A Rapid and Efficient Protocol for Adventitious Shoot Regeneration and Genetic Transformation of Manchurian Ash (*Fraxinus mandshurica* Rupr.) using Hypocotyl Explants

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Research article

Keywords: Adventitious shoots, Fraxinus mandshurica, hypocotyl explants, tissue culture, Agrobacterium-mediated transformation

Posted Date: September 17th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-60987/v1

Abstract

Background: Manchurian ash (*Fraxinus mandshurica* Rupr.) is an endangered hardwood tree species, providing both economic and medicinal benefits. However, observations such as browning of adventitious shoot buds and high rate of somatic embryo abnormality, were presented in protocols of *F. mandshurica* regeneration. Therefore, a rapid and high-efficiency regeneration system is demanded for mass propagation and genetic transformation of *F. mandshurica*.

Results: We have developed an efficient regeneration system through adventitious shoot organogenesis in F. mandshurica using hypocotyl explants, with which the adventitious shoots are able to elongate and were obtained in an affordable time. Hypocotyls excised from embryos were pre-cultured in the dark on woody plant medium (WPM) supplemented with 6 g L -1 potassium citrate, and then inoculated on WPM medium supplemented with different concentrations of plant growth regulators (PGRs) to induce adventitious shoot bud formation. The induction medium supplemented with a single PGR of 1.0 mg L -1 thidiazuron (TDZ) was the best treatment, showing 86.67% shoot bud induction with a 15-day initial dark culture, followed by culture under a low light condition. The survival rate of regenerated shoot buds reached 70.97% on WPM medium supplemented with 0.025 mg L -1 TDZ and 1.0 mg L -1 gibberellic acid (GA3). Based on this regeneration system, By using the sonication plus vacuum-infiltration method,a protocol for Agrobacterium tumefaciens -mediated transformation of hypocotyls was established,the transformation rate was determined to be 3.57%.

Conclusions: Key factors, such as the potassium citrate pretreatment, wound treatment on explants, variable light conditions, and significant PGR interactions, were revealed to affect the induction and elongation of adventitious shoots from *F. mandshurica* hypocotyls in this study. The adventitious shoots, tissue culture plantlets, and rooted plantlets were obtained at 40, 80-100, and 160 days, respectively. This regeneration system shortens the period of traditional regeneration methods, which require months to induce callus from leaves or stems, and additional several months for organ differentiation. In addition, the *Agrobacterium*-mediated transformation protocol established on the basis of this regeneration system provides a foundation for breeding, genetic improvement and genomic studies of *F. mandshurica*.

Background

Manchurian ash, *Fraxinus mandshurica* Rupr. (family Oleaceae), is a species of *Fraxinus* native to northeastern Asia in northern China (Gansu, Hebei, Heilongjiang, Henan, Hubei, Jilin, Liaoning, Shaanxi, and Shanxi Province), Korea, Japan, and southeastern Russia[1]. It is a large deciduous tree reaching 30 m tall or above (the tallest one reaches beyond 35 m), with a straight trunk up to 2 m in diameter. *F. mandshurica* is tolerant of many soil conditions and does not require particular soil pH except for showing poor growth at high pH. It is occasionally cultivated as an ornamental tree in parts of Canada and the United States. *F. mandshurica* is widely used for furniture and special building materials because of its good timber quality and beautiful texture. However, adult trees of *F. mandshurica* are rarely found to spread across large areas due to long-term overexploitation and widespread deforestation. *F.*

mandshurica is now a threatened species and has therefore been declared a national endangered tree species in China [2, 3]. *F. mandshurica* is also a commonly used species for afforestation. It can form a mixed coniferous-deciduous forest with a variety of coniferous and broad-leaved trees, which is conducive to improving the stability of the forest ecosystem [4].

The propagation of *F. mandshurica* mainly relies on seeds, which can be produced from seed banks or excellent strains for seedling nursery and afforestation. However, it takes approximate 20 to 30 years for *F. mandshurica* to grow from seedlings to adult trees, and another 3 to 5 years for adult *F. mandshurica* to bloom and bear fruit once or twice. Low rates of pollen formation and dispersal due to low efficiency of fertilization and rainfall, as well as the delayed flowering caused by low temperature, greatly affected the fruit set rate of *F. mandshurica*. In addition, seeds of *F. mandshurica* have the characteristics of hard pericarp, thick waxy layer, high oil content, deep dormancy, low germination and propagation rates [5]. *F. mandshurica* seed germination must undergo a vernalization treatment for up to 6 months [6], otherwise it takes two to three years for natural germination. Taken together, these increase the difficulty of *F. mandshurica* seedling raising and limit its reproduction in forest development [7, 8]. During the grafting process, the degree of joining between scions and rootstocks, the quality of scions, and the method of grafting all affect the final survival rate of *F. mandshurica* [9]. Studies on tissue culture of *F. mandshurica*, such as callus culture[10], somatic embryogenesis [11–13], and axillary bud proliferation[14], all demonstrated certain limitations including high rates of adventitious buds browning and somatic embryo deformity, and low rate of shoot elongation. A variety of materials including the vegetative organs and reproductive organs of Fraxinus mandshurica are cultivated, but the best results can only obtain the growth of a single bud, and have not obtained any hyperplastic plants[12].Genetic engineering technologies provide an attractive way to effectively improve current varieties of *F. mandshurica*[15]. However, the low regeneration efficiency has always led to the failure of obtaining transgenic plantlets. Therefore, it is urgent to establish a rapid and high-efficiency regeneration system of *F. mandshurica*.

Flowering time of plants is a complex physiological response induced by various environmental signals. The proteins encoded by *LIGHT-REGULATED1* (*LWD1*) and *LWD2* genes play a crucial role in the photoperiodic pathway for flowering time control in Arabidopsis[16]. However, there are currently very limited reports on the genes related to flowering time in *F. mandshurica*, and no report on successful genetic transformation of *F. mandshurica*. Therefore, we aim to (1) establish a rapid, high-efficiency, and cost effective regeneration system of *F. mandshurica*, (2) develop a protocol for genetic transformation of the *FmLWD1* gene in *F. mandshurica* by taking advantage of the regeneration system.

Results

Induction of adventitious shoot buds from hypocotyls

Six introduction media with different combinations of 6-benzyladenine (BA) and thidiazuron (TDZ) were tested (Table 1). After incubation in the dark for 5 days, visible protuberances were formed on most hypocotyl explants (Fig. 1a-f), with relatively more protuberances on those hypocotyls pre-treated with

potassium acid (Fig. 1g). However, explants without hypocotyls produced no protuberance except for the enlargement in size (Fig. 1h). After 11 days, protuberances and multiple Y-shape outgrowths, which subsequently developed into adventitious shoots, were observed on all hypocotyl explants (Fig. 2a). While those radicle (entire or partial) explants produced only calluses that were white, transparent, globular, and loose (Fig. 2h). Explants were then cultured under light conditions after 15 days due to some protuberances were observed in vitrification.

Table 1
Effect of Plant growth regulator on adventitious shoots regeneration from hypocotyls of *Fraxinus mandshurica*

Medium number	PGR (mg/L)		Hypocotyl
	TDZ	BA	Shoot formation (%)
WY1	0.4		37.50 ± 12.50
WY2	0.6		63.33 ± 5.77
WY3	0.8		56.67 ± 5.77
WY4	1.0		82.22 ± 5.88
WY5	0.08	1.0	80.00 ± 10.00
WY6	0.8	1.0	63.33 ± 5.77
N-WY4	1.0		86.67 ± 5.77
N-WY4: explants were pretreated with potassium citrate for 5 days before inoculated on WY4 medium			

Although all combinations of BA and TDZ in the induction medium showed some level of adventitious shoot bud formation on hypocotyls, the response of hypocotyl explants was variable based on the relative concentrations of the two PGRs (Table 1). The percent of explants with shoot bud induction ranged from 37.50–82.22% on media containing TDZ and zero BA, and increased with an increase in TDZ concentration (Table 1). While an opposite was observed for media supplemented with 1.0 mg L^{-1} BA and TDZ of various concentrations (Table 1). With comparison of the germination rates, a significant difference ($P = 0.009$) was observed among TDZ concentrations, indicating that TDZ is a significant factor in adventitious shoot bud formation from hypocotyls of *F. mandshurica*. We also compared the germination rates between explants pretreated with and without potassium citrate (Table 1), and found, however, no significance ($P = 0.634$).

