

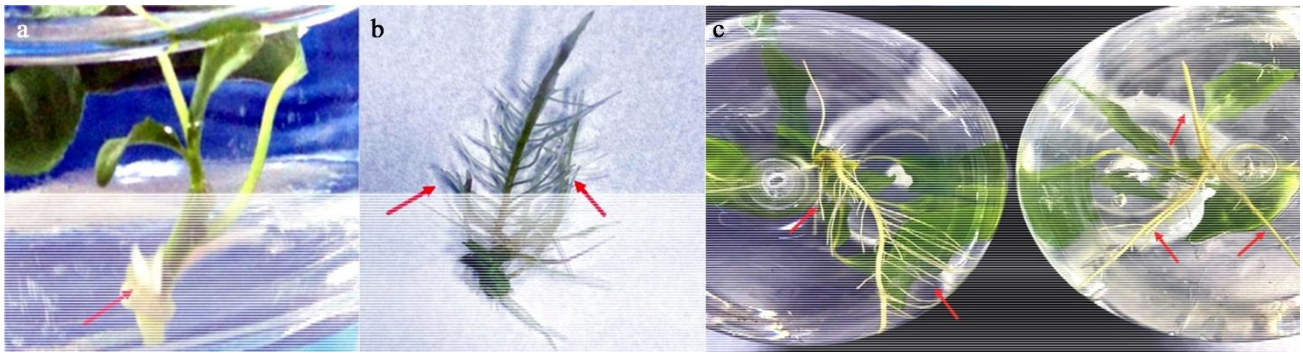

Fig. 3. Morphology of transgenic poplar 'Nanlin895' hairy roots: a, emergency of hairy roots on the selection medium at ten days post-infection (dpi); b, growth of the hairy roots approximately 3 weeks post-infection; c, comparison of root morphology between *A. rhizogenes* transformed poplar (left) and the non-transformed control (right). Arrows indicate the characteristic feature of the hairy roots or the normal roots.

