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Optimization of the regeneration and *Agrobacterium*-mediated transformation in pear

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Abstract

Conventional breeding in pears is inefficient due to a long juvenile phase and self-incompatibility. Genetic transformation offers a promise to expedite the breeding process. However, the frequencies of regeneration and genetic transformation in most *Pyrus* spp. are relatively low. This study investigated various factors influencing regeneration and genetic transformation using leaves from *Pyrus ussuriensis* Maxim "Shanli" and *P. communis* L. "Conference" as explants. The optimum regeneration medium for "Shanli" and "Conference" was NN69 containing 3.0 mg L^{-1} thidiazuron (TDZ) and 0.3 mg L^{-1} indolybutyric acid (IBA) for the former or 1.0 mg L^{-1} TDZ and 0.5 mg L^{-1} naphthalene acetic acid (NAA) for the latter. Sectioning the leaves from 30-day-old plantlets transversely and placing them with their abaxial side facing downward could significantly improve the regeneration ratio in both accessions. Moreover, a two- or four-week culture under the dark was beneficial for the regeneration of "Shanli" and "Conference" respectively. The optimal infection time was 12 and 8 min, while the time of the delayed screening test was two and one day for "Shanli" and "Conference" respectively. Moreover, a coculture of two days was recommended for both accessions. Post-transformation, the optimal concentrations of antibiotics were 16 mg L^{-1} kanamycin (Kan), 150 mg L^{-1} timentin (Tim), and 300 mg L^{-1} cefotaxime (Cef). The optimized regeneration and transformation system can be an effective alternative for either gene function analysis or genetic improvement in pear.

Keywords Pear, *Pyrus ussuriensis*, *Pyrus communis* L., Plant regeneration, Genetic transformation

Introduction

Pear is a member of Rosaceae and is extensively found in Asia and Europe (Wu et al. 2013). It comprises at least 22 primary spp. and ten naturally occurring interspecific hybrid taxa worldwide (Wu et al. 2018). Among them, *Pyrus bretschneideri*, *P. pyrifolia*, *P. ussuriensis*, *P. sinkiangensis* and *P. communis* are the five major cultivated spp. (Wu et al. 2018). Except for *P. communis*, the remaining four spp. are primarily distributed in Asia (Bao et al. 2007, Wu et al. 2018). Pear is a woody plant characterized by a lengthy juvenile phase and high levels of heterozygosity (da Silva et al. 2018), factors that present challenges to genetic improvement through conventional breeding, which is a laborious and time-consuming process (Hacket 1985). Additionally, the lack of uniformity in rootstock seedlings can significantly impact the growth of scion varieties and the quality of fruits (Mourgues et al. 1996). In China, *P. ussuriensis* is predominantly found in the northern, northeast and northwest regions, and to a lesser extent, in Siberia and Korea (Cao et al. 2012). This spp. exhibited higher cold-hardiness and resistance to fire blight, making it an appealing candidate

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for developing new stress-tolerant germplasm resources (Yang et al. 2017). Due to its robust stress resistance and compatibility for grafting, the *P. ussuriensis* Maxim wild accession "Shanli" is commonly utilized as a rootstock for scion grafting in Northeast China, where propagation is mainly achieved through seeds (Cao et al. 2012). Conversely, *P. communis* L., also known as the European pear, is a widely cultivated species indigenous to Europe and has been grown for thousands of years (Wolko et al. 2010). "Conference", a variety of *P. communis*, can be distinguished by its elongated shape and yellow-green skin, which may develop a reddish blush. These attributes contribute to its worldwide popularity, and its reputation continues to grow due to its distinctive flavor and versatility in culinary applications (Mourgues et al. 1996).

The advancement of genetic transformation and regeneration technologies has revolutionized plant biotechnology. These technologies have made precise and efficient genetic modification in plants possible (Maren et al. 2022, Ramkumar et al. 2020). Genetic transformation allows the introduction of new genes into plants, leading to the development of desirable traits such as improved crop productivity, disease resistance and stress tolerance (Anjanappa and Gruissem 2021). On the other hand, regeneration plays a vital role in the recovery and propagation of genetically modified plants by enabling the production of whole plants from single cells or tissues (Perez-Garcia and Moreno-Risueno 2018). These technologies have found extensive applications in both traditional agriculture and modern biotechnology. They have been utilized for crop improvement, production of pharmaceutically important compounds, and environmental remediation (Newell 2000). Micropropagation, a rapid technique for the uniform multiplication of stock-plant material, involves various steps, such as shoot-tip culture, shoot proliferation, rooting and acclimatization of the plantlets (Loyola-Vargas and Ochoa-Alejo 2018). This technique facilitates the efficient production of large numbers of genetically identical plantlets.

Constructing efficient regeneration and genetic transformation systems for pear is crucial for obtaining new transgenic varieties with desirable traits. The initial report of micropropagation in *Pyrus* involved the rootstock OH×F51 and the scion variety "Bartlett" (Lane 1979). Since then, numerous efficient micropropagation systems have been developed for various *Pyrus* spp. and varieties, focusing on identifying the factors that influence their effectiveness. Successful induction of adventitious shoots has been achieved in cultivated spp. such as *P. communis* (Matsuda et al. 2005, Mourgues et al. 1996, Yancheva et al. 2006), *P. ussuriensis* (Yang et al. 2017), *P. betulaefolia* (Xiao et al. 2022), and *P. pyraster* (Palombi et al. 2007). While the regeneration frequency of *P. communis* reached 100% in specific genotypes, those of other spp. were relatively low, indicating that a successful induction of regeneration was generally genotype-dependent (Maren et al. 2022). Several studies have reported the genetic transformation of *Pyrus* building upon successful regeneration, with the majority focusing on *P. communis* (Freiman et al. 2012, Righetti et al. 2014, Yancheva et al. 2006, Zheng et al. 2022). However, fewer reports on the transformation of Asian pear spp., including *P. pyrifolia* (Nakajima et al. 2013), *P. ussuriensis* (Yang et al. 2017) and *P. betulaefolia* (Xiao et al. 2022) are available. Furthermore, pears have been successfully transformed with a limited number of genes. Overexpressing *ARABINOGALACTAN PROTEIN 7–1* (*PcAGP7-1*) enhanced cell wall thickness, affected cell morphogenesis, and reduced the brassinolide content, resulting in dwarfing (Zheng et al. 2022). Resistance to diseases and drought tolerance was promoted in pear varieties by introducing *Rvi6* (formerly *HcrVf2*, homologue of the *Cladosporium fulvum* resistance genes of the Vf region) and *Pyrus bretschneideri WRKY transcription factor 53* (*PbrWRKY53*), respectively (Liu et al. 2019, Perchepied et al. 2021). Transforming pears with additional foreign genes may contribute to improving rootstocks, producing dwarfs and breeding new varieties with better disease resistance.