Both buds (Fig. 3a) and hypocotyls (Fig. 3b) began to turn red after being cultured under light with an intensity of 80 μmol m^{-2} s^{-1} for 3 days. Given the observation of compact calluses (Fig. 3b), we reduced the light intensity to 10 μmol m^{-2} s^{-1} because strong lights may promote the aging of hypocotyls. The buds then turned to green as a consequence, and formed adventitious shoots at around 20 days (Fig. 3c, d). The callus continued expanding (Fig. 3d) even cultured under low light conditions, probably due to

having been incubated under strong lights at an early stage. To confirm this, we cultured the hypocotyl explants, which having been incubated in the dark for 15 days, under low light condition for 20 days, and found no callus was produced (Fig. 4a).

Elongation And Transplanting Of Adventitious Shoots

The explants with adventitious shoots (Fig. 4a) were transferred to elongation media and cultured for 30 days. Eight elongation media with different combinations of PGRs were tested in this study (Table 2). To better elucidate which medium would produce a higher percentage of shoot elongation, we present the percent shoot elongation and percent explant survival (Table 2). The medium supplemented with 0.025 mg L^{-1} TDZ and 1.0 mg L^{-1} gibberellic acid (GA3) proved to be the best elongation medium overall. Even though the percent explant survival (53.33%) appears low, this medium produced the highest percentage (53.33%) of shoots able to elongate (Fig. 4b). On media with higher explant survival rates (medium supplemented with 0.6 mg L^{-1} TDZ and 1.0 mg L^{-1} naphthaleneacetic acid (NAA), and medium supplemented with 0.025 mg L^{-1} TDZ and 0.6 mg L^{-1} BA), the shoots either produced callus that inhibited the elongation, or grew leaves (Fig. 4d). In comparison of the elongation and survival rates of adventitious shoots, a significant difference (P = 0.029) was observed among media with different PGR combinations, indicating that PGR interactions can affect the activity of adventitious shoots during the first round elongation.

Table 2
Effect of Plant growth regulator on shoots elongating

Medium number	PGR (mg/L)				Elongation rate(%)	Survival rate(%)
	TDZ	GA3	BA	NAA		
A1	0.025	1.0			53.33 ± 23.09	53.33 ± 11.55
A2	0.05	1.0			33.33 ± 23.09	33.33 ± 23.09
A3	0.05	2.0	0.6		40.00 ± 20.00	20.00 ± 34.64
A4	0.05	2.0			40.00 ± 34.64	26.67 ± 30.55
A5	0.025		0.6		46.67 ± 30.55	66.67 ± 30.55
A6	0.025		1.5		26.67 ± 11.55	46.67 ± 30.55
A7	1.0		5.0		13.33 ± 11.55	33.33 ± 11.55
A8	0.6			1.0	13.33 ± 11.55	73.33 ± 11.55

All survived explants from the first-round elongation were therefore transferred on the best elongation medium (with 0.025 mg L^{-1} TDZ and 1.0 mg L^{-1} GA3) for an 18-d second round elongation. The mean number of shoots able to elongate per explant were all greater than one (the highest was 2.5) except for

explants from two first-round media (Table 3). There is no significant difference ($P = 0.796$) in the mean number of shoots per explant among different first-round media, indicating the current elongation medium is suitable for explants from various culture conditions. We then cultured another 155 *F. mandshurica* hypocotyls on this elongation medium. Approximate 70.97% explants survived and were able to elongate 3–4 cm after two to three rounds of subculture (Fig. 4c), confirming the stability of this protocol.

Table 3
Elongation sub-culture of adventitious shoots

Shoots source	Elongation rate(%)	Survival rate(%)	Mean No. Seedlings per explant
A1	75.00 ± 12.50	87.50 ± 12.50	1.86 ± 0.15
A2	100.00 ± 0.00	100.00 ± 0.00	2.00 ± 0.20
A3	66.67 ± 33.35	100.00 ± 0.00	0.67 ± 0.34
A4	100.00 ± 0.00	100.00 ± 0.00	2.50 ± 0.25
A5	40.00 ± 10.00	80.00 ± 10.00	0.50 ± 0.25
A6	71.40 ± 14.30	85.70 ± 14.30	1.83 ± 0.17
A7	83.33 ± 15.28	80.00 ± 20.00	2.25 ± 0.25
A8	54.53 ± 9.05	72.70 ± 9.10	1.00 ± 0.25

The rootless plantlets were then transplanted in pods for rooting. Lateral roots were visible after cultured in sealed plastic bags for 10 days (Fig. 5b). The plastic bags were removed after cultured for a total of 30 days (Fig. 5c). After another 30-d culture under 25 °C and 40% humidity, the survival rate of the plantlets reached 72% (Fig. 5d).

Determining the concentration of kanamycin on hypocotyl activity

To determine the appropriate concentration of kanamycin to select putative transformants, hypocotyls were horizontally inoculated to WPM supplemented with different concentrations of kanamycin (Table 4). The mortality rate increased as the concentration of kanamycin increased, reached 93.33% at the kanamycin concentration of 50 mg L^{-1}, and reached 100.00% at the kanamycin concentration of 50 mg L^{-1}. Significant difference was observed among various kanamycin concentrations in mortality rates ($P = 0.001$). Therefore, 30 mg L^{-1} kanamycin was used as the supplement for the selection medium.

Table 4
Effect of kanamycin concentration of *Fraxinus mandshurica* hypocotyls

Kanamycin (mg/L)	death rate (%)
0	13.33 ± 11.55
20	40.00 ± 20.00
30	93.33 ± 5.77
50	100.00 ± 0.00
60	76.67 ± 40.41

Impact of Agrobacterium **infection time on hypocotyl regeneration**

Infection time is an important factor that affects transformation efficiency. To determine the infection time for *Agrobacterium*-mediated transformation, we tested 10, 15, and 20 min using the traditional *Agrobacterium* infection method (Table 5). The highest germination rate (71.62%) was observed in the period of 15 min, with a mortality rate of 21.07%. Neither the induction rate ($P = 0.886$) nor the mortality rate ($P = 0.906$) was observed to be significantly different among different infection times. However, the infection time showed a significant difference in the formation of callus ($P = 0.009$). A larger number and healthier adventitious shoot buds were formed per explant (Fig. 6) if treated for 15 min compared to those treated for 10 or 20 min. Therefore, 15 min was used as the infection time in the following *Agrobacterium*-mediated transformation.

Table 5
Effect of infection time on hypocotyl differentiation of *Fraxinus mandshurica*

Time(min)	Shoot formation (%)	Callus formation (%)	Explants mortality (%)
10	66.67 ± 14.43	33.33 ± 8.34	15.28 ± 13.39
15	71.62 ± 11.51	77.30 ± 9.20	21.07 ± 21.88
20	66.99 ± 14.82	59.08 ± 15.09	22.46 ± 25.38

Agrobacterium **-mediated transformation of** F. mandshurica

In this study, two methods were used for *Agrobacterium* infection. Compared to traditional *Agrobacterium* infection method, hypocotyls treated by using the sonication plus vacuum-infiltration method produced more buds (72.70%) on induction media (Table 6; Fig. 8). All transformants infected by using traditional method showed albino plantlets and finally died (Fig. 7) after cultured on selection media supplemented

with 30 mg L^{-1} kanamycin and 500 mg L^{-1} cephalosporin for 20 days, indicating a lower transformation rate of the traditional *Agrobacterium* infection method in *F. mandshurica*. While for explants infected using the sonication plus vacuum-infiltration method, 3.80% survived after cultured on the same selection medium for 40 days (Table 6). The histochemical staining analysis of infected and cultured hypocotyl for 3 days showed that the wound and surface of the injured hypocotyl explant were blue (Fig. 8), and the transformation rate of transient infection was 93.33%. This suggested that the hypocotyls infected by sonication plus vacuum-infiltration had GUS activity and the surface tissue of hypocotyls was destroyed, which could increase the probability of infection by *Agrobacterium*.The kanamycin-resistant plantlets were subjected to PCR analysis. Target fragments (Fig. 9) were detected in 4 out of 112 plantlets (3.57%), suggesting that the regeneration system from hypocotyl meets the requirements of genetic transformation of *F. mandshurica*.

