An Efficient and Stable Agrobacterium-Mediated Transformation System for Rubber Grass (Taraxacum Spp.)

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Research

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Abstract

Background

Rubber grass is excellent alternatives to *Hevea* and ideal model plants for natural rubber research through genetic transformation. However, the transformation is unstable due to the high heterozygosity of rubber grass and the efficiency of the existing transformation system could not well satisfy the needs of the studies on key gene functions of natural rubber biosynthesis.

Results

Here, we report an efficient and stable *Agrobacterium*-mediated transformation system of rubber grass species using young leaves as explants. An excellent rubber grass species, named as 12-16, that is suitable for tissue culture was selected from a wild germplasm repository collected from Xinjiang. The total regeneration efficiency of 12-16 which cultivated on shoot regeneration medium (Murashige and Skoog (MS) medium plus 1.5 mg/L 6-benzylaminopurine (6-BA) and 0.1 mg/L naphthaleneacetic acid (NAA)) was 100%, and the excellent regeneration shoots reached in 74.1%. Finally, two genes, rubber elongation factors 138 (*REF138*) and rubber elongation factors 258 (*REF258*) driven under an enhanced 35S promoter were transformed into 12-16 by *Agrobacterium tumefaciens* GV3101 mediated genetic transformation. The final transformation efficiency exceeded 20% and the total transformation cycle from leaf explants to hygromycin-resistant seedling lasted approximately 3–4 months.

Conclusions

We provides a highly efficient transformation system based on rubber grass germplasm 12-16 for the introduction of interest genes into rubber grass and provides important technical support for rubber biosynthesis research.

Background

Natural rubber is a secondary metabolite of rubber-yielding plants and an industrial raw material of global importance that synthetic rubber cannot replace. The rubber tree (*Hevea brasiliensis*) has been an important economic crop, with large scale cultivation in tropical and subtropical regions, which provides 99% of all natural rubber [1]. Nevertheless, *Hevea* is susceptible to *Agrobacterium tumefaciens* and resistant to genetic modification due to its poor regeneration capacity [2, 3]. Although there are a few reports on successful genetic transformation of *H. brasiliensis*, it is still very difficult to verify the function of key genes and promote genetic breeding of this species [4, 5].

Together with *H. brasiliensis* and guayule, rubber grass is one of the world's three major gum plants. Rubber grasses are plants of *Taraxacum*, which contains a variety of plants including *Taraxacum kok-saghyz* Rodin (Russian dandelion, TK) and *Taraxacum brevicorniculatum* (TB). The roots and leaves of TK and TB have ductal systems, and the structure and properties of rubber are similar to those of the *H.*

brasiliensis [6]. The natural rubber of TK can account for up to 20% of its dry weight in the perennial roots [7]. In addition, TK grows in a wide range of temperate areas, while *H. brasiliensis* grows only in the tropics. Therefore, TK is an excellent alternative to *H. brasiliensis* and has good development prospects as a rubber production plant. Moreover, TK is herbaceous plant with a short life-cycle, a relatively simple genome (approximately 1.4 Gb, forming 2n = 16 chromosomes), and is relatively easy to culture and manipulate in transgene operations. These characteristics make TK an ideal model for natural rubber research [8, 9]. TB is a vigorous apomictic rubber producing dandelion. Although the latex content of TB is lower than that of TK, the growth activity and regeneration ability of TB in tissue culture are better than that of TK. Therefore, TB has been used as a model plant for rubber biosynthesis studies [10, 11]

There are some previous reports on the regeneration of different types of dandelion including *Taraxacum officinale* [12], *Taraxacum platycarpum* [13], *Taraxacum brevicorniculatum* [14, 15] and *Taraxacum kok-saghyz* [12, 16]. However, the transformation is unstable due to the high heterozygosity [17] and the efficiency is insufficient for use as a model plant for studies on key gene functions of natural rubber [13, 15].

In this study, we collected numerous wild rubber grass species from Xinjiang, China. According to the regeneration efficiency, growth rate and growth status of regenerative shoots, we selected 12–16 as an excellent variety suitable for tissue culture. In this study, we established an efficient and stable *Agrobacterium*-mediated transformation system of 12–16. Through optimization of the tissue culture media and improvement of genetic transformation methods, we successfully induced and obtained complete transgenic plantlets from leaves using a highly efficient, simple and rapid technique. Based on this system, we introduced and expressed two key genes in the *Hevea* rubber synthesis pathway into 12–16. The method reported here allows rubber grass regeneration and transformation using leaves as explant material and is applicable to the study of gene function of natural rubber biosynthesis.