This study aimed to optimize the *Agrobacterium*-mediated transformation and regeneration systems in *P. ussuriensis* Maxim "Shanli" and *P. communis* L. "Conference". The various factors that influence these systems, including culture medium, plant growth regulators, age of explant, wounding methods, positioning of leaves, period of dark incubation, antibiotic concentration, infection time, cocultivation time and time of delayed screening test, were evaluated. The results of this study can provide valuable technical insights for developing genetically modified pears with desirable traits.

Materials and methods

Plant material

The test materials comprised *P. ussuriensis* Maxim wild accession "Shanli" and the cultivated *P. communis* L. variety "Conference". Plantlets of these varieties were obtained through tissue culture following established protocols (Mourgues et al. 1996; Yang et al. 2017). The plantlets were subcultured on a shoot propagation medium consisting of basal MS salts, supplemented with 0.5 mg L^{-1} 6-Benzylaminopurine (6-BA) (A6007743; Sangon Biotech, Shanghai, China), 0.1 mg L^{-1} NAA (N108489; Sangon Biotech), and 7.5 g L^{-1} agar (BS195; Biosharp, Hefei, Anhui Province, China), pH 5.8 every four weeks to obtain leaves used as explants for this study. The growth conditions were 25°C, a photoperiod

of 16 h of light / 8 h of darkness, and a light intensity of 25 μmol m^{-2} s^{-1}.

Callus induction and regeneration

Various approaches were used to optimize the regeneration conditions for the explants, which have been detailed in the results section. The completely unfolded leaves from the 30-day-old in vitro-propagated seedlings of "Shanli" and "Conference" were used. During the initial dark incubation period, leaf explants were cultured on an optimum medium at 25°C for three weeks to initiate callus induction and shoot propagation. Leaf explants were moved to a 16 h light / 8 h dark photoperiod at the same temperature. The regeneration ratio of explants and adventitious bud growth was measured after 35 days post-subculturing. The numbers of leaves with calli and regenerating buds were counted as surviving explants. The regeneration rate, number of regenerated buds/number of viable explants × 100% was scored. Each treatment consisted of thirty leaves, and the experiments were conducted in three biological replicates.

Screening for antibiotic-based selection pressure

In order to ascertain the suitable antibiotic concentration for selecting transgenic plants, leaf explants were cultured on an optimal regeneration medium supplemented with different concentrations of kanamycin (Kan) (A506636; Sangon Biotech), timentin (Tim) (T8660; Beijing Solarbio Science & Technology Co. Ltd., Beijing, China) and cefotaxime (Cef) (A600469; Sangon Biotech) under darkness at 25°C. Each treatment consisted of thirty leaves, and the regeneration rate of explants was ascertained after three weeks of culture.

Agrobacterium tumefaciens-mediated transformation

To create the 35S::*GUS* plasmid, the pRi101 vector was utilized to insert the *Beta-glucuronidase* (*GUS*) reporter gene, which encodes the β-glucuronidase enzyme. A single colony of the *A. tumefaciens* strain EHA105 harboring the constructed plasmid was cultured while being shaken at 200 rpm, 28°C, overnight in a liquid lysogeny broth medium containing 50 mg L^{-1} Kan and 50 mg L^{-1} rifampicin. After centrifugation, *Agrobacterium* cells were collected and resuspended in a liquid MS medium supplemented with 100 μM acetosyringone (AS) till the OD_{600} reached 0.4–0.5, which is useful for infection. The leaves designated for infection were precultured on a regeneration medium under darkness at 25°C for two days. The leaves were immersed in the resuspended *Agrobacterium* solution for 4, 8 or 12 min with gentle shaking. Subsequently, the infected leaves were placed on a regeneration medium with the abaxial surface facing upward for cocultivation under darkness at 25°C for one, two or three days. The experiment was repeated thrice, with at least thirty leaves utilized for each repetition.

GUS staining

After the cocultivation and delayed screening test period, leaf explants were cultured on a selection medium, collected seven days post-infection, and rinsed with a staining buffer lacking X-Gluc. Subsequently, the explants were stained using a GUS staining buffer consisting of 50 mM sodium phosphate buffer (pH 7.0), 10 mM Na_2EDTA, 0.5 mM $K_4[Fe(CN)_6]\cdot 3H_2O$, 0.5 mM $K_3[Fe(CN)_6]$, 0.1% Triton X-100, and 1 mg mL^{-1} X-Gluc. The staining was terminated using 70% ethanol. The GUS staining efficiency was evaluated by calculating the percentage of stained leaves out of the total number of leaves, expressed as the number of stained leaves/total number of leaves × 100%.

Confirmation of transformation

Genomic DNA and total RNA were extracted from the Kan-resistant calli using the cetyltrimethylammonium bromide (CTAB) method (Allen et al. 2006). Fragments of *GUS* were amplified from the genomic DNA using a T100 thermal cycler (Bio-Rad, Hercules, CA, USA). The PCR products were separated on a 0.8% agarose gel through electrophoresis and visualized by staining with ethidium bromide. cDNA was synthesized using a one-step gDNA removal and cDNA synthesis kit (TransGen Biotech Co. Ltd., Beijing, China). qPCR was performed using an ABI Q3 system (Thermo Fisher Scientific, Waltham, MA, USA). The primers used are shown in Supplemental Table 1. The *PbrGAPDH* (*Pyrus bretschneideri glyceraldehyde-3-phosphate dehydrogenase*) gene was employed as a reference gene to determine the relative expression level of *GUS*. The $2^{-\Delta\Delta Cp}$ method was utilized for the calculation.

Statistical analysis

The statistical processing of the data was performed using GraphPad Prism 9.0 software (GraphPad, L.A., California, USA). Duncan's multiple range test ($P < 0.05$) and Student's *t*-test were used to perform the statistical significance.