Table 6
Traditional method and Sonication plus vacuum-infiltration assisted agrobacterium-mediated transformation

	20d Shoot formation(%)	40d Shoot formation(%)	Callus formation (%)	Selective survival rate (%)
Control	88.33 ± 6.03	88.33 ± 6.03	11.76 ± 5.89	0.00 ± 0.00
Traditional method	51.57 ± 9.61	71.62 ± 11.51	77.30 ± 9.20	0.00 ± 0.00
Sonication plus vacuum-infiltration	64.69 ± 15.36	72.70 ± 4.98	2.74 ± 2.54	3.80 ± 2.30

Discussion

TDZ is widely used in *in vitro* plant tissue culture to induce shoot growth[17], somatic embryogenesis[18, 19], protoplast division[20], and protocorm-like body morphogenesis [21]. Zeng et al. [22]found that the frequency of shoot organogenesis of explants cultured with TDZ (71.16%) far surpassed those cultured without TDZ (30.42%). We have showed in this study that the germination of the adventitious buds from hypocotyls of *F. mandshurica* was affected by TDZ concentration. The highest germination rate (82.22%) of adventitious shoot buds from hypocotyls was obtained on the induction medium supplemented with 1.0 mg L^{-1} TDZ, indicating TDZ may play a key role in regulating the germination of adventitious shoot buds on *F. mandshurica* hypocotyls. However, on the induction medium supplemented with both TDZ and BA, the germination rate was decreased with an increase in TDZ concentration (Table 1), suggesting that TDZ alone is able to induce direct organogenesis of hypocotyls. This result is consistent with the previous reports that TDZ can substitute auxins and cytokinins[23, 24], and Murashige and Skoog (MS) medium supplemented with TDZ instead of BA increased the proportion of *F. excelsior* hypocotyl explants that produced adventitious shoots[25]. We also observed that hypocotyls produced more adventitious shoot buds if incubated in the dark, while they aged and formed calluses (inhibited the explant regeneration) under light conditions (Fig. 3). Our result is similar with those of Jain *et al.* [26], who

reported that the basal segment from hypocotyl of *Linum usitatissimum* produced a large number of shoots in a short period with a low concentration of TDZ and dark incubation.

Reports have shown that different portions of the hypocotyls had different regenerative capacities. The hypocotyls with root and apical meristems removed exhibited the highest regenerative capacity[27]. In this study, all explants with hypocotyl could form adventitious buds(Fig. 2a), while explants which only have radicles could only form calluses(Fig. 2h). In addition, difference in germination rate was also observed between hypocotyls with and without potassium citrate pretreatment. Ajay *et al.* [28] revealed in *Sesbania restrata* that adventitious shoot formation from 12-day-old hypocotyl explants had a higher regeneration rate than 14- or 18-day-old hypocotyl explants. Hypocotyl explants of 3-5-day-old (> 80% regeneration rate) Chinese cabbage had more potential to regenerate than 7-day-old (51.1% regeneration rate) explants[29]. Du *et al.*[27] found that with an increasing age of explants, organogenesis potential of explants was decreased in *F. pennsylvanica*. There was no adventitious shoot regeneration when 15-day-old hypocotyls and 7-, 10- or 15-day-old cotyledons were used as explants. It was reported that organic acids were able to enhance the embryogenesis of callus. The potassium citrate pretreatment may cause an alteration in cellular metabolism towards cell differentiation and away from cell proliferation, which promoted the regeneration of peppers [30]. In this study, explants pretreated with 6 g L^{-1} potassium citrate for 3–5 days showed a higher regeneration rate (86.7%) than those explants without pretreatment. Meanwhile, the number of adventitious shoot buds per explant were greater on pretreated explants (Fig. 2g), and it further increased if explants were infected using sonication plus vacuum-infiltration during the *Agrobacterium*-mediated transformation (Fig. 8). This finding is different from those obtained from the regeneration of other *F. mandshurica* [13] or ashes [27], indicating that the regeneration rate of *F. mandshurica* hypocotyls not only depends on the starting hypocotyl explants and time of pretreatment, but also depends on the supplement used in pretreatment and other external stimulations.

In many plants, the whole plant individuals are able to regenerate from small tissues, such as leaves, stems, and roots[31]. An important aspect of organ regeneration is the reactivation of cell proliferation at wound sites, leading to the formation of callus and subsequent establishment of shoot or root apical meristems [32]. In some cases, organogenesis occurs without the formation of callus. This may be caused by genetic regulation of plant cellular reprogramming induced by wounds or plant hormones[33]. In this study, the highest germination rate (86.67%) was achieved for explants pretreated with potassium citrate, while an even higher germination rate (88.33%) was observed during the *Agrobacterium*-mediated transformation when wounds were created on explants by using the sonication and vacuum-infiltration method. This further demonstrates that wounds promoted plant regeneration, which may not necessarily induce callus formation. In addition, the requirement of light with low intensity for producing adventitious shoots suggests that plant regeneration is not only affected by wounds or plant hormones, but also by light intensities.

Conclusions

In this study, a rapid and efficient regeneration system of *F. mandshurica* was developed. The adventitious shoots able to subsequently elongate to vigorous plantlets were obtained from *F. mandshurica* hypocotyls in an affordable time. On the basis of this regeneration system, a protocol of *A. tumefaciens*-mediated transformation of hypocotyl explants was established and positive transgenic plantlets were obtained. The regeneration system, along with the transformation protocol, allowed us to produce the adventitious shoots, tissue culture plantlets, and rooted plantlets in 40, 80–100, and 160 days. Moreover, this regeneration system also established the foundation for forest development, genetic improvement, and gene function verification of *F. mandshurica*.

Methods

Plant materials

Fraxinus mandshurica seeds were collected from the elite varieties of Heilongjiang Province, China: annual seeds obtained from the families of Fraxinus mandshurica No. 70, 46, 76, 64 and 72 and excellent hybrid combinations The seeds were soaked in tap water for 24 hours, and rinsed with running tap water for 1 h. After removal of the pericarps, the seeds were agitated in 75% (v/v) ethanol for 30 s, and then rinsed 3 times with sterile distilled water. The seeds were then again surface sterilized in 10% (v/v) $NaClO_2$ for 10 - 15 min, and rinsed 5 times with sterile distilled water.

Pre-culture of explants

The turgid embryos were extracted from the sterilized seeds under aseptic conditions, and placed on either the WPM (30 g L^{-1} sucrose and 5.6 g L^{-1} agar; pH = 6.0) (Lloyd and McCown 1980) without any supplement or that supplemented with 6 g L^{-1} potassium citrate. The embryos were pre-cultured in the dark at 25 ± 2 ℃ for 3 to 5 days.

Induction of adventitious shoot buds

Radicles and cotyledons were removed from embryos, and the hypocotyls were horizontally inoculated on the different combinations of PGRs media supplemented with WPM to induce adventitious shoot bud formation. The hypocotyls were incubated at 25 ± 2 ℃ for an initial 2 weeks in the dark, followed by cultured under a low light with intensity of 10 μmol m^{-2} s^{-1} for another 20 days. The induction medium is:

WY1：0.4mg·L^{-1}TDZ+WPM+30 g ·L^{-1} sucrose +5.6 g ·L^{-1} agar

WY2：0.6mg·L^{-1}TDZ+WPM+30 g ·L^{-1} sucrose +5.6 g ·L^{-1} agar

WY3：0.8mg·L^{-1}TDZ+WPM+30 g ·L^{-1} sucrose +5.6 g ·L^{-1} agar

WY4：1.0mg·L^{-1}TDZ+WPM+30 g ·L^{-1} sucrose +5.6 g ·L^{-1} agar

WY5：0.08mg·L^{-1}TDZ+1.0 mg·L^{-1}BA+WPM+30 g ·L^{-1} sucrose +5.6 g ·L^{-1} agar

WY6：0.8mg·L^{-1}TDZ+1.0 mg·L^{-1}BA+WPM+30 g ·L^{-1} sucrose +5.6 g ·L^{-1} agar

Elongation of adventitious shoots

All explants initiating shoot buds were transferred to different elongation media. To test the effect of different plant growth regulator combinations, WPM medium was supplemented with different combinations of GA3, TDZ, BA, and NAA. Cultures were incubated under a 16-h photoperiod with a light intensity of 80 μmol m^{-2} s^{-1} at 25 ℃ for 30 days. The elongation and survival rates of shoot buds, and the shoot number on each explant were recorded. A suitable elongation medium was selected for secondary elongation. The elongation medium is:

A1：0.025mg·L^{-1}TDZ+1.0mg·L^{-1}GA3+WPM+30 g ·L^{-1} sucrose +5.6 g ·L^{-1} agar