Results

Selection of excellent germplasm suitable for tissue culture

We collected wild rubber grass germplasms from Xinjiang area, and selected the most suitable germplasm for tissue culture of cluster shoots. After 60 days in culture on regeneration medium A, the number of induced shoots and regeneration efficiency of 15 wild rubber grass species were calculated. The plants were then divided into four screening levels based on the status of the leaves of the regenerated shoots on the explants. Level I was regarded as the best level. The screening criteria were leaf color (ranging from light green to green), leaf length > 3 cm and no vitrification. In this study, 12–16 yielded the highest regeneration efficiency of 74.1% at level I, with a total regeneration efficiency of 100%. Thus, 12–16 was obviously superior to the other germplasms and was chosen as the plant material for subsequent *Agrobacterium*-mediated transformation studies (Table 1).

Table 1
Analysis of regeneration shoots of different wild rubber grass species.

Germplasm number	I		II		III		IV		Number of total explants
	A	B(%)	A	B(%)	A	B(%)	A	B(%)	
12 – 1	-	-	-	-	4	20	-	-	20
12 – 2	-	-	2	15.2	5	38.5	-	-	13
12 – 4	-	-	3	30	1	10	-	-	10
12 – 6	-	-	3	23.1	-	-	-	-	13
12 – 7	3	11.1	6	22.2	4	14.8	-	-	27
12 – 9	3	17.6	14	82.4	-	-	-	-	17
12 – 10	-	-	-	-	7	43.8	-	-	16
12 – 11	-	-	15	60	-	-	-	-	25
12–12	-	-	1	7.7	-	-	-	-	13
12–13	1	8.3	4	33.3	3	25	-	-	12
12–14	1	9.1	2	18.2	2	18.2	-	-	11
12–15	3	21.4	5	35.7	6	42.9	-	-	14
12–16	40	74.1	10	18.5	4	7.4	-	-	54
12–17	-	-	-	-	-	-	17	100	17
12–18	-	-	-	-	-	-	14	100	14

Note: I, Excellent regeneration shoots, leaf color ranging from light green to green, leaf length > 3 cm, no vitrification; II, Good regeneration shoots, leaf color ranging from light green to green, 3 cm > leaf length > 1.5 cm, no vitrification; III, Medium regeneration shoots, leaf color ranging from light green to green, 1.5 cm > leaf length > 0.5 cm, no vitrification; IV, Poor regeneration shoots, leaf color ranging from yellow or brown, leaf length < 0.5 cm, vitrification; A, Mean number of regeneration shoots; B, Mean regeneration efficiency.

Identification of wild rubber grass species of Xinjiang

In this study, cleaved amplified polymorphic sequences (CAPS) were used as species-specific markers to distinguish four eugonic wild rubber grass species, 12 – 7, 12 – 9, 12 – 11 and 12–16, from TO. The C3 (667 bp) and C5 (462 bp) markers were amplified from the genomic DNA of all four rubber grass species and *Taraxacum officinale* (TO). In restriction enzyme digestion of the C3 and C5 markers with *Kpn* I and *Taq* I, respectively, the C3 marker of TO was digested, while the C5 marker of rubber grass species was not (Fig. 1A). In addition, the C5 marker of TO was not digested, while the C5 marker of rubber grass species was digested (Fig. 1B). These results are consistent with the TK identification results [18] and confirmed that these germplasms were rubber grass, not TO.

Comparison of different regeneration media

Various types of regeneration medium have been reported previously. To achieve the highest regeneration efficiency in pieces of 12–16 leaves collected from Xinjiang, we analyzed the ability of medium B [14], medium C [16] and medium A, formulated in our laboratory, to induce shoots clusters. After 20 days in culture, clustered shoots began to appear from the main veins of the leaf disc. The regeneration efficiency was determined after two subcultures and total 60 days in culture. Among the three regeneration media, medium A, containing 1.5 mg/L 6-BA and 0.1 mg/L NAA, provided the highest regeneration efficiency for 12–16 explants (Table 2).

Table 2
Efficiency of regeneration of 12–16 leaf pieces using different regeneration media.

Regeneration medium	Regeneration efficiency (%)	Mean regeneration efficiency (%)
A	85	87.33 ± 2.52^{a}
	90	
	87	
B	25	26.67 ± 2.89^{c}
	30	
	25	
C	75	72.33 ± 2.52^{b}
	70	
	72	

Note: Regeneration media were MS media supplemented with (A) 1.5 mg/L 6-BA + 0.1 mg/L NAA, (B) 1.0 mg/L 6-BA + 0.2 mg/L NAA and (C) 1.0 mg/L 6-BA and 0.2 mg/L IAA. Data were collected after two subcultures and 60 days in culture. Data represent the means ± standard error of n = 3 biological replicates. Means denoted by the same letter do not significantly differ as determined by the Student's t test.