Results

Determining the in vitro regeneration potential on different basal media

To construct a highly efficient regeneration system and obtain a substantial yield of adventitious shoots in "Shanli" and "Conference", at least 30 leaves of each were used as explants to investigate the effects of MS and NN69 basal media supplemented with 3.0 mg L^{-1} TDZ and 0.3 mg L^{-1} IBA. As depicted in Fig. 1 and

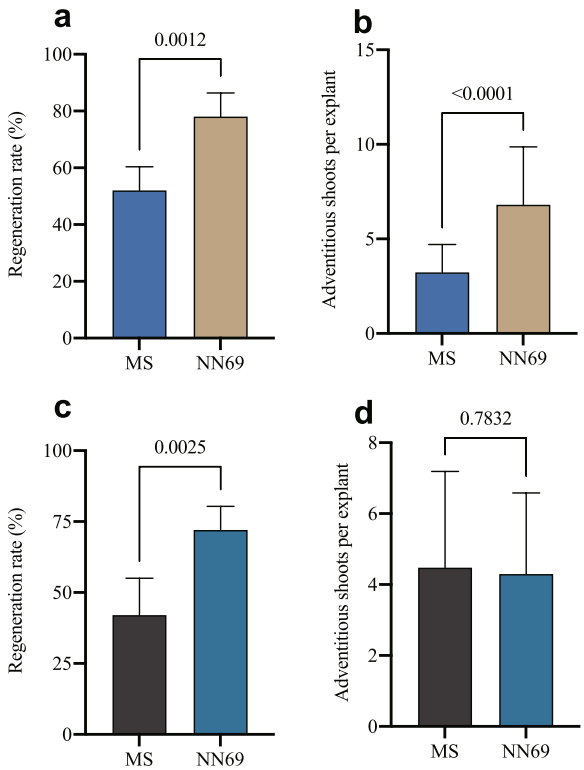


Fig. 1 Effect of culture medium on in vitro regeneration. **(a)**-**(d)** Regeneration rate and adventitious shoots per explant in "Shanli" (**a**, **b**) or "Conference" (**c**, **d**) leaves were measured after 35 days of cultivation. MS, Murashige and Skoog; NN69, Nitsch and Nitsch 1969. *P* values were determined using Student's *t*-test

Supplemental Fig. 1, at the same concentrations of plant growth regulators, relatively higher regeneration rates and a greater number of adventitious shoots on NN69 were observed for "Shanli". Conversely, for "Conference", the regeneration rates of explants were slightly enhanced by NN69, but not the number of adventitious shoots.

The influence of plant growth regulators on the in vitro regeneration of "Shanli" and "Conference" explants

Next, the effects of varying concentrations of plant growth regulators on the initiation of adventitious shoots on NN69 were studied. The results indicate that in "Shanli" higher regeneration rates were achieved with 2.0 mg L^{-1} TDZ, while 1.0 mg L^{-1} TDZ resulted in a more significant number of adventitious shoots (Table 1 and Supplemental Fig. 2). Similarly, in "Conference", the utilization of 1.0 mg L^{-1} TDZ exhibited the highest rates of regeneration and number of adventitious shoots per explant.

MS medium containing 3.0 mg L^{-1} TDZ and 0.3 mg L^{-1} IBA significantly induced the formation of callus in "Shanli" (Yang et al. 2017). Based on this report, MS medium was also tested with the same concentration of plant growth regulators as with NN69. Interestingly, the NN69 culture supplemented with 3.0 mg L^{-1} TDZ and 0.3 mg L^{-1} IBA displayed higher rates of regeneration and a greater number of adventitious shoots per explant compared to the culture supplemented with 2.0 mg L^{-1} TDZ and 0.5 mg L^{-1} NAA (Table 1, Supplemental Figs. 1 and 2). Consequently, the optimal regeneration medium for "Shanli" was NN69 containing 3.0 mg L^{-1} TDZ and 0.3 mg L^{-1} IBA, resulting in a regeneration frequency of ~ 76.67% and an average of 5.05 shoots per leaf. The optimal regeneration medium for "Conference" was NN69 containing 1.0 mg L^{-1} TDZ and 0.5 mg L^{-1} NAA, yielding a regeneration frequency of ~ 76.67% and an average of 5.71 shoots per leaf.

Plantlet age influences on the in vitro regeneration

To study the influence of plantlet age on the in vitro regeneration of "Shanli" and "Conference" explants, leaves from plantlets of different ages: 25, 30 and 35 days old, were examined for ascertaining the regeneration ratio and the number of adventitious shoots. The explants from 30-day-old plantlets exhibited the most prominent callus growth compared to those from 25- or 35-day-old plantlets, both in "Shanli"

Table 1 Effects of different combinations of thidiazuron (TDZ), naphthalene acetic acid (NAA) or indolybutyric acid (IBA) on regeneration of *Pyrus ussuriensis* Maxim "Shanli" and *P. communis* L. "Conference" leaves

Hormone concentration (mg L^{-1})				Shanli		Conference	
TDZ	NAA	IBA	No. of explants	Regeneration rate (%)	Adventitious shoots per explant	Regeneration rate (%)	Adventitious shoots per explant
1	0.5		30	36.67 ± 5.77 b	4.14 ± 0.47 a	76.67 ± 7.55 a	5.71 ± 0.62 a
2	0.5		30	46.67 ± 5.05 b	2.88 ± 0.33 ab	70.00 ± 9.00 a	3.19 ± 1.06 b
4	0.5		30	43.33 ± 2.50 b	3.22 ± 0.20 b	33.33 ± 5.28 b	3.43 ± 1.29 b
3		0.3	30	76.67 ± 3.44 a	5.05 ± 0.08 a	60.00 ± 5.00 a	4.55 ± 1.13 ab

The statistical significance of the differences was determined by Duncan's multiple range test ($P < 0.05$), and the lowercase letters indicate the statistically significant differences within a column

and "Conference" (Fig. 2, Supplemental Fig. 3). The regeneration ratios were ~ 79.3% and ~ 80.2% for the 30-day-old plantlets of "Shanli" and "Conference" respectively, significantly higher than those observed with the 25- or 35-day-old plantlets. Additionally, the number of regenerated adventitious shoots was also higher in the 30-day-old plantlets of "Conference". These findings suggest that the leaves from 30-day-old plantlets serve as better explants for inducing callus formation in both "Shanli" and "Conference".

Effects of wounding method on in vitro regeneration

The impact of different wounding methods, such as transverse and bisect leaf sections, on the in vitro regeneration of the explants from "Shanli" and "Conference" were investigated. The survival rates did not significantly differ among these methods. However, callus growth from the transverse leaf sections was more pronounced than from the bisect leaf sections in both "Shanli" and "Conference" (Fig. 3, Supplemental Fig. 4). The regeneration ratios for transverse leaf sections were ~ 87.2% and ~ 72.5%, whereas those for the bisect leaf sections were ~ 28.9% and ~ 32.5% in "Shanli" and "Conference" respectively. The regeneration frequency of the transverse leaf section wastwo- to three-fold that of the bisect leaf sections. On the other hand, there were no obvious differences in the number of regenerated adventitious shoots between the transverse and bisect leaf sections. These findings suggest that transverse leaf sectioning was the optimal wounding method for promoting regeneration.