A2：0.05mg·L^{-1}TDZ+1.0mg·L^{-1}GA3+WPM+30 g ·L^{-1} sucrose +5.6 g ·L^{-1} agar

A3：0.05mg·L^{-1}TDZ+2.0mg·L^{-1}GA3+0.6mg·L^{-1}BA+WPM+30 g ·L^{-1} sucrose +5.6 g ·L^{-1} agar

A4：0.05mg·L^{-1}TDZ+2.0mg·L^{-1}GA3+WPM+30 g ·L^{-1} sucrose +5.6 g ·L^{-1} agar

A5：0.025mg·L^{-1}TDZ+0.6mg·L^{-1}BA+WPM+30 g ·L^{-1} sucrose +5.6 g ·L^{-1} agar

A6：0.025mg·L^{-1}TDZ+1.5mg·L^{-1}BA+WPM+30 g ·L^{-1} sucrose +5.6 g ·L^{-1} agar

A7：1.0mg·L^{-1}TDZ+5.0mg·L^{-1}BA+WPM+30 g ·L^{-1} sucrose +5.6 g ·L^{-1} agar

A8：0.6mg·L^{-1}TDZ+1.0mg·L^{-1}NAA+WPM+30 g ·L^{-1} sucrose +5.6 g ·L^{-1} agar

Rooting and acclimatization of plantlets

The rootless plantlets were transplanted into （9x13cm） plastic pots containing a substrate with autoclaved peat soil and vermiculite (3:1, v/v), and the WPM medium supplemented with 3 mg L^{-1} indole-3-butyric acid (IBA) was added. The pods were placed in sealed （16 x24cm）plastic bags to provide a high relative humidity and grown at 25 °C and 80 μmol m^{-2} s^{-1} photoperiod for 30 days. The plastic bags were then progressively opened and plantlets were cultured under 25°C and 40% humidity for another 30 days.

Agrobacterium tumefaciens strain and plasmid

The *FmLWD1* gene (gifted by Dr. Yang Cao from our laboratory) was inserted into the Proke2 plasmid vector (gifted by Dr. Yang Cao from our laboratory), which carries β-glucuronidase (GUS) fusion gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Figure 10) by using *KpnI* restriction enzyme (*Thermo Fisher* Scientific) digestion. The vector was then introduced into *Agrobacterium tumefaciens* strain LBA4404 (Freeze storage at -80℃ in our laboratory) and used for plant transformation.

Effect of kanamycin on hypocotyl regeneration

To determine the optimal concentration of kanamycin for screening out positive plantlets, 15-day-old hypocotyls were inoculated on WPM media containing kanamycin in a concentration gradient of 0, 20, 30, 40, 50, or 60 mg L^{-1}. Kanamycin was dissolved in sterile distilled water, filter-sterilized (0.22 μm), and added to the medium after autoclaving. The medium without adding kanamycin was set as the control group. For each treatment, 30 hypocotyls were inoculated, and results were recorded after 40 days of being cultured.

Effect of *Agrobacterium* infection time on hypocotyl regeneration

A. tumefaciens was grown in 50 mLYEB liquid medium supplemented with 50 mg L^{-1} rifampicin and 50 mg L^{-1} kanamycin . The culture was incubated overnight at 28 ℃ with shaking (250 rpm). When the culture was at density of 0.6 OD_{600}, bacterial cells were resuspended in the inoculation medium. Hypocotyl segments were immersed in 50 mL *Agrobacterium* suspension for 10, 15, or 20 min. Explants were blotted on sterile filter paper to remove excess bacterial solution before transfer to co-cultivation media (WY4) and incubated in the dark for 3 days. After 3 days co-cultivation, hypocotyl segments were washed 2min times with sterile, distilled water to remove excess bacteria, blotted on sterile filter paper, transferred to the WY4 medium supplemented with 500 mg L^{-1} cephalosporin, and cultured for 3 days. The germination rate of adventitious shoot buds and the mortality rate of explants were recorded after 40 days of culture following the adventitious shoot induction protocol described previously.

Agrobacterium-mediated transformation

Following the optimized infection time (15 min), two methods were used for *Agrobacterium*-mediated transformation. With the traditional method, hypocotyl segments were immersed in 50 mL *Agrobacterium* suspension for 15 min. While with the sonication plus vacuum-infiltration method [34], the explants were first sonicated for 90 s and then vacuum-infiltrated under the pressure of 0.8 Mpa for 15 min. Explants were blotted on sterile filter paper to remove excess bacterial solution before being transferred to co-cultivation media and incubated in the dark for 3 days. After 3 days of co-cultivation, the explants were washed with sterile distilled water, transferred to induction medium. After being cultured for 20 days, explants with adventitious shoots initiated were transferred to selection medium 30 mg L^{-1} kanamycin and 500 mg L^{-1} cephalosporin.

Histochemical GUS assay

Hypocotyl explants were first sonicated for 90 s in liquid induction medium and then vacuum-infiltrated under the pressure of 0.8 Mpa for 15 min in 0.6 OD_{600} Agrobacterium resuspension. After being shaken for 16h, the explants were inoculated into solid induction medium for 2 days. Take out and immersed in GUS buffer solution (25ml 0.2M Na_3PO_4, 6.2ml 0.2M Na_2HPO_4 and 28ml 0.2M NaH_2PO_4 were mixed to form 0.2M phosphate buffer (pH 7.0). The 25ml phosphate buffer was mixed with 0.25ml 0.1M K_3 [Fe

(CN) $_6$], 0.25ml 0.1M K_4 [Fe (CN) $_6$], 0.5ml 0.1M Na_2EDTA and 24ml ddH_2O) in dark at 24 ℃ for 1 h, then GUS buffer solution was sucked out with a pipette.

PCR analysis

Genomic DNA was isolated from leaves of six inde-pendent putative transgenic lines and from one control (non-transformed) plant following either the protocol of the Plant DNA Lsolation Reagent (TaKaRa Da Lian, China).PCR was performed primer set (forward primer MCS1-F 5' CACTATCCTTCGCAAGACCCT 3' and reverse prime MCS1-R 5' CCAGACTGAATGCCCACAGG 3') was designed to amplify a 1353bp PCR product containing CaMV 35S promoter, GUS gene, and 968 bp full length of *FmLWD1* gene. PCR analysis was carried out in a reaction volume of 25 μl containing 12.5 μl of 10 × T5 buffer (TaKaRa Da Lian, China), 1 μl of 10 μmol L^{-1} MCS1-F, 1 μl of 10 μmol L^{-1} MCS1-R , and 1 μl of inde-pendent putative transgenic lines or non-transformed DNA template. DNA from non-transformed plantlets and the recombinant plasmid (pROK2: *FmLWD1-GUS*) were used as negative and positive controls, respectively. The PCR reaction included a pre-denaturation step of 98 °C for 3 min, followed by 30 cycles of denaturation of 98 °C for 10 s, annealing of 55 °C for 10 s, and extension of 72 °C for 30 s, and a final cycle at 72 °C for 4 min. Products from PCR amplification was electrophoresis on 1.0% agarose gel.

Statistical analysis

Data statistics use SPSS 17.0 software analysis.

Abbreviations

TDZ
Thidiazuron
GA3
Gibberellic Acid
BA
6-Benzylaminopurine
NAA
α-Naphthalene acetic acid
IBA
Indole-butyric acid
WPM
Woody Plant Medium

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are availablefrom the corresponding author on reasonable request

Competing interests

The authors declare no competing interests.

Funding

This study was co-funded by Heilongjiang Provincial R&D Project on Provincial Applied Technology (GA19B201) and Open Projects of the State Key Laboratory of Tree Genetics and Breeding. The funders had no role in the study design, analysis, or preparation of the manuscript.

Authors' contributions

LL did the genetic transformation, CY constructed the vectors, ZYG designed the experiment, QFH designed the experiment, analyzed the data and wrote the manuscript. All authors have read and approved the manuscript.

Acknowledgements

We acknowledged Dr. Jing Tianzhong (Northeast Forest University) for help on data analysis.