Optimization of hygromycin concentration for selection of 12–16 transformants

To identify the exact concentration of hygromycin for selection of 12–16 regeneration shoots, we screened 12–16 explants cultured in regeneration medium A containing 0, 4, 6, 8, 10, 12, 14, and 16 mg/L hygromycin. After 30 days in culture, many regenerated shoots were induced, and lengths of approximately 1–1.5 cm in the control group (0 mg/L hygromycin) (Fig. 2A). In the group treated with 4–6 mg/L hygromycin, the number of regenerated shoots was reduced, although the length still reached 8 mm (Fig. 2B–C). In the group treated with 8–10 mg/L hygromycin, there was a small amount of shoot regeneration, although the growth of the shoots was significantly inhibited, with an average length of < 5 mm (Fig. 2D–E). In the group treated with hygromycin at 12–16 mg/L, almost all the explants were dead and brown in color (Fig. 2F–H). The regeneration of shoots was more significantly decreased and

the growth of shoots was more seriously inhibited, with only a few regenerated shoots < 2 mm in length (Fig. 2C–D). Based on the combination of the regeneration efficiency and growth status in the different treatment groups, 8–10 mg/L hygromycin was selected as the best concentration for screening and selection of the shoots regenerated in subsequent genetic transformation experiments.

Agrobacterium-mediated transformation of 12–16

Agrobacterium-mediated transformation was performed using the established efficient tissue culture system. After 2 days of preculture, the leaf pieces were co-cultivated with the *Agrobacterium* containing binary vectors for 3 days (Fig. 3A). To restore the cells injured during *Agrobacterium* infection, the leaf pieces were cultured for 7 days in regeneration medium A containing 400 mg/L carbenicillin. During this period, a small amount of callus developed on the leaf pieces (Fig. 4B). Subsequently, the pieces were transferred to medium A containing 10 mg/L hygromycin and 400 mg/L carbenicillin to screen hygromycin-resistant regenerated shoots. Induction of shoot regeneration began after 5–15 days in culture in the screening medium (Fig. 3C). Then the hygromycin-resistant regenerated shoots continued to grow and even form clustered shoots in the screening medium, while the growth of other non-resistant regenerated shoots was severely inhibited, with increased vitrification and browning and necrosis observe at the leaf tips (Fig. 3C). Screening of the hygromycin-resistant regenerated shoots during this period was easily accomplished. After 75–90 days, when the hygromycin-resistant regenerated shoots reached 3–5 cm in length, they were separated and transferred to rooting medium for root induction. In this study, rooting medium consisted of 1/2 MS medium with 8 mg mg/L hygromycin and without any hormones [19]. All the hygromycin-resistant regenerated shoots produced a robust root system after 30–45 days in culture (Fig. 3D) and the survival rate of the regenerated seedlings after transplanting into soil was 100% (Fig. 3E–F, Table 3).

Table 3
Transformation efficiency and rooting and seedling survival rate analysis of regenerated shoots using different binary vectors.

Vectors	Explants	Resistant plantlets	Positive rate (%)	Transformation Efficiency (%)	Rooting rate (%)	Survival rate (%)
pHbREF138	132.67	33.67	94.83	23.98 ± 2.01[a]	100	100
pHbREF258	123	30.67	93.10	23.06 ± 2.09[a]	100	100

Note: Data represent the means ± standard error of n = 3 biological replicates. Means with the same letter are not significantly different. Positive rate (%) = (the number of positive transgenic plantlets/the number of putative transgenic plantlets) × 100%. Transformation efficiency (%) = (the number of positive transgenic plantlets/the number of infected explants) × 100%. Rooting rate (%) = (the number of rooted seedlings/the number of putative transgenic plantlets) × 100%. Survival rate (%) = (the number of the surviving seedlings/the number of the rooted seedlings. Data represent the means ± standard error of n = 3 biological replicates

Molecular evaluation of transgenic lines

Insertion of the exogenous genes into the putative transgenic plantlets genome was confirmed by polymerase chain reaction (PCR) and quantitative PCR (qPCR) analyses. In this study, two different binary expression vectors, *pHbREF138* and *pHbREF258*, were transformed into 12–16. PCR analysis using specific primers yielded bands of the expected sizes for 600 bp of *REF138* and 1027 bp of *REF258* in the most of the putative transgenic plantlets; the results for 22 of the represented lines are shown in Fig. 4A. The PCR-positive detection rate reached 94.83% for *REF138* and 93.10% for *REF258*. The data showed a very low rate of false positive results for the resistant plantlets. The final transformation efficiencies using the transformation method established in this study were 23.98% for *REF138* and 23.06% for *REF258* (Table 3). All the positive transgenic plantlets form robust root systems in the rooting medium containing 8 mg/L hygromycin, and all survived after transplantation (Table 3).