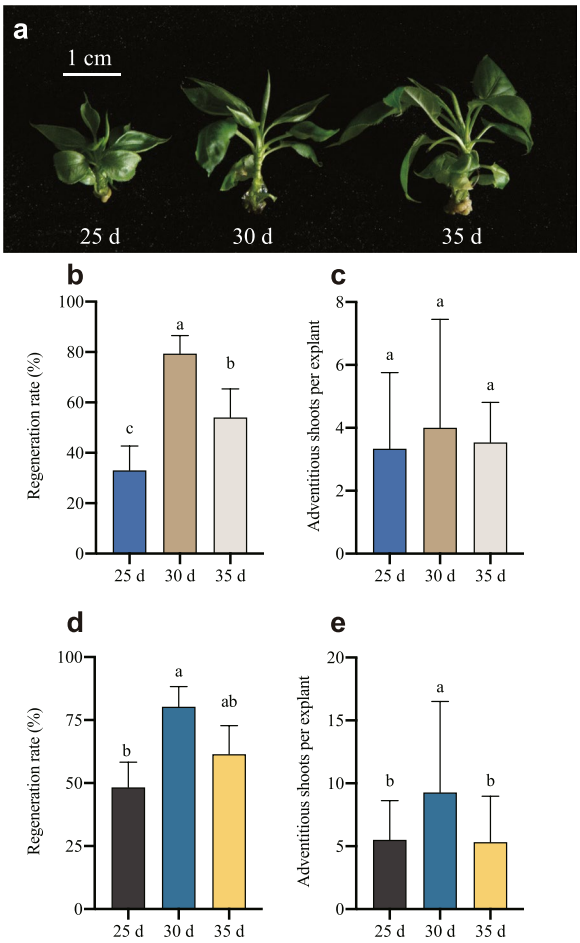


Fig. 2 Effect of plantlet age on in vitro regeneration. **(a)** Phenotypes of plantlets at different developmental stages; leaves were used as explants. **(b)-(e)** Regeneration rate and adventitious shoots per explant in *Pyrus ussuriensis* Maxim "Shanli" (**b**, **c**) or *P. communis* L. "Conference" (**d**, **e**) leaves were measured after 35 days of cultivation. The statistical significance of the differences was determined by Duncan's multiple range test, and the lowercase letters indicate the statistically significant differences between each treatment ($P < 0.05$)

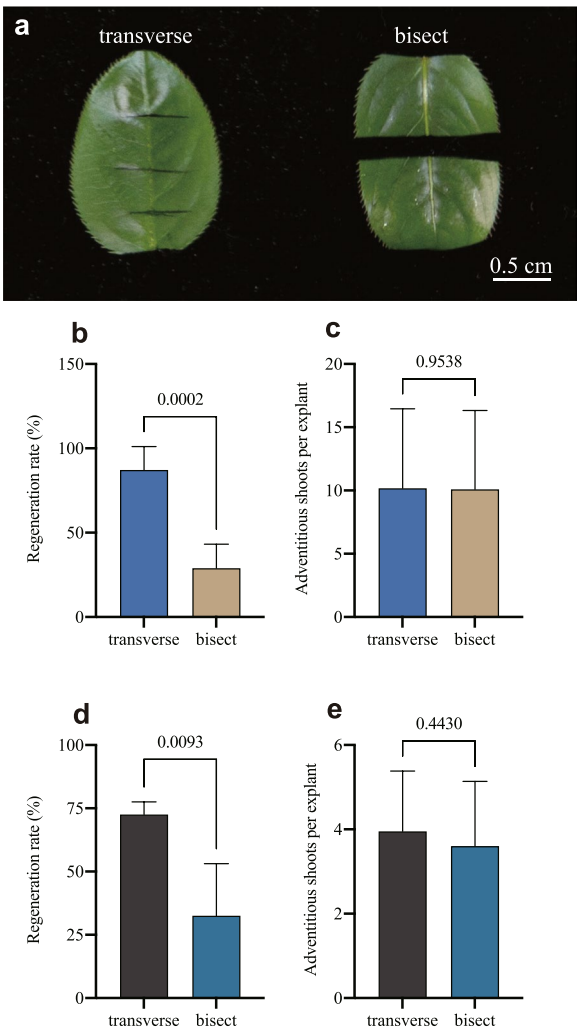


Fig. 3 Effect of wounding methods on in vitro regeneration. **(a)** Schematic diagram of wounding methods. **(b)-(e)** Regeneration rate and adventitious shoots per explant in *Pyrus ussuriensis* Maxim "Shanli" (**b**, **c**) or *P. communis* L. "Conference" (**d**, **e**) leaves were measured after 35 days of cultivation. *P* values were determined using Student's *t*-test

Positioning of the leaf section influences callus formation

The leaf sections were positioned in different orientations after pretreatment in the dark to investigate the influence of leaf position on callus formation. The results indicated that the regeneration rate was slightly higher when the leaf section was positioned with the abaxial side facing downwards on the medium than with the adaxial side in both "Shanli" and "Conference". However, the number of adventitious shoots per explant in either orientation did not vary prominently (Fig. 4 and Supplemental Fig. 5). In conclusion, placing the abaxial side of the leaf downwards modestly affected callus formation in both "Shanli" and "Conference".

Effect of incubation time under darkness on adventitious shoot formation

The effects of the duration of the dark treatment on adventitious shoot induction and regeneration rates were examined in both accessions. In "Shanli", a three-week dark- pretreatment did not markedly elevate the regeneration rate or the formation of adventitious shoots compared to a two-week pretreatment (Fig. 5, Supplemental Fig. 6). However, in "Conference", a four-week pretreatment remarkably enhanced both compared to those after two or three weeks. In summary, pretreatment in darkness for two weeks was an excellent choice for callus formation in "Shanli", while a four-week pretreatment was better in "Conference".

Antibiotic concentration influences leaf regeneration

Three antibiotics at varying concentrations, namely, Kan at 0, 8, 16 and 24 mg L^{-1}; Tim at 0, 150, 300 and 450 mg L^{-1}; and Cef at 0, 300, 400 and 500 mg L^{-1}, were used to determine the antibiotic concentration suitable for leaf regeneration in both "Shanli" and "Conference". The results indicated that 16 mg L^{-1} Kan significantly inhibited leaf regeneration in both "Shanli" and "Conference" (Table 2). In "Shanli", only 450 mg L^{-1} Tim demonstrated a negative impact. In the case of "Conference", 150 mg L^{-1} Tim showed the highest regeneration rates (Table 2). Additionally, 300 mg L^{-1} Cef resulted in a regeneration rate > 80% in both "Shanli" and "Conference" (Table 2).