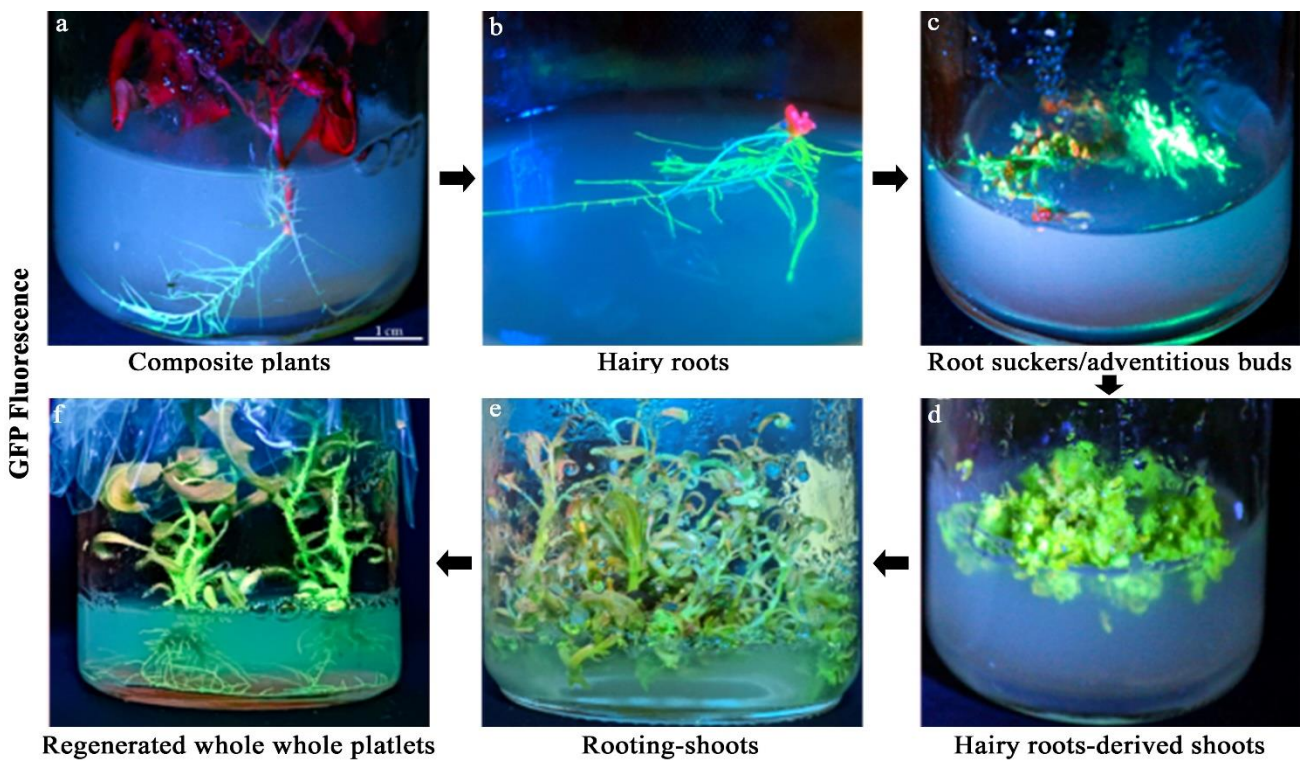

Fig. 4. Tracking records of *A. rhizogenes*-mediated poplar transformation at different development stages in poplar 'Nanlin895'. UV light images (GFP fluorescence) of hairy-root transformants: a, Composite plant with transformed roots and untransformed shoots; b, Hairy roots; c, Root suckers/adventitious buds generated from the hairy roots; d, Shoots developed from hairy roots; e, Rooting shoots; f, Regenerated transgenic plants. Green fluorescence indicates the parts expressing the GFP transformed via *A. rhizogenes*. Red color indicates the parts untransformed. Scale bar represents 1 cm.

Determination of the co-transformation probability: The target gene and genes contribute to hairy roots induction, namely *rolA*, *rolB*, *rolC* and *rolD*, are carried by separate plasmids: the mini Ti plasmid (transformed vector) carrying the gene of interest, and the Ri plasmid harboring the *rolA*/*B*/*C*/*D* genes in the T-DNA region. Therefore, the co-integration rate of the two fragments has become an important factor deciding the efficiency of *A. rhizogenes* to generate transgenic hairy roots (Daspute *et al.*, 2019; Pistelli *et al.*, 2010). To estimate the efficiency of the *A. rhizogenes*-mediated poplar gene transformation, the co-integration rate of our target gene (*AspAT::GFP*) as indicated by the occurrence of GFP fluorescence signal and the generation of hairy roots as revealed by the presence of *rolB* gene was determined. The results (Table 5) showed that on average 97.5% of explants (10 randomly selected independent explants) developed hairy roots, among which 90% regenerated plantlets carrying our gene of interest. Finally, an average co-integration rate of more than 92.2% was assessed, indicative of a high transformation efficiency of the present *A. rhizogenes*-mediated gene transformation method, in comparison with that described in previous reports (Table 1).

Utilization of hairy root transformation method for protein cellular localization and gene expression analysis in poplar: The signal of the GFP fluorescent protein in the transgenic hairy roots was further verified by a Laser-scanning Confocal Microscope imaging system (Zeiss; LSM710). From the microscope observation result (Fig. 6), it can be seen that the protein product of AspAT5 predominantly located in the plastids, whereas AspAT10 was enriched in the cytoplasm as indicated by the GFP signal, which was consistent with corresponding bioinformatic predictions (Su *et al.*, 2019).