To further verify gene expression in the heterologous transgenic lines, six transgenic lines of *REF138* and *REF258* were randomly selected and analyzed by qPCR. The results showed that the exogenous genes were successfully expressed in the transgenic plants, while none of these genes were expressed in the 12–16 control (Fig. 4B).

Discussion

For many years, *Agrobacterium*-mediated transformation of various *Taraxacum* species has been reported in studies of gene function in natural rubber synthesis and this model has gradually developed as an important research tool [12, 13, 14, 16]. But there are few reports on genetic transformation of dandelions, including TK, TB and TO. Due to high heterozygosity and complex germplasm sources [9, 20], this results in significant differences in tissue regeneration capacity and transformation efficiency of rubber grass species from different sources, and has made it difficult to repeat published results. Moreover, the transformation efficiency is low and insufficient for use of this as a model plant for investigations of natural rubber synthesis. In this study, we identified an excellent rubble grass species from Xinjiang and established an efficient and stable *Agrobacterium*-mediated transformation system using leaf discs as explants.

The identification of excellent rubber grass species represents an important breakthrough in the development of a model plant for research purposes. Xinjiang is a vast territory with a large number of wild rubber grass resources. In 2016, Yang et al. used 23 simple-sequence repeats (SSR) markers to analyze the genetic diversity of wild rubber grass materials collected from Russia (n = 2), the USA (n = 11) and Xinjiang (n = 83). The results showed widespread genetic polymorphisms and complex genetic relationships among the wild rubber grasses collected in Xinjiang [21]. In addition, correlation analysis of eight phenotypic traits in three wild rubber grass populations collected from Yili in Xinjiang revealed rich phenotype diversity rich within the populations, with significant differences in the phenotypic characteristics among the populations [22]. The rich diversity of wild rubber grass resources indicates the existence of many genetic variations that can be explored and utilized. We collected several batches of wild rubber grass species from Xinjiang, and selected four eugonic germplasms for further species-specific identification using CAPS markers after phenotypic characteristic screening. The results showed

that all four germplasms were rubber grasses, which was consistent with the phenotypic identification. This, in turn, confirmed that phenotypic traits are the most basic method and approach to the identification of rubber grass species.

The selection of excellent germplasms suitable for tissue culture is the important material basis of efficient and stable genetic transformation system. In this study, after screening for excellent germplasms collected from Xinjiang, we focused on 12–16. This germplasm yielded the highest regeneration efficiency at level I (Table 1) and selected the leaf material for subsequent genetic transformation experiments. In subsequent experiments, we found that 12–16 had stable and high regeneration ability, as well as the advantages of rapid and continuous growth activity. These characteristics also facilitated genetic transformation, with significantly higher (2–4-fold) transformation efficiency (Table 3) than that reported previously [13, 15]. Furthermore, previous reports did not show a link with germplasm screening, which we have included in this study for the first time.

The simple tissue media developed in this study reduces the experimental workload and more efficiency. The entire tissue culture cycle requires only shoot regeneration medium and rooting medium, while elongation medium is unnecessary. The formula for the two media is very simple. Shoot regeneration medium consists of MS plus 1.5 mg/L 6-BA and 0.1 mg/L NAA, and rooting medium comprises 1/2 MS without any plant hormones. The reduction in the experiment steps and the simplification of the medium formulation not only reduces the experimental cost, and workload, but also improves the practical operability and probability of success, providing a guarantee of a large number of genetic transformants.

The short tissue culture cycle also accelerates the research. In this study, it took 7–15 days from leaf preparation (I) to callus induction (□), when a small amount of callus was produced. The shoot regeneration stage (□) occurred from 15–75 days, during which a large number of shoot clusters was induced. During the rooting stage (□) of the regenerated seedlings on days 75–105, sufficient numbers of roots could be grown to ensure survival of the seedlings after transplantation (Fig. 5). Therefore, the total tissue culture cycle from leaf to integrated seedling was completed in 3 months, which is consistent with previous reports [13].

The appropriate concentration of hygromycin for screening not only ensures sufficient regeneration efficiency, but is also beneficial for screening of resistant regenerated shoots. In this study, 10 mg/L hygromycin was used to select transformed regenerated shoots. This concentration is lower than that used for *Taraxacum platycarpum* [13]. The callus and shoots induced by explants that failed in transformation showed multiple symptoms: (1) severe inhibition of growth; (2) easy vitrification; and (3) browning and necrosis of the tips of seedling leaves (Fig. 3B–C). Based on these characteristics, the regenerated buds that failed in transformation were removed, and the final positive detection rate of putative transgenic plantlets was over 90% (Table 3). The results showed that 10 mg/L hygromycin was suitable for screening resistant regeneration shoots.