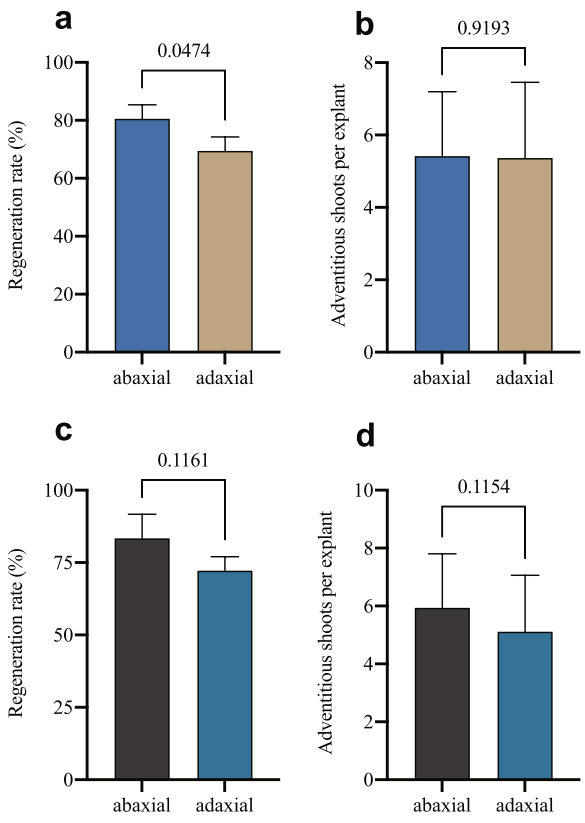


Fig. 4 Effect of the positioning of the leaf section on callus formation. **(a)-(d)** Regeneration rate and adventitious shoots per explant in *Pyrus ussuriensis* Maxim "Shanli" (**a**, **b**) or *P. communis* L. "Conference" (**c**, **d**) leaves were measured after 35 days of cultivation. *P* values were determined using Student's *t*-test

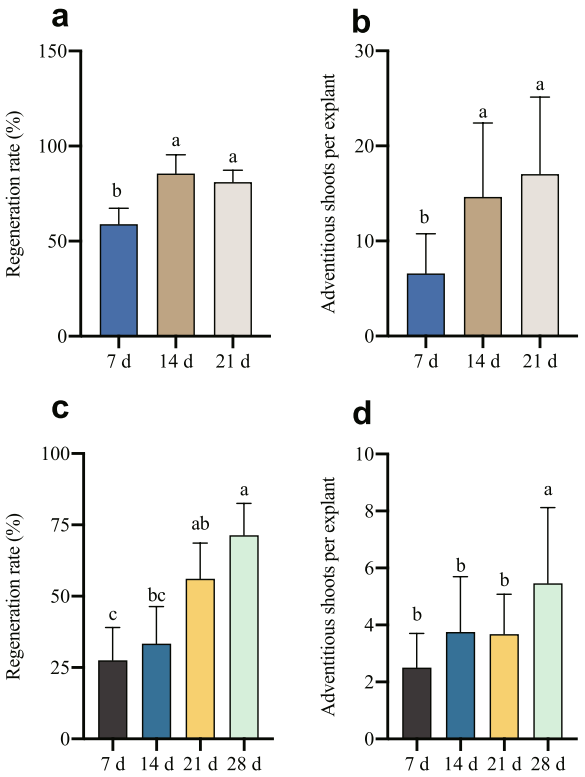


Fig. 5 Effect of duration of darkness on in vitro leaf regeneration. **(a)-(d)** Regeneration rate and adventitious shoots per explant in *Pyrus ussuriensis* Maxim "Shanli" (**a**, **b**) or *P. communis* L. "Conference" (**c**, **d**) leaves were measured. The statistical significance of the differences was determined by Duncan's multiple range test, and the lowercase letters indicate the statistically significant differences between each treatment ($P < 0.05$)

Therefore, for leaf regeneration post-transformation in "Shanli" and "Conference", 16 mg L^{-1} Kan, 150 mg L^{-1} Tim and 300 mg L^{-1} Cef were used. However, in cases where the leaves of transformed plants were contaminated with *Agrobacterium*, it is advisable to increase the concentrations of Tim and Cef within a reasonable limit while ensuring the survival of the transformed plants.

Transformation and regeneration of calli in "Shanli" and "Conference"

To construct a genetic transformation system and obtain the transgenic calli in "Shanli" and "Conference" the leaves of both varieties were infected with *Agrobacterium* strain EHA105 carrying the 35S::*GUS* vector. Different combinations of infection time (4, 8, 12 or 16 min), cocultivation (1, 2 or 3 days), and delayed culture time (0, 1, 2 or 3 days) were tested to determine the optimal conditions. After the cocultivation and delayed screening test period, leaf explants were cultured on a screening medium and collected seven days post-infection for GUS staining. The infected leaves of "Shanli" exhibited a higher GUS staining rate when treated with the resuspended *Agrobacterium* for ~ 12 min, but for 8 min in "Conference". Moreover, a cocultivation period of 2 days led to a GUS staining rate of > 50% in both "Shanli" and "Conference" Furthermore, the optimal time for the delayed screening test in "Shanli" was one day, while in "Conference" was two days, resulting in a higher GUS staining rate of > 60% in both (Table 3). Therefore, the recommended infection time was 12 min for "Shanli" and 8 min for "Conference", while the optimal cocultivation time for both accessions was two days.

Confirmation of transgenic lines

PCR was used to identify the presence of the exogenous *GUS* gene and confirm the successful integration of the transgene. Genomic DNA was isolated from the Kan-resistant lines and wild-type plants of both "Shanli" and "Conference" and used as the template. PCR yielded products of the length as expected with *GUS* in the GUS-positive transformants. In contrast, no bands were detected in the nontransformed controls (Fig. 6a, lane NC). qPCR was performed to ascertain the expression levels of *GUS* in transgenic calli, which were higher in the transgenic lines than in the nontransformed controls (Fig. 6b and c). These results affirm that the Kan-resistant lines derived from both "Shanli" and "Conference" were genuine transformants and did not merely escape the transformation process.

Discussion

Pear is a vital fruit tree with a long history of cultivation, resulting in abundant germplasm resources. However, when applied to fruit trees such as pear, traditional breeding methods are time-consuming and inefficient due to a long fruit development period, high heterozygosity and self-incompatibility. Genetic transformation is a promising approach that can rapidly expedite the breeding process. Previous studies have successfully established the regeneration and *Agrobacterium*-mediated transformation systems for certain varieties of pears. In this study, such systems were optimized in the wild spp. *P. ussuriensis* Maxim "Shanli" and the cultivated spp. *P. communis* L. "Conference" leaves were used as explants.