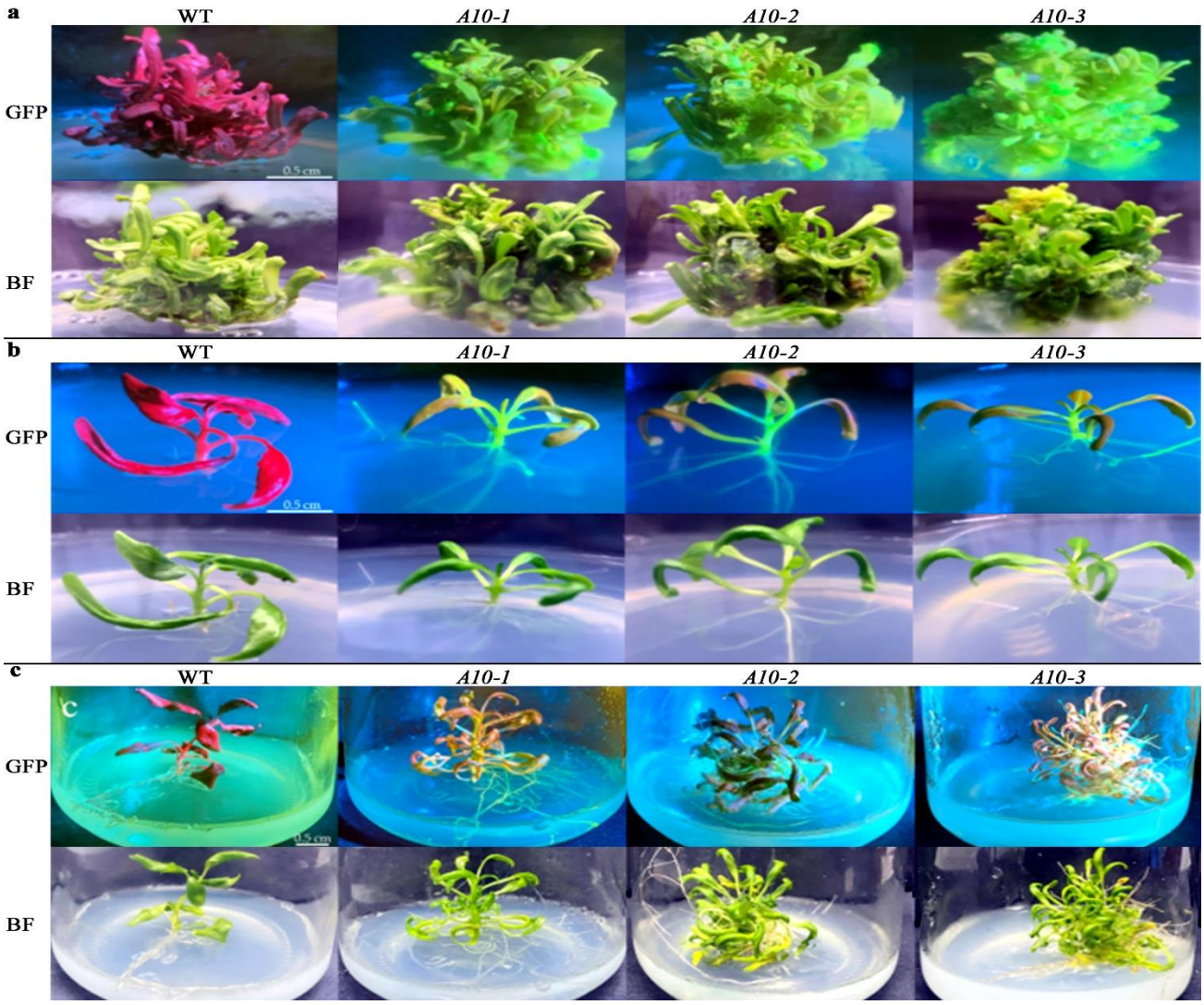

Fig. 5. Fluorescence (GFP) and bright field (BF) images of transgenic poplars 'Nanlin895' from several lineages at different transformation stages: a, Root suckers/adventitious buds on the shoot elongation medium; b, The rooted plantlets on the rooting medium; c, The regenerated transgenic plants 30 days after rooting. From left to right: untransformed WT control, independent transgenic lines: *AspAT10*::*GFP* line 1 (*A10-1*), line 2 (*A10-2*) and line 3 (*A10-3*), respectively. Scale bars represent 0.5 cm.