The system established in this study provides the advantage of high transformation efficiency. The final transformation efficiency of three transformation events was stable in more than 20% of transformants

(Table 3), which is higher than previous reports of 10% [15] and 2–5% [13]. High transformation efficiency is one of the most important conditions for a model plant suitable for natural rubber research and the transformation efficiency of our system fully meets this requirement.

Conclusions

In brief, we screened an excellent rubber grass species suitable for tissue culture from Xinjiang. Based on this germplasm 12–16, by further optimizing the culture medium and simplifying the genetic transformation, we developed an efficient and stable *Agrobacterium*-mediated transformation system using leaves pieces as explants. The study also provides a transgenic platform for future natural rubber biosythesis research.

Methods

Plant material and cultivation conditions

Seeds of wild rubber grass were collected from the Tekes river basin in Xinjiang, China (LNG/LAT: 81°11'33"/42°52'58"). The seeds were sterilized by washing in 70% ethanol for 30 s, followed by washing with sterile water. The seeds were then sterilized by washing in 1% sodium hypochlorite solution for 15 min, followed by five rinses with sterile water. Subsequently, the seeds were germinated on solid half strength Murashige and Skoog (1/2 MS) medium (1/2 strength MS micro- and macro-salts (Hope, Qingdao, China), supplemented with 20 g/L sucrose, and 8 g/L agar, pH 5.8) in the culture flask [19]. The flasks were placed in a plant light incubator under cultivation conditions of a 16-h light/8-h dark photoperiod, 22 °C and approximately 70% relative humidity (RH) for 2 weeks. The seedlings were transferred to 1/2 MS medium and cultured under the same conditions for a further 8 weeks. Healthy young leaves were cut from seedling and placed on sterile filter paper. The main veins and 2/3 width of the leaves were cut at 1 cm intervals with an aseptic scalpel. The leaf pieces were then moved to regeneration medium for regeneration of seedlings or transformation mediated by *Agrobacterium tumefaciens.*

To retain juvenility and expand the propagation of seedling, root fragments were cut from vigorous plants and incubated on solid 1/2 MS medium without any hormones [19]. The adventitious shoots began to be induced from roots in a plant light incubator under the same conditions (16-h photoperiod, 22 °C, approximately 70% RH) for two weeks. After 30–45 days in culture, the adventitious shoots were detached from the roots and moved to root induction medium to induce the root growth.

Molecular identification of wild rubber grass species

To discriminate the wild rubber grass species and the common dandelion (*Taraxacum officinale*, TO) at the molecular level, PCR analysis of the genomic DNA of both species was performed using cleaved amplified polymorphic sequences (CAPS) as species-specific markers [18]. The primers and restriction enzymes used for marker detection are included in Supplementary Table S1.

Binary vector and Agrobacterium strain

The binary expression vectors *pSuper1300*, *pHbREF138* and *pHbREF258*, which contain the prokaryotic kanamycin resistance gene. The T-DNA of the plasmid contained the hygromycin phosphotransferase (Hpt) screening gene that provides hygromycin resistance, with expression driven by the *CaMV35S* promoter. The exogenous genes *REF138* (KR076812) and *REF258* (KR076814) cloned from *H. brasiliensis* were inserted into the multiple cloning sites between *Xba*I and *Sac*I, with expression driven by an enhanced 35S promoter (Fig. 6). Finally, the empty vector control, *pSuper1300*, and the *pHbREF138* and *pHbREF258* overexpression vectors were transformed into *Agrobacterium tumefaciens* strain GV3101.

Optimization of regeneration system and explants

The leaf pieces were cultured on the following three types of regeneration media consisting of basic MS medium (20 g/L sucrose, 8 g/L agar, pH 5.8) supplemented with phytohormone plus: Medium A, 1.5 mg/L 6-BA and 0.1 mg/L NAA; Medium B, 1.0 mg/L 6-BA and 0.2 mg/L NAA [14]; Medium C, 1.0 mg/L 6-BA and 0.2 mg/L indole acetic acid (IAA) [16]. As the optimal regeneration medium, a total of 15 wild rubber grass species were cultivated in medium A. After 60 days, the number and status of regenerated shoots were recorded to calculate regeneration efficiency. The shoots were then divided into four screening levels to select the most suitable germplasm for tissue culture and transformation.