Table 2 Effect of antibiotics on the leaf regeneration rate in "Shanli" and "Conference"

Antibiotic	Concentration (mg L^{-1})	No. of explants	Shanli		Conference	
			Regeneration rate (%)	Adventitious shoots per explant	Regeneration rate (%)	Adventitious shoots per explant
Kanamycin	0	30	76.67 ± 5.77 a	11.08 ± 1.95 a	60.00 ± 0.00 a	3.50 ± 0.50 a
	8	30	40.00 ± 9.00 b	5.19 ± 1.79 b	23.33 ± 5.77 b	1.83 ± 0.76 b
	16	30	0.00 ± 0.00 c	0.00 ± 0.00 c	0.00 ± 0.00 c	0.00 ± 0.00 c
	24	30	0.00 ± 0.00 c	0.00 ± 0.00 c	0.00 ± 0.00 c	0.00 ± 0.00 c
Timentin	0	30	70.00 ± 6.00 ab	7.93 ± 3.01 b	73.33 ± 3.54 a	3.43 ± 0.44 a
	150	30	76.67 ± 5.77 a	19.92 ± 5.27 a	76.67 ± 2.68 a	3.50 ± 0.57 a
	300	30	83.33 ± 3.55 a	17.43 ± 5.21 a	63.33 ± 4.33 b	2.80 ± 0.22 ab
	450	30	50.00 ± 0.00 b	12.93 ± 3.64 a	53.33 ± 5.04 c	2.40 ± 0.53 b
Cefotaxime	0	30	56.67 ± 9.55 c	9.40 ± 2.96 b	73.33 ± 3.21 a	2.67 ± 0.34 b
	300	30	86.67 ± 5.77 a	23.97 ± 3.66 a	80.00 ± 0.00 a	3.83 ± 0.56 a
	400	30	66.67 ± 5.28 b	18.51 ± 6.94 a	60.00 ± 8.00 b	4.23 ± 0.56 a
	500	30	66.67 ± 3.34 b	22.06 ± 3.93 a	56.67 ± 5.77 c	2.58 ± 0.68 b

The statistical significance of the differences was determined by Duncan's multiple range test ($P < 0.05$), and the lowercase letters indicate the statistically significant differences within a column

Table 3 Evaluation of the effects of infection time, cocultivation time and delayed cultured time on the transformation rates in *Pyrus ussuriensis* Maxim "Shanli" and *P. communis* L. "Conference" leaves

Progress		No. of explants	Shanli		Conference	
			Leaf browning rate (%)	GUS staining rate (%)	Leaf browning rate (%)	GUS staining rate (%)
Infection time (min)	4	30	13.33 ± 2.58 b	25.00 ± 0.24 b	16.66 ± 1.23 c	26.67 ± 1.01 b
	8	30	20.00 ± 3.43 ab	31.67 ± 1.09 b	23.33 ± 2.44 b	73.33 ± 2.22 a
	12	30	26.67 ± 1.04 a	53.33 ± 2.45 a	26.67 ± 3.21 b	63.33 ± 3.31 ab
	16	30	36.67 ± 2.33 a	50.00 ± 1.24 a	40.00 ± 2.34 a	66.67 ± 2.52 a
Coculture time (day)	1	30	26.67 ± 1.00a	31.67 ± 2.23 b	30.00 ± 2.00 a	30.00 ± 2.02 b
	2	30	23.33 ± 0.00ab	58.33 ± 3.12 a	20.00 ± 0.18 b	56.67 ± 1.34 a
	3	30	33.33 ± 1.11a	43.33 ± 2.45 b	33.33 ± 0.00 a	36.67 ± 0.89 b
Delayed culture time (day)	0	30	43.33 ± 2.94 a	43.33 ± 1.23 b	36.67 ± 1.56 a	40.00 ± 0.34 b
	1	30	36.67 ± 2.50 a	43.33 ± 2.12 b	26.67 ± 3.23 b	66.67 ± 1.11 a
	2	30	33.33 ± 3.11 ab	60.00 ± 0.90 a	30.00 ± 2.22 ab	46.67 ± 1.56 b
	3	30	40.00 ± 1.23 a	33.33 ± 2.24 c	30.00 ± 2.01 ab	40.00 ± 0.00 b

The statistical significance of the differences was determined by Duncan's multiple range test ($P < 0.05$), and the lowercase letters indicate the statistically significant differences within a column. GUS, Beta-glucuronidase

Various factors, including culture medium, plant growth regulators, age of the explant, wounding methods, positioning of leaves, dark incubation period, infection time, and cocultivation time, were evaluated and optimized. Based on the findings, an optimized system of regeneration and *Agrobacterium*-mediated transformation for both accessions was successfully developed (Fig. 7).

In our study, regeneration frequencies as high as 87.23% were achieved using leaf explants of *P. ussuriensis* Maxim "Shanli", consistent with previous studies on this variety (Yang et al. 2017). Additionally, an average of 14.63 shoots per leaf were regenerated, significantly higher than those obtained previously (Yang et al. 2017). In "Conference", a maximum regeneration frequency of 83.33% was obtained by placing leaf explants with the abaxial side in contact with the culture medium, resulting in an average of 5.93 regenerated shoots per leaf. These values were, however, lower than those in a previous report (Leblay et al. 1991). Notably, all three tested durations of dark incubation resulted in higher shoot formation rates and a higher average number of regenerated shoots compared to previous studies on leaf regeneration (Yang et al. 2017). A dark culture time of 14 days using "Shanli" leaves as explants yielded the highest shoot formation rate of 85.54% and an average of 14.63 regenerated shoots per leaf, making it the most effective approach in terms of the number of shoots obtained in our protocol.