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Figures

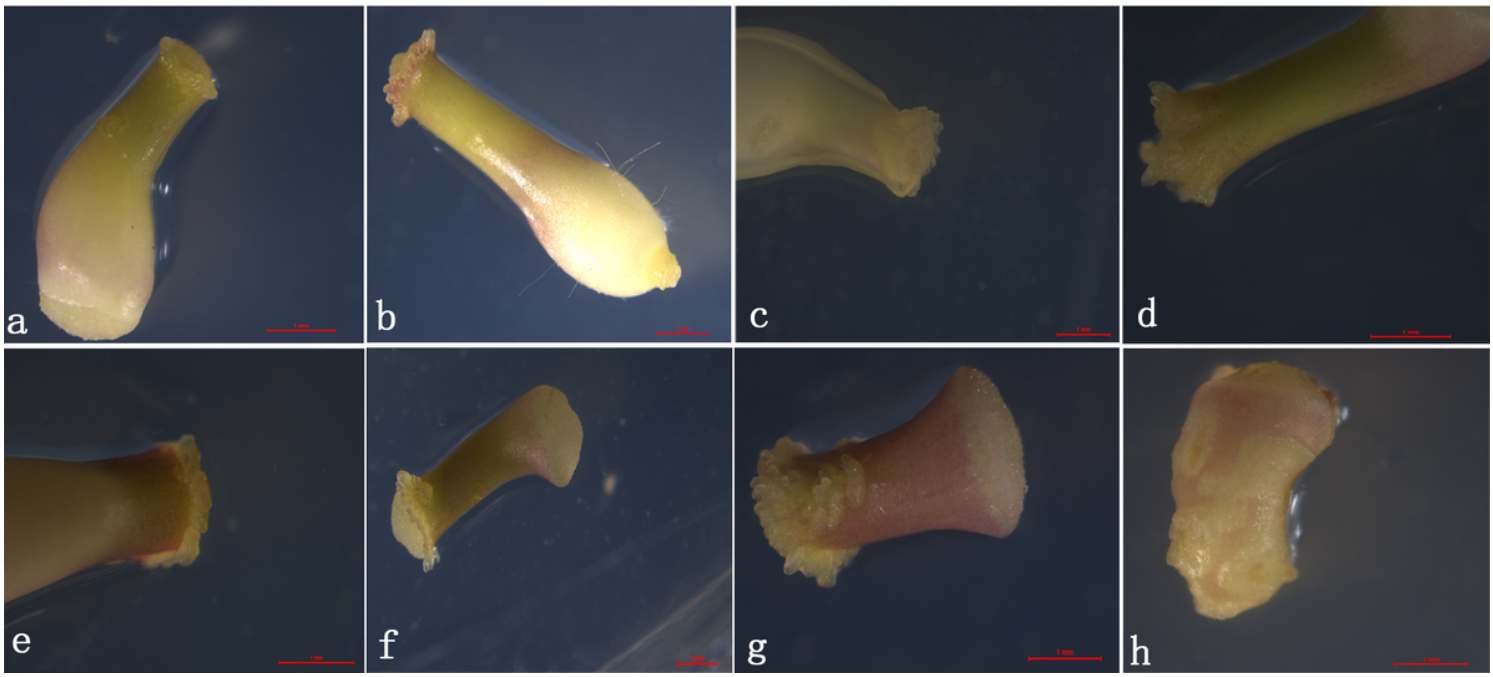

Figure 1

Hypocotyl after dark induction for 5d. a-f Growth situation in induction medium, respectively (bar 1 mm). g Growth situation in contain 1.0mg·L-1 TDZ medium after potassium citrate pretreatment (bar 1 mm). h Growth situation of explants with only the radicle (bar 1 mm)

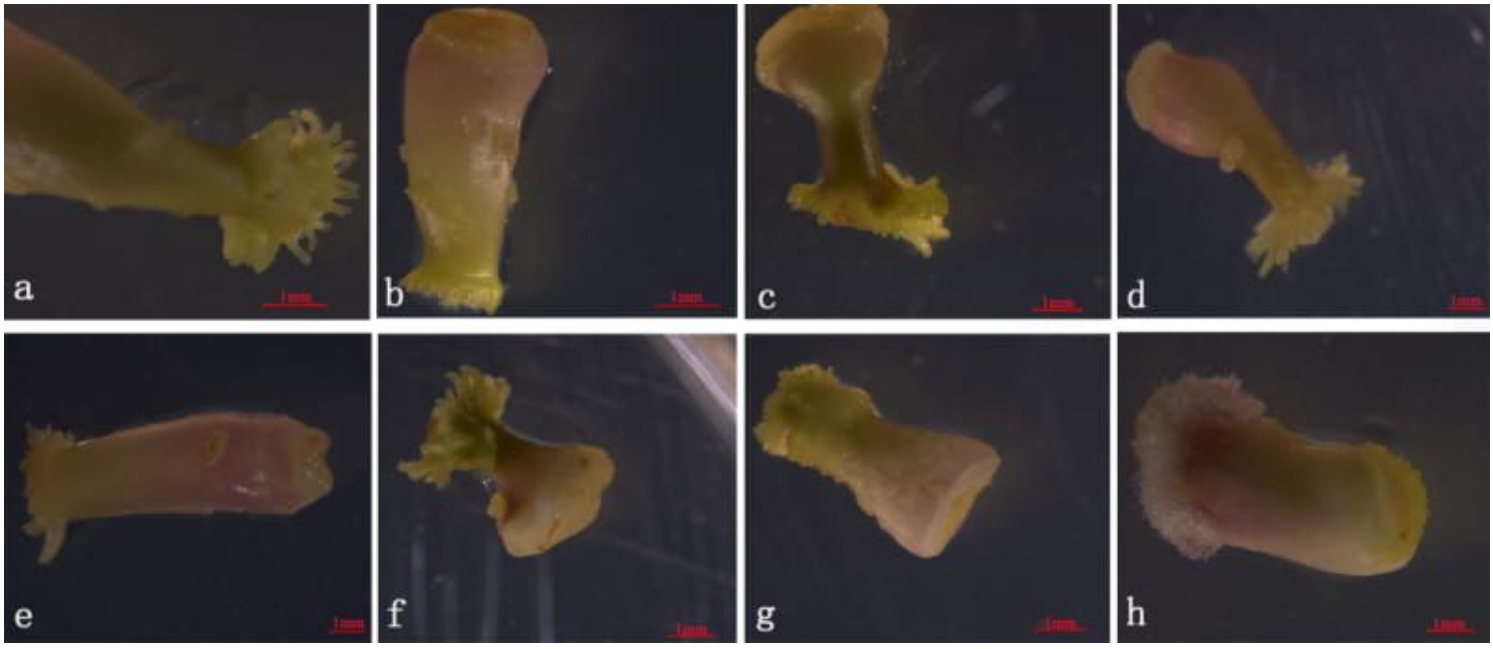

Figure 2

Hypocotyl after induction for 11d. a-f Growth situation in induction medium, respectively(bar 1 mm). g Growth situation in contain 1.0mg·L-1 TDZ medium after potassium citrate pretreatment (bar 1 mm). h Explants with radicles have a white, loose callus(bar 1 mm)

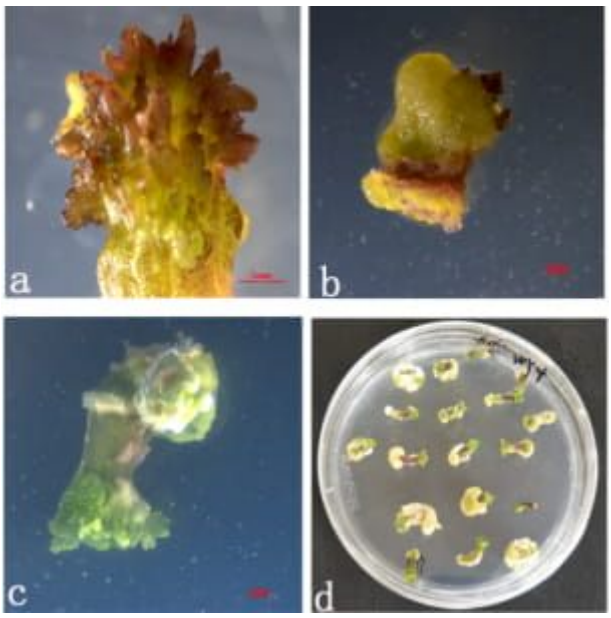

Figure 3

Adventitious shoots formation. a Hypocotyl turns red in light culture (bar 1 mm). b Callus formation(bar 1 mm). c Shoot points of hypocotyl turn green and adventitious shoots formation preliminarily(bar 1 mm). d Adventitious shoots and callus