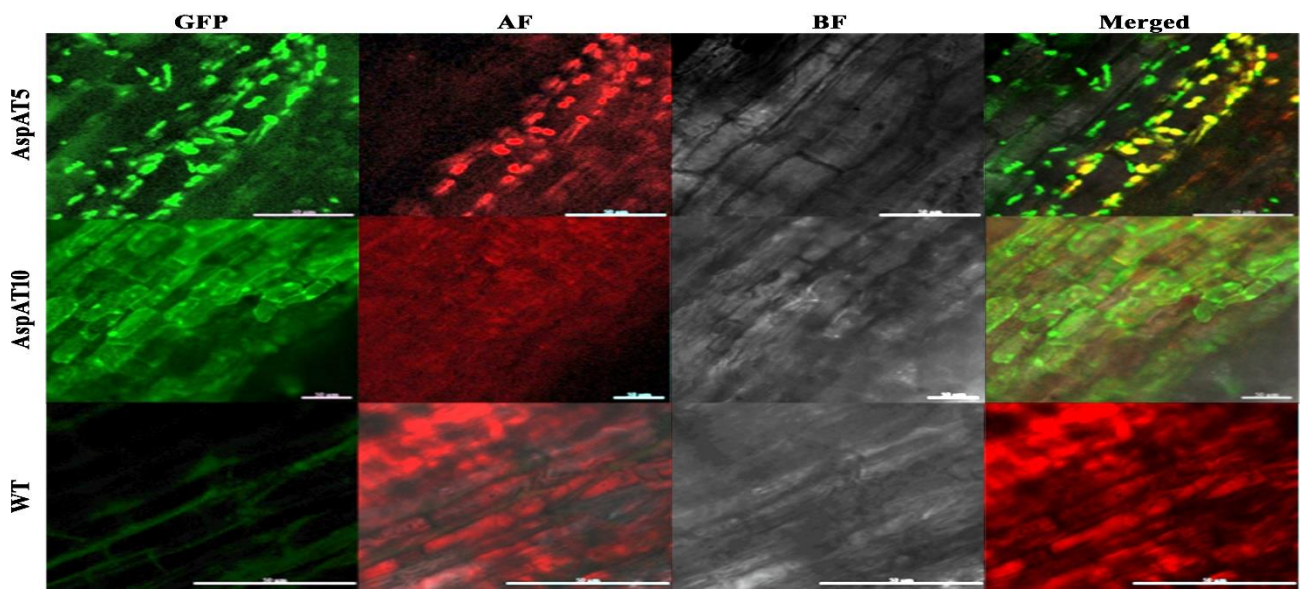

Fig. 6. Fluorescence of AspAT: GFP in the roots of WT and transgenic poplars 'Nanlin895'. From left to right, the signal was collected under different channels: emission wavelength at 505-525 nm (GFP); auto-fluorescence (AF); bright field (BF) and the merged images; a, *AspAT5* transgenic roots; b, *AspAT10* transgenic roots; c, Control roots. All scale bars represent 50 μm.