Concentration of hygromycin for the selection of putative transgenic plants

We used eight hygromycin treatments (0, 4, 6, 8, 10, 12, 14, 16 mg/L) in regeneration medium A to optimize the concentration of hygromycin for selection of 12–16 in the regeneration system. Based on the regeneration efficiency and status of the induced shoots, the appropriate concentration of hygromycin for screening of 12–16 was determined after 30 days in culture.

Transformation mediated by Agrobacterium tumefaciens and regeneration

A single fresh *Agrobacterium tumefaciens* GV3101 colony was picked from the Petri dish and placed in 10 mL YEP liquid medium supplemented with 50 mg/L kanamycin and 25 mg/L rifampicin. The bacterial culture was incubated at 28°C overnight in the dark with shaking at 200 rpm. Then, 1 mL bacterial suspension was diluted in 100 mL fresh YEP liquid medium containing 50 mg/L kanamycin and incubated overnight under the same conditions. The bacterial culture was then centrifuged at 6,000 rpm for 10 min at 4°C. The *Agrobacterium* pellet was washed with 40 mL MS liquid medium (20 g/L sucrose, 4.4 g/L MS supplemented with micro- and macro-nutrients, pH 5.8) to remove trace amounts of antibiotics and resuspended in MS liquid medium until an OD_{600} value reached 0.6.

The leaf pieces cut from the aseptic seedling were precultured on the regeneration medium A for 2 days before soaking in *Agrobacterium* solution containing 100 mM acetosyringone for 30 min. The leaf pieces

were then transferred to sterile filter paper to remove as much of the *Agrobacterium* solution as possible, and cultured in medium A (pH 5.2) in the dark for 3 days.

The pieces were then washed twice with liquid MS medium and once with liquid MS medium containing 400 mg/L carbenicillin to remove *Agrobacterium*. After that, the pieces were transferred to sterile filter paper to remove the washing solution, and cultured in medium A containing 400 mg/L carbenicillin for 7 days. Subsequently, the leaf pieces were transferred to fresh medium A to induce shoot formation. During this time, the medium was changed every 14 days. The medium A was supplemented 400 mg/L carbenicillin and 10 mg/L hygromycin to prevent the growth of *Agrobacterium* and select transgenic regeneration shoots. When the shoots were approximately 5 cm high, they were then transferred to root induction medium (1/2 MS medium with micro- and macro-nutrients, 20 g/L sucrose, 9 g/L agar, 400 mg/L carbenicillin, 8 mg/L hygromycin, pH 5.8). During this time, the plants were kept in a light chamber with 16-h/8-h photoperiod at 33 μmol $m^{-2}s^{-1}$ and temperature of 22 °C/18 °C (day/night).

Molecular analysis of transgenic plants using PCR and qPCR

The transgenic plants were identified by PCR and qPCR. Briefly, plant tissue was collected from 60 putative transformed lines and one wild-type as a negative control. The tissue was frozen in liquid nitrogen and then ground to a fine powder. Genomic DNA from transformed and non-transformed plants (100 mg each) was isolated with a plant genomic DNA kit (Tigen, Beijing, China). The open reading frame sequences of the REF138 and REF258 coding regions were amplified to confirm the transgenic plants by PCR using the corresponding universal primers (Supplementary Table S2). In the PCR, a negative control consisting of DNA from wild-type 12–16, and a positive control consisting of the *pHbREF138* and *pHbREF258* plasmids were included in addition to the 22 different samples from the putative transformed lines.

Total RNA from transformed plants was isolated with RNAprep Pure Plant Plus Kit (Tiangen, Beijing, China). The first strand cDNA was synthesized using 1 μg total RNA with RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher, Waltham, USA) and used as a template for qPCR analysis. The primers of REF138 and REF258 used in qPCR were provided in Supplementary Table S2. The *actin* gene (JX425362.1) was used as an internal control for normalization. The primers for amplification of the *actin* gene are also shown in Supplementary Table S2. Triplicate PCR assays were performed for three biological replicates with SYBR Green PCR Master Mix (Thermo, Waltham, USA) using the StrataGene Mx3005P qPCR system (Agilent, Palo Alto, USA). The results were analyzed with MxPro software (version 4.10) according to the instruction manual.

Statistical analysis

All experiments were repeated three times. Data were presented as the mean ± SD and differences between means were located Student's t test using the Microsoft Excel.

Abbreviations

6-BA
6-benzylaminopurine
CaMV
cauliflower mosaic virus
CAPS
cleaved amplified polymorphic sequences
Hpt
hygromycin phosphotransferase
HygR
hygromycin resistance
IAA
indole acetic acid
MS
Murashige and Skoog
MCS
multiple cloning site
NAA
naphthaleneacetic acid
PCR
polymerase chain reaction
qPCR
quantitative PCR
REF138
rubber elongation factors 138
REF258
rubber elongation factors 258
RH
relative humidity
SSR
simple-sequence repeats
TB
Taraxacum brevicorniculatum
TK
Taraxacum kok-saghyz Rodin
TO
Taraxacum officinale

Declarations

Availability of data and materials

Not applicable.