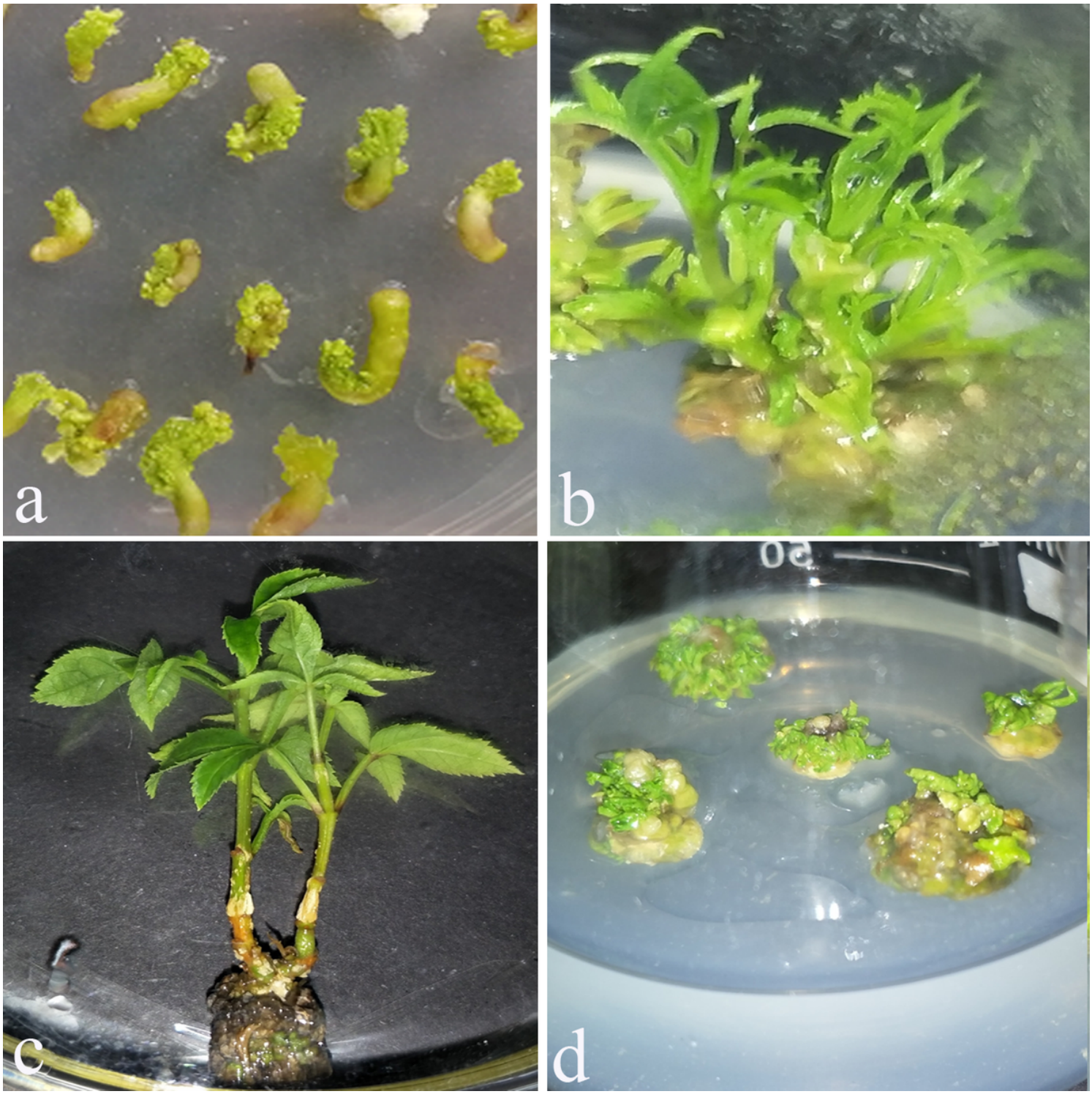

Figure 4

Elongating of Adventitious shoots. a Adventitious shoots of Fraxinus mandshurica under low light condition (see Fig. S1 in Additional file 1 for the original image). b Adventitious shoots elongation in medium supplemented with 0.025 mg·L-1 TDZ and 1.0 mg·L-1 GA3 (see Fig. S2 in Additional file 1 for the original image). c Fraxinus mandshurica regenerated seedlings after 2-3 elongated subculture. d Adventitious shoots fail to elongate

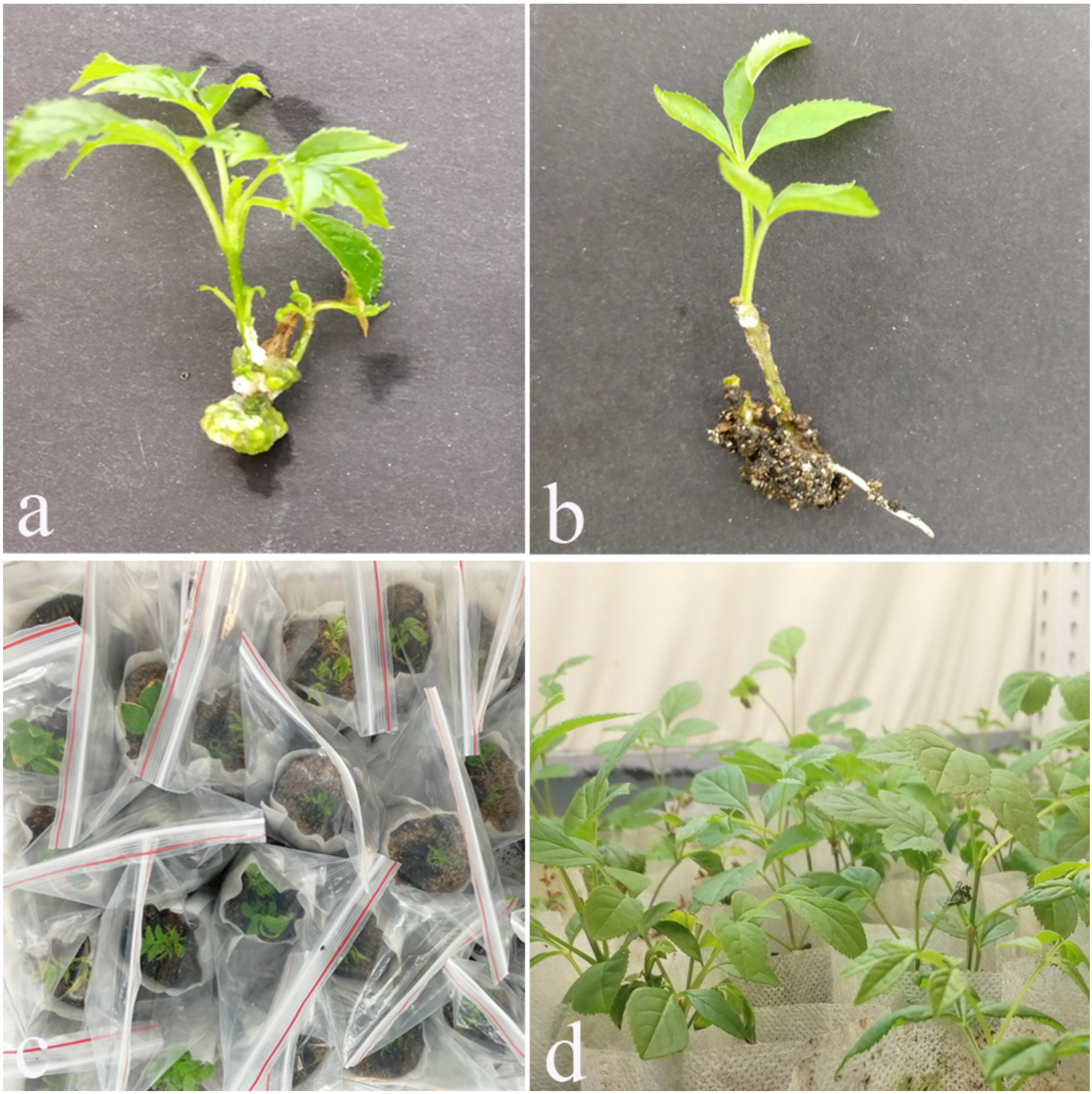

Figure 5

Transplanting and domestication of Fraxinus mandshurica regenerated seedlings. a Healthily-growing rootless regenerated seedling (see Fig. S3 in Additional file 1 for the original image). b in vitro root production. c Transplanted growth of a plant (see Fig. S4 in Additional file 1 for the original image). d Acclimatizatio of Fraxinus mandshurica plants rooted in the culture room (see Fig. S5 in Additional file 1 for the original image)