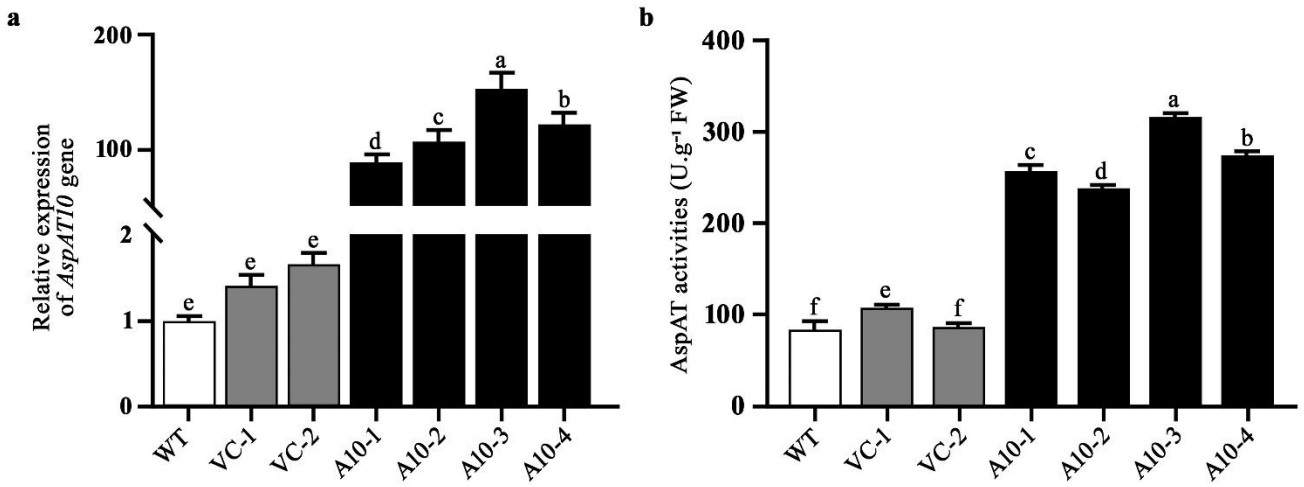

Fig. 7. Transcriptional and enzymatic analyses of AspAT in the poplar 'Nanlin895' transgenic lines: a, relative gene expression of *AspAT10* by qRT-PCR in the plantlets of the transgenic plant lines regenerated from *A. rhizogenes*-induced hairy roots compared with the WT control; b, Enzyme activity assay of AspAT. WT, wild type; VC, vector control; *A10*, *AspAT10* overexpressing lines. Populus *UBIC* was taken as an internal control for the qRT-PCR analysis. Error bars indicate the mean values ± SD for three replicates. The experiment was repeated thrice, with similar results. Different letters above the bars represent significance of differences based on the Duncan test at $p<0.05$.

Transcriptional and enzymatic analyses of AspAT in the transgenic plant lines: To determine the mRNA levels of the *AspAT* genes being transferred in the regenerated plantlets, quantitative real-time PCR (qRT-PCR) was implemented by using poplar housekeeping gene *UBIC* as an internal reference. The qRT-PCR result showed high mRNA expression levels of the *AspAT10* genes in the plantlets of the *AspAT10::GFP* independent transgenic lines (line *A10-1*, *A10-2*, *A10-3* and *A10-4*) compared to the WT control (Fig. 7a). The average expression levels of *AspAT10* in the transgenic plant lines were 87.9- to 152.8- fold higher than the WT control. By contrast, the transcript abundance of *AspAT10* in the vector control (VC) lines was insignificantly changed compared to that in the WT. From the gene expression pattern, it can be concluded that the candidate gene *AspAT10* was actually transferred into the poplar genome and successfully transcribed into mRNA in the regenerated plants derived from *A. rhizogenes*-induced hairy roots.

To further verify that the introduced *AspAT* genes were indeed integrated into poplar plants and produced respective enzymes, the activity of AspAT enzymes was measured by the chemical colorimetry assay (Fig. 7b). Our results revealed that the AspAT activity level in the VC lines was comparable to that in the WT control, whereas in the *A10* lines it was approximately three times higher than the WT control. This result demonstrated that the *AspAT10* genes delivered by *A. rhizogenes* were truly producing enzymes in the regenerated poplar *A10* lines.

Discussion

Gene transformation approach is important for gene functional investigation and crop improvement. Remarkable progress in poplar transformation has been made in the last decades (Kutsokon, 2011), which dramatically advanced the research of poplar functional genomics, genetic engineering, and physiology. Among various gene transformation methods, *A. tumefaciens*-mediated poplar transformation method is one of the most widely used technique. However, its shortcomings of low transformation efficiency, multifarious preparation steps, time-consuming process (Chabaud *et al.*, 2006), and the formation of chimera have gradually emerged. In the current study, we present an optimized and detailed procedure for *Agrobacterim*-mediated transformation of poplars by using the root-inducing pathogen *A. rhizogenes*, which provides a useful tool for the genetic manipulation of poplar.

Directly injecting the bacteria into the explants or dipping the explants in the bacterial solution have been the most frequently used methods for *A. rhizogenes* infection (Patel *et al.*, 2021), however, this method is not practicable for poplar high-throughput transformation. We therefore improved the *A. rhizogenes*-mediated poplar transformation method, taking poplar apical buds with two to four leaves from the *In vitro* culture as explants, using its freshly cutting end to prick the *A. rhizogenes* colonies on the agar plate for infection. The modified procedure did not involve agrobacteria liquid culture, OD adjustment or agrobacteria solution preparation, thus accelerated the transformation process. It took less than one minute for the inoculation implementation with *A. rhizogenes* on poplar bud cuttings, and no more than three weeks post *A. rhizogenes* infection to obtain a considerable number and length of transgenic hairy roots (Fig. 2). Our method significantly speeded up and simplified the progress of poplar gene transformation.