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Contributions

CZP, ZT and BQX conceived and designed the experiments. CZP and LLC performed the experiments. QLX collected wild plant materials. DW and XMZ analyzed the data. CZP wrote the paper. All authors read and approved the final manuscript.

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Ethics declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors have declared that no competing interests exist.

Additional information

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Figures

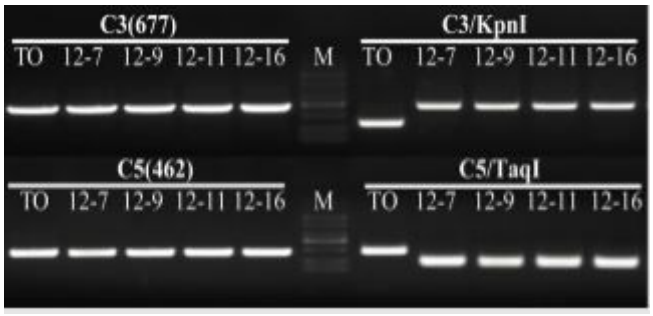

Figure 1

PCR identification of wild rubber grass species using species-specific marker. TO and wild rubber grass species (12-7, 12-9, 12-11 and 12-16) were identified using the species-specific markers of CAPS of C3 and C5. The numbers in parentheses indicate the size of the CAPS marker. Kpn I and Taq I are restriction enzymes used to digest the CAPS marker. TO, Taraxacum officinale; M, DL2000 DNA marker.

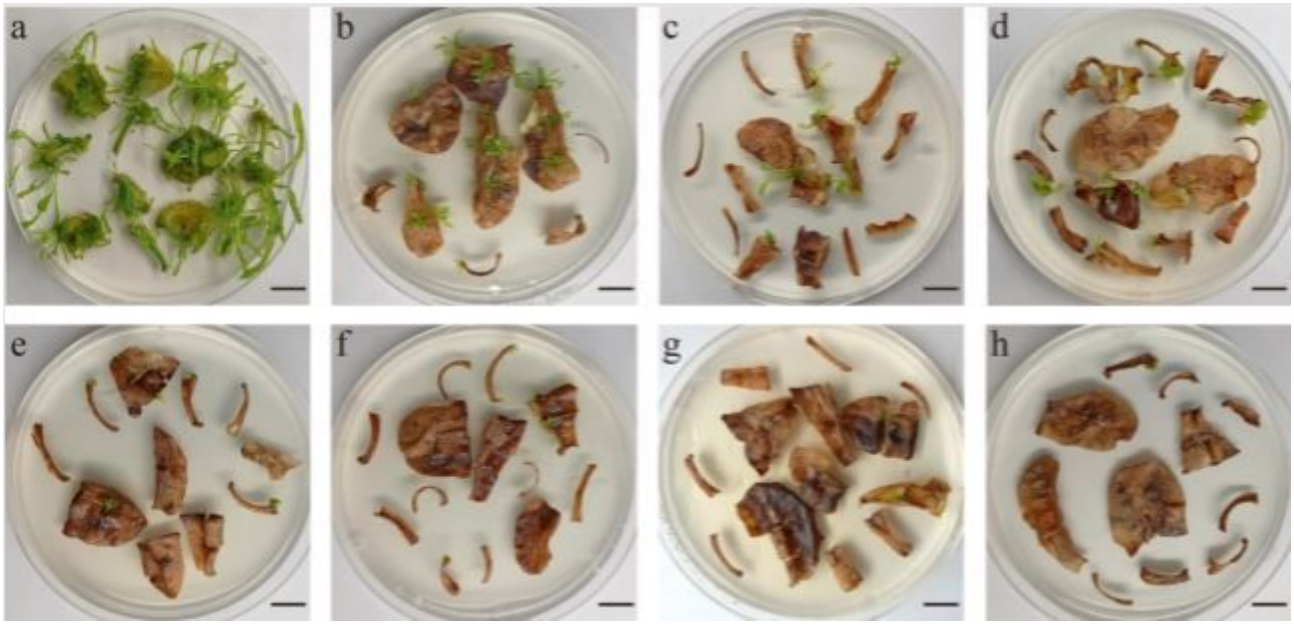

Figure 2

Hygromycin sensitivity tests. Leaf pieces of 12-16 were cultured in medium A containing a 0 mg/L, b 4 mg/L, c 6 mg/L, d 8 mg/L, e 10 mg/L, f 12 mg/L, g 14 mg/L and h 16 mg/L hygromycin to determine the optimal antibiotic concentration for selection of transformed regeneration shoots. Scale bar = 1 cm