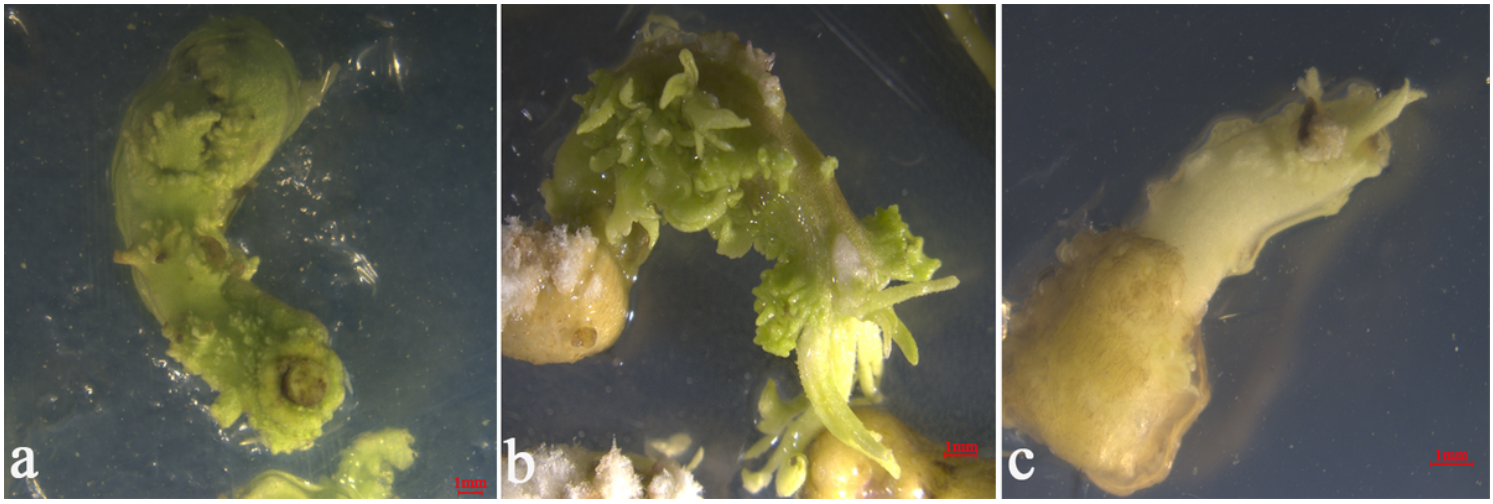

Figure 6

Effect of Agrobacterium infection time on adventitious shoots regeneration induced by hypocotyls of Fraxinus mandshurica. a The infection of 10 min (bar 1 mm). b The infection of 15 min (bar 1 mm). c The infection of 20 min (bar 1 mm)

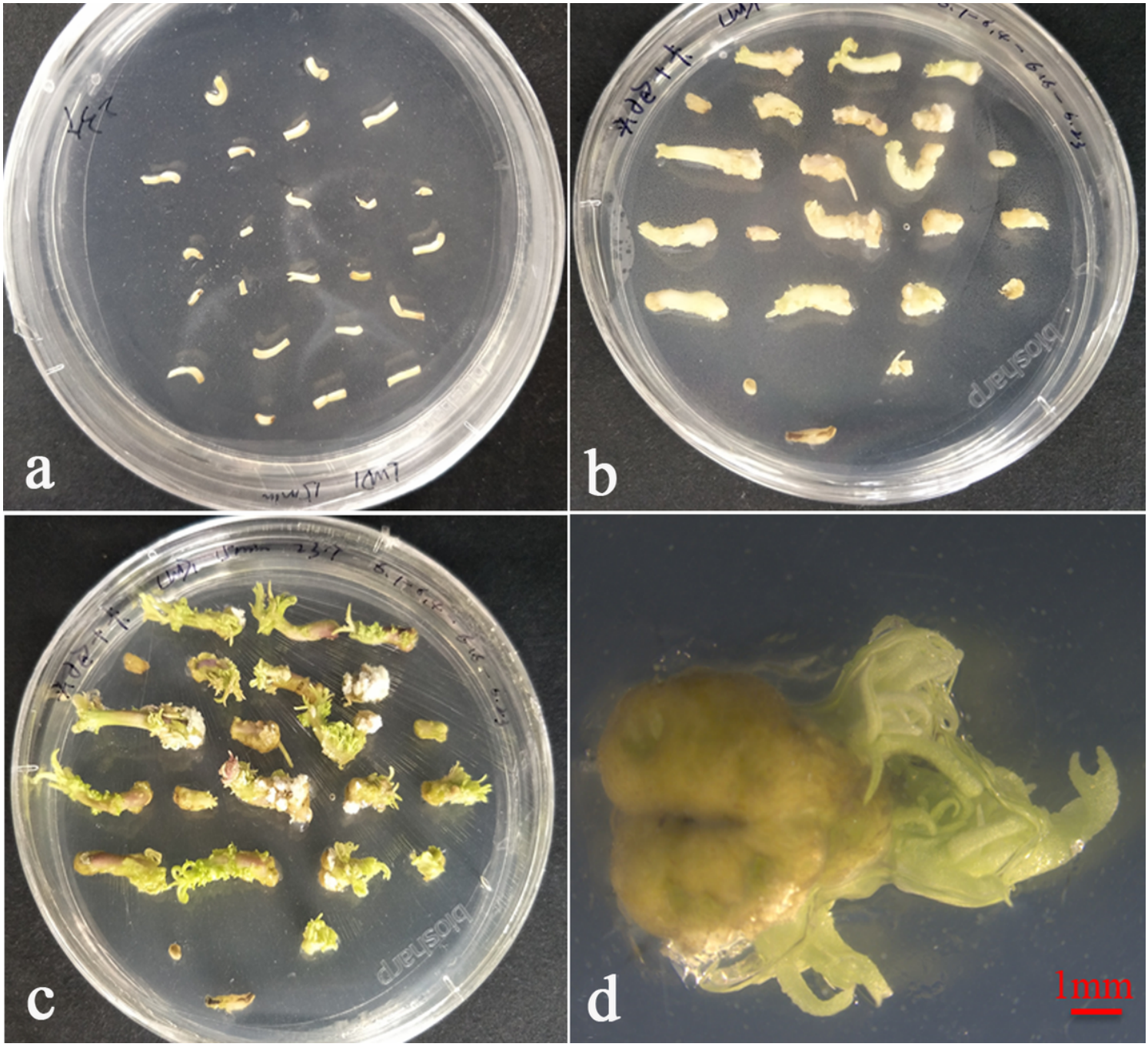

Figure 7

Traditional agrobacterium transformation. a Infection of Fraxinus mandshurica hypocotyl by agrobacterium. b After dark culture and low light induction, transferred to selective medium. c Selective cultivation for 20 days under light conditions. d Albino adventitious shoots (bar 1 mm)