Auxin is an effective regulator of multiple biological process, including cell division, differentiation, and expansion (Enders & Strader, 2016). Extensive studies showed that auxin displayed an essential role in root growth (Ameen *et al.*, 2017; Amri *et al.*, 2010). IBA in particular can stimulate root initiation (Korasick *et al.*, 2013). As an active component of plant propagation media, such as Rootone®, IBA has been applied to trigger adventitious rooting from the stem cuttings (Frick & Strader, 2018). In the current study, we applied IBA to the

selection medium, and found that under optimal concentration (i.e., 0.1 mg L^{-1}) of IBA, the emergency and elongation rate of poplar hairy-root was pronouncedly elevated (Table 3).

In addition, we took the advantage of the hairy roots phenotype and GFP marker to screen the positive transformants by visual observation of hairy-root morphology and detection of GFP fluorescence signals using a portable fluorescence lamp. This not only added great convenience to the experiment operation but also improved the transformation efficacy since the non-transgenic hairy roots could be eliminated at an early stage. With our modified protocol, more than 90% of the candidate poplar buds developed transgenic hairy roots (Table 5). Moreover, in agreement with the findings reported in previous studies in *Arachis hypogaea* (Liu *et al.*, 2016), and *Salix* spp. (Gomes *et al.*, 2019), the transgenic hairy roots of poplars in the current study grew rapidly with no requirement of a complex cultured process. Intriguingly, they regenerated into whole plantlets spontaneously (Desmet *et al.*, 2020). Further enzyme activity and gene transcriptional analysis showed that our target gene *AspAT10* in poplars transformed were significantly induced (Fig. 7), confirming that the modified transformation system by means of *A. rhizogenes*-induced hairy root was effective in expressing and producing active enzymes from the foreign genes in poplar plants. Hence, the method reported in the present study was proven simple, speedy and straightforward to apply, and was of high efficacy.

With this developed *A. rhizogenes*-mediated poplar transformation system, both composite plants (carrying transgenic hairy roots) and hairy-root-regenerated transgenic plants were obtained. While the stable-transgenic plant allows for functional genomics study and agronomic traits improvement, the composite plant also shows good applicability in research relevant to root systems, such as root-microbe interaction, nutrition, secondary metabolism production and stress response (Kereszt *et al.*, 2007; Priya *et al.*, 2023; Veena & Taylor, 2007; Yu *et al.*, 2020). Moreover the composite plants can be developed within a very short period (i.e., two to three weeks with roots length > 1 cm), and able to be maintained and multiplied in agar plates or hydroponic culture using the *A. rhizogenes*-mediated transformation method, further investigations implemented on the root systems is possible at a precocious stage without the need to regenerate whole transgenic plants (Guo *et al.*, 2018; Pistelli *et al.*, 2010; Song *et al.*, 2021; Tomasz *et al.*, 2020; Yoshida *et al.*, 2015). As such, the current method provides a versatile platform for gene function investigation in poplars.

Conclusions

In the present study, we reported a simple, fast, straightforward, and efficient method to produce transgenic poplars by using poplar apical buds as explants and *A. rhizogenes* (K599 strain) to stimulate hairy roots. The advantages of our method are: (1) It omitted several redundant operation steps, such as agrobacteria liquid culture, OD adjustment and agrobacteria solution preparation, hence it dramatically accelerates and simplifies the experiment operation. (2) The supplement of IBA (0.1 mg/L) significantly promoted hairy root induction. (3) The feature of hairy roots and GFP reporter were used to screen for the transgenic composite plants, as well as the hairy-root-regenerated shoots and hairy-root-regenerated whole plants. The visible hairy-root phenotype combined with GFP fluorescence signals detected by a handy fluorescence lamp made it easy and rapid to identify the positive transformants. With these optimized procedures, high efficiency of poplar transformation was achieved. Our study provides a convenient and simple approach to generate a considerable number of hairy roots and hairy-root-regenerated poplars within a short time period. This modified method is conducive to poplar functional genomics studies, agronomic traits improvement and root biology research.

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