Additionally, the effects of NN69 basal medium supplemented with different concentrations of plant growth regulators on the induction of adventitious shoots were studied. TDZ, a cytokinin analog, is a new-generation plant growth regulator crucial in inducing calli from explants. It can mimic their function while chemically different from auxins and cytokinins (Ali et al. 2022). In "Shanli", the addition of 2 mg L^{-1} TDZ resulted in a relatively high regeneration rate of 46.67%, whereas the inclusion of 1 mg L^{-1} TDZ along with a constant concentration of NAA yielded a greater number of adventitious shoots, with an average of 4.14 shoots per explant. However, in a previous study, the highest regeneration rate (100%) and the greatest number of adventitious shoots per explant (6.30) were obtained on NN69 supplemented with 2 or 3 mg L^{-1} TDZ and 0.2 mg L^{-1} IAA using leaves as explants (Yang et al. 2017). These findings suggest that differences in the concentrations of cytokinins, auxins and other factors in culture media across experiments may account for the variation in regeneration rates. Wounding promoted the biosynthesis of endogenous hormones, inducing callus formation (Ikeuchi et al. 2013). Compared to longitudinal leaf sectioning, needle-piercing of leaves or transverse stem sectioning, the transverse sectioning of apple leaves exhibited a higher frequency of in vitro regeneration (Liu et al. 2022). Consistent with these findings, this study also corroborated the superiority of transverse leaf sectioning as the most suitable method for inducing regeneration in "Shanli" and "Conference". Furthermore, the positioning of the leaf section on the culture medium also influenced regeneration frequency (Song et al. 2019), which was higher when the adaxial side was in contact with the culture medium (Liu et al. 2022). However, the results of this report revealed that placing the leaf with the abaxial side facing downwards modestly affected callus formation in both varieties of pear. This unexpected outcome may be attributed to the loose packing of the cuticle and the more significant number of stomata on the abaxial leaf

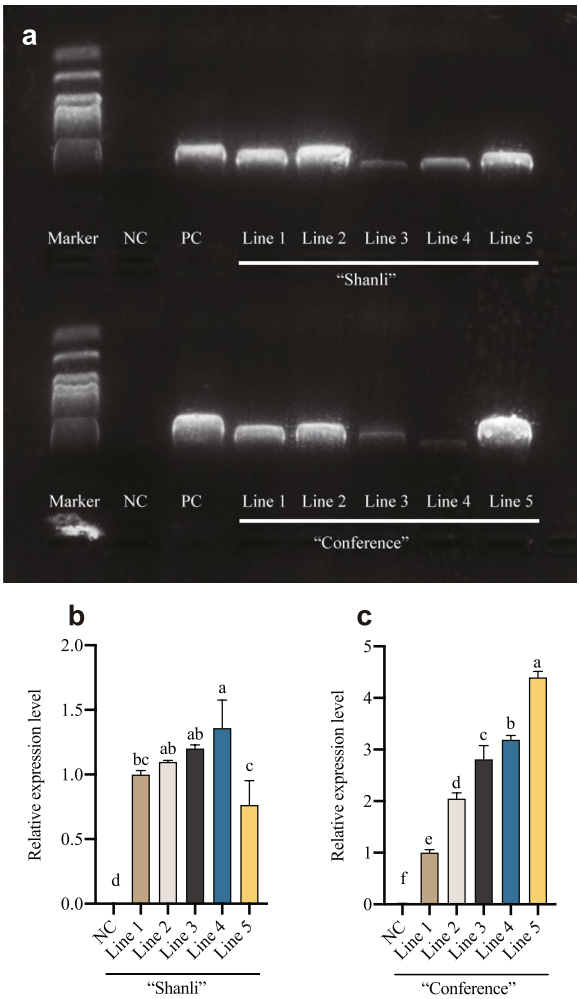


Fig. 6 Molecular confirmation of transgenic lines. **(a)** Putative transgenic calli of the leaf explants were collected from the screening medium for PCR. **(b)**, **(c)** qPCR was carried out to ascertain the expression levels of *GUS* in the putative transgenic lines. NC, nontransformed control; PC, positive control; lanes, independent transformants from *Pyrus. ussuriensis* Maxim "Shanli" and *P. communis* L. "Conference"

surface, factors that promote the absorption of nutrients from the culture medium. In this study, "Shanli" exhibited a higher regeneration frequency and average number of regenerated shoots than "Conference", which could be attributed to the modified regeneration conditions used in this experiment. Furthermore, it is essential to establish regeneration systems for pear varieties with different genotypes, as this can lay the groundwork for further genetic transformation studies.

Establishing an optimal system for producing transgenics is crucial in generating new germplasms through gene modification. While the number of transgenic lines in European pear varieties has been increasing, there have been limited advancements in Asian varieties (Maren et al. 2022). Consequently, it is vital to develop a stable transformation system tailored explicitly to Asian varieties, considering the unique characteristics of different spp. and cultivars. Providing an appropriate transformation method for a particular spp. can serve as a valuable reference for future studies. This study achieved a successful generation of transgenic "Shanli" and "Conference" calli with a GUS staining rate > 60%. However, the regeneration efficiency of the transgenic lines was generally low, and a solution is needed to overcome the barriers to producing transgenic plants in pear. Inspiration can be drawn from advancements in other fruit crops, particularly apple (*Malus domestica* Borkh.), where the efficiency in the production of transgenic plants was successfully elevated through the ectopic expression of *Malus domestica Baby boom 1* (*MdBBM1*) (Chen et al. 2022). In monocots and species recalcitrant to transformation, many developmental regulators, such as *WUSCHEL* (*WUS*), *WUSCHEL-RELATED HOMEOBOX* (*WOX*), *Growth-Regulating Factor* (*GRF*) and chimeric *GRF-INTERACTING FACTOR* (*GRF-GIF*) have been utilized to enhance transformation efficiency, which has shown promise in reprogramming the somatic cells for embryogenesis (Chen et al. 2022, Debernardi et al. 2020, Lowe et al. 2016, Wang et al. 2022). Consequently, this has generated renewed interest in the exploration of specific developmental regulators for crop transformation. Some of these regulators have the potential to enhance the transformation efficiency in pear.

In contrast to annual crops such as wheat, maize, rice and soybean, fruit crops are typically clonally propagated to ensure genetic stability. Unfortunately, the explant sources available for developing regeneration and genetic transformation systems are often limited to leaf tissues, while research on the use of calli, somatic embryos, and protoplasts is relatively scarce (Song et al. 2019). Although successful regeneration through callus tissue has been achieved in *P. domestica* L. (Petri et al. 2012) and *M.s prunifolia* (Liu et al. 2022), a callus-based regeneration system in fruit trees is still underdeveloped. Transformation using embryo culture has been reported in cherry (*P. avium*×*P. pseudocerasus*) (Gutièrrez-Pesce et al. 1998), peach (Pérez-Clemente et al. 2004), and apricot rootstocks (Câmara and Laimer da CÂmara Machado M, 1995), along with partial regeneration from protoplasts (Reyna-Llorens et al. 2023). As protoplasts demonstrate the characteristic features of single-cell-based regeneration, all plants generated through protoplast induction are obtained by inducing tissue and organ differentiation (Reed and Bargmann 2021, Yadava et al. 2016). However, the study of regenerating and transforming materials using these alternative methods in pear is limited to leaves, thus preventing the establishment of stable regeneration and