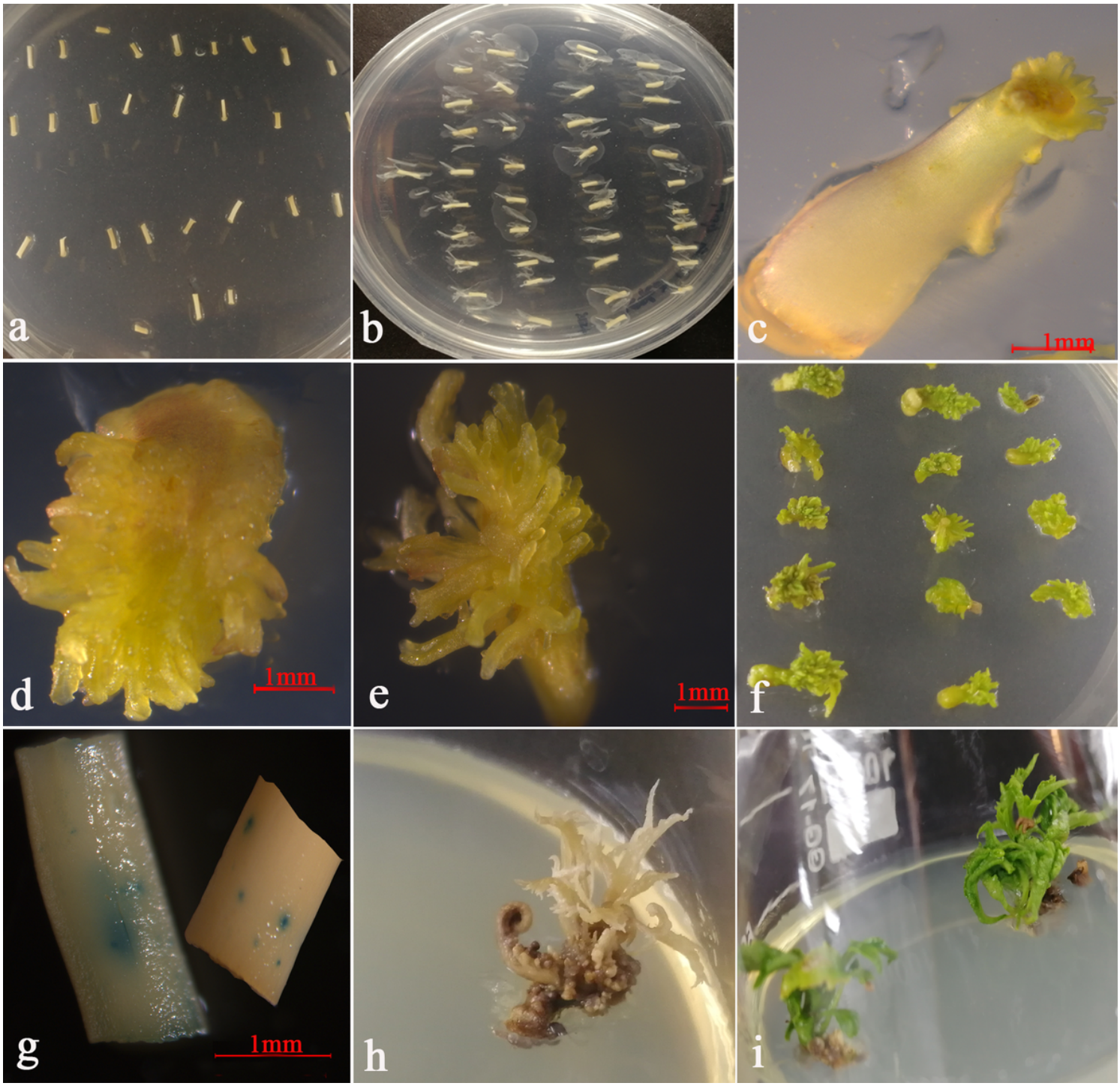

Figure 8

Sonication with vacuum-infiltration assisted agrobacterium-mediated transformation and transient GUS expression assay. a Infecting hypocotyl 1d. b Co-cultivation for 3 days. c Without sonication with vacuum-infiltration treatment of non-transformed material induced for 12 days (bar 1 mm). d Sonication with vacuum-infiltration treatment of non-transformed material induced for 12 days(bar 1 mm). e Sonication with vacuum-infiltration assisted agrobacterium-mediated transformation to induce hypocotyl for 12d(bar 1 mm). f Sonication with vacuum-infiltration assisted agrobacterium-mediated transformation to induce hypocotyl for 22d to obtain regenerated adventitious shoots (see Fig. S6 in

Additional file 1 for the original image). g transient GUS expression assay (bar 0.5 mm) .h Albino death of negative control in selective medium (see Fig. S7 in Additional file 1 for the original image). i Transgenic shoots surviving in selective medium (see Fig. S8 in Additional file 1 for the original image)

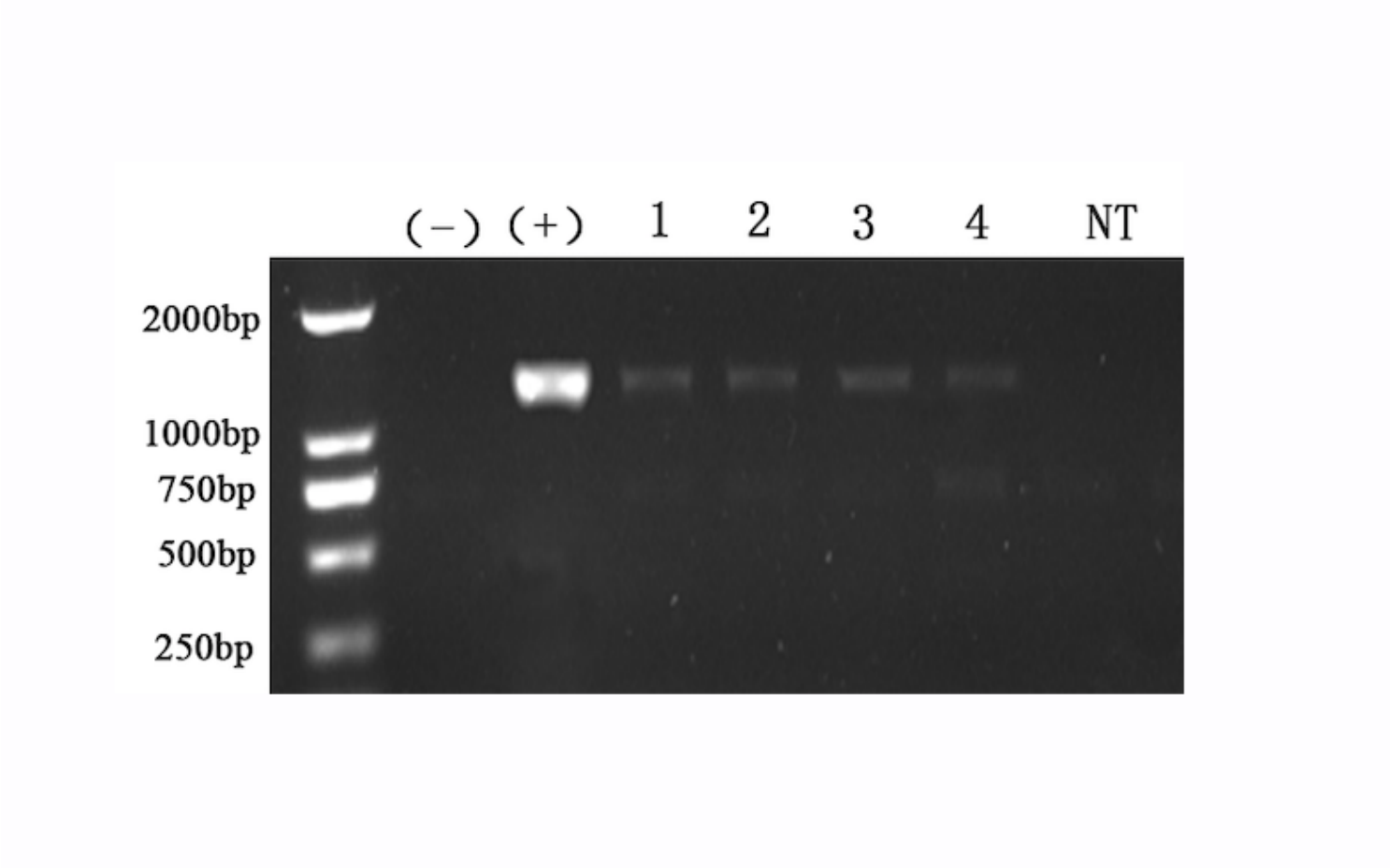

Figure 9

PCR analysis (see Fig. S9 in Additional file 1 for the original image). PCR analysis of non-transformed and transgenic resistant Fraxinus mandshurica for amplification of 1353 bp. M 2000 bp molecular marker. - water control. + positive control. lanes 1–4 transgenic resistant shoots. NT negative control (non-transformed plant)

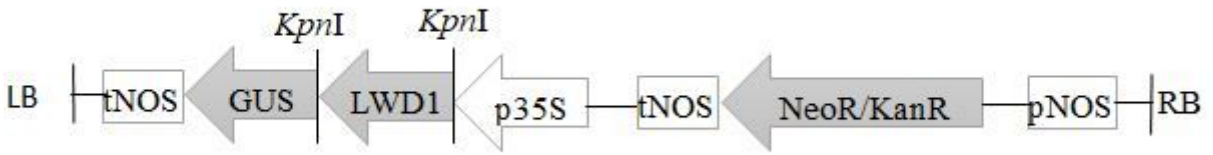

Figure 10

Schematic diagram of T-DNA regions of GUS and Proke2-LWD1 constructs.

Supplementary Files

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