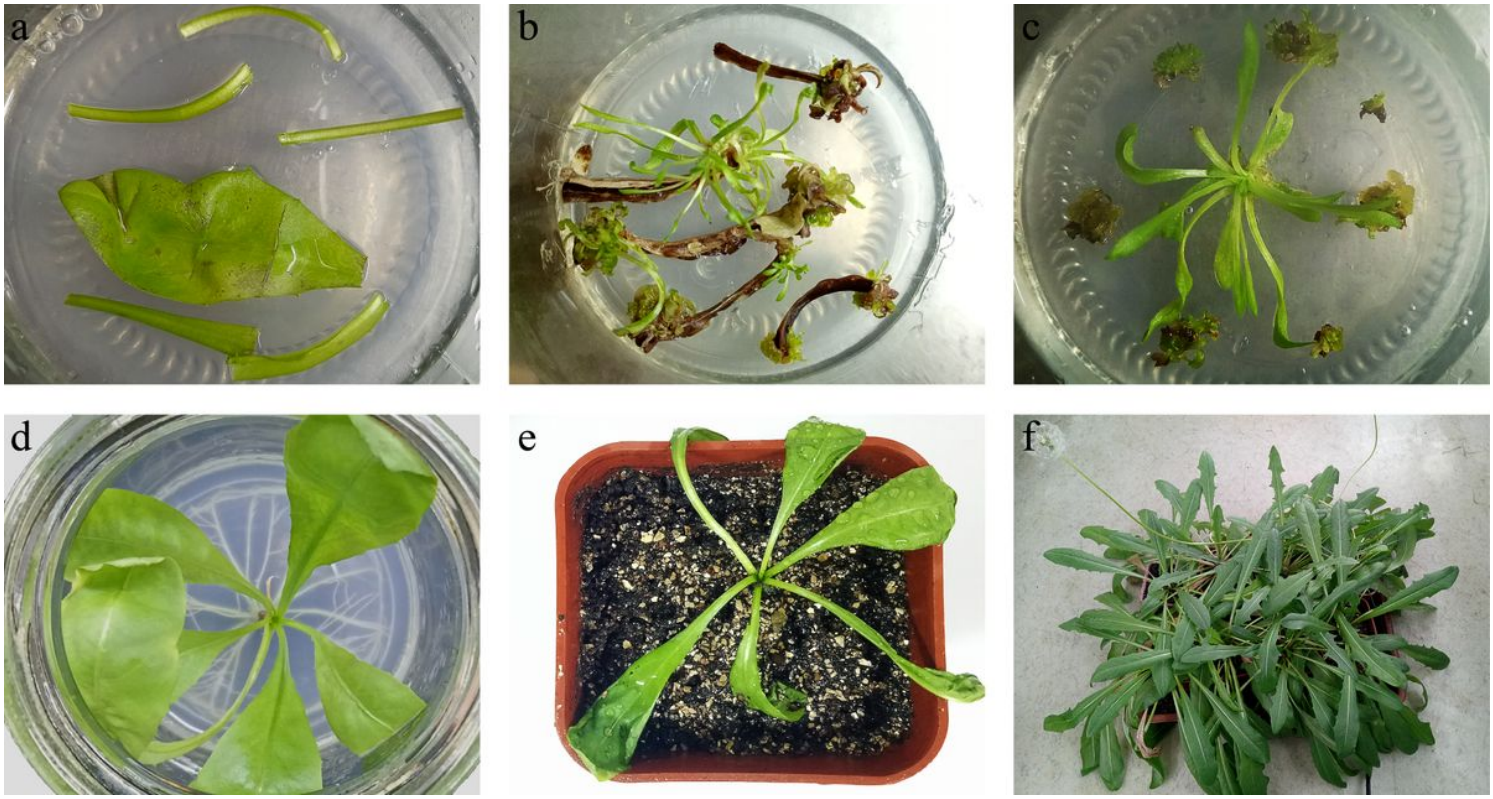

Figure 3

Agrobacterium-mediated transformation of 12-16. a Restorative culture of leaf pieces in regeneration medium after Agrobacterium infection. b Induction of hygromycin-resistant shoots in regeneration medium containing 10 mg/L hygromycin. c Continuous growth of hygromycin-resistant shoots in regeneration medium containing 10 mg/L hygromycin. d Rooting of transformed shoots in 1/2 MS medium containing 8 mg/L hygromycin without any hormone. e Transplantation of transformed shoots. f Transgenic lines entering the flowering stage.