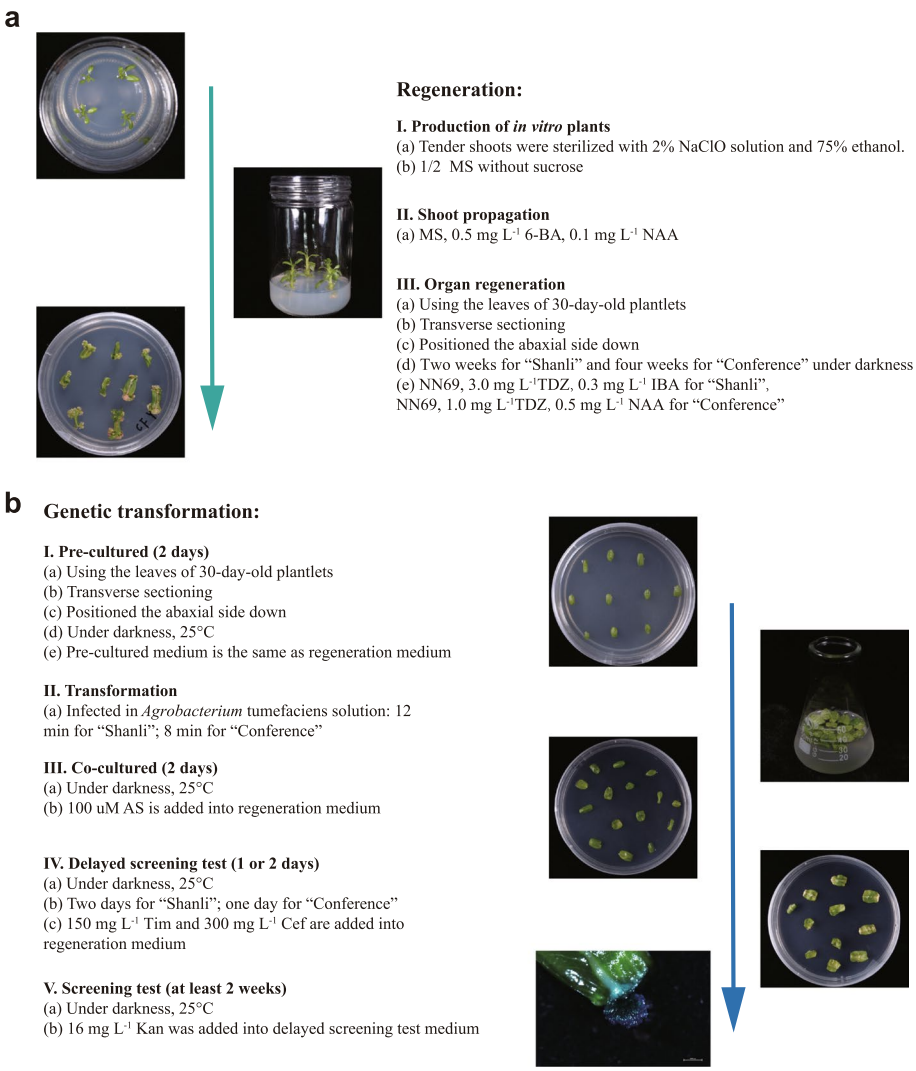


Fig. 7 Schematic model of the regeneration and genetic transformation systems. *Pyrus. ussuriensis* Maxim "Shanli"; *P. communis* L. "Conference"; AS, acetosyringone; NaClO, sodium hypochlorite; Cef, cefotaxime; IBA, indolybutyric acid; Kan, kanamycin; NAA, 1-naphthaleneacetic acid; TDZ, thidiazuron; Tim, timin

transformation systems. Recent advancements in *P. betulifolia Bunge* "Duli" have led to the establishment of effective transformation systems using cotyledons and hypocotyls as viable explants (Xiao et al. 2022). These developments underscore the importance of future research projects exploring transformation methods and creating efficient regeneration systems utilizing various tissue types.

Conclusions

In conclusion, this study on the regeneration and genetic transformation of *P. ussuriensis* Maxim "Shanli" and *P. communis* L. "Conference" has provided valuable insights into the conditions suitable for leaf regeneration and genetic modification in pear. A comparison of the regeneration-associated factors revealed that "Shanli" exhibited a higher regeneration frequency and an average number of regenerated shoots than "Conference". These findings can be a reference for optimizing regeneration and *Agrobacterium*-mediated transformation systems in other pear accessions. By understanding the factors that contribute to successful regeneration and genetic transformation, researchers can work toward improving the efficiency and applicability of these techniques in different pear cultivars.

Abbreviations

6-BA	6-Benzylaminopurine
Cef	Cefotaxime
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GUS	Beta-glucuronidase
IBA	Indolybutyric acid

Kan	Kanamycin
MS	Murashige and Skoog
NAA	Naphthalene Acetic Acid
NN69	Nitsch and Nitsch 1969
TDZ	Thidiazuron
Tim	Timentin

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1007/s44281-023-00017-0.

Additional file 1: Supplemental Figure 1. Regeneration of in vitro leaves on different medium type supplemented with 3.0 mg·L^{-1} TDZ and 0.3 mg·L^{-1} IBA. *Pyrus. ussuriensis* Maxim "Shanli"; *P. communis* L. "Conference"

Additional file 2: Supplemental Figure 2. Regeneration of in vitro leaves with different plant growth regulators. *Pyrus. ussuriensis* Maxim "Shanli"; *P. communis* L. "Conference"

Additional file 3: Supplemental Figure 3. Regeneration of in vitro leaves using different plantlet age. *Pyrus. ussuriensis* Maxim "Shanli"; *P. communis* L. "Conference"

Additional file 4: Supplemental Figure 4. Regeneration of in vitro leaves using different wounding methods. *Pyrus. ussuriensis* Maxim "Shanli"; *P. communis* L. "Conference".

Additional file 5: Supplemental Figure 5. Regeneration of in vitro leaves by different positioning of leaf section. *Pyrus. ussuriensis* Maxim "Shanli"; *P. communis* L. "Conference".

Additional file 6: Supplemental Figure 6. Regeneration of in vitro leaves with different duration of darkness. *Pyrus. ussuriensis* Maxim "Shanli"; *P. communis* L. "Conference".

Additional file 7: Supplemental Table 1. List of primers used in this study.

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Authors' contributions
JW conceived and designed the experiments. SG and MX performed the experiments. CX and SG performed the data analysis. JW and KG provided suggestions for experiments. CX and JW wrote and revised the manuscript.

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Availability of data and materials
The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
Authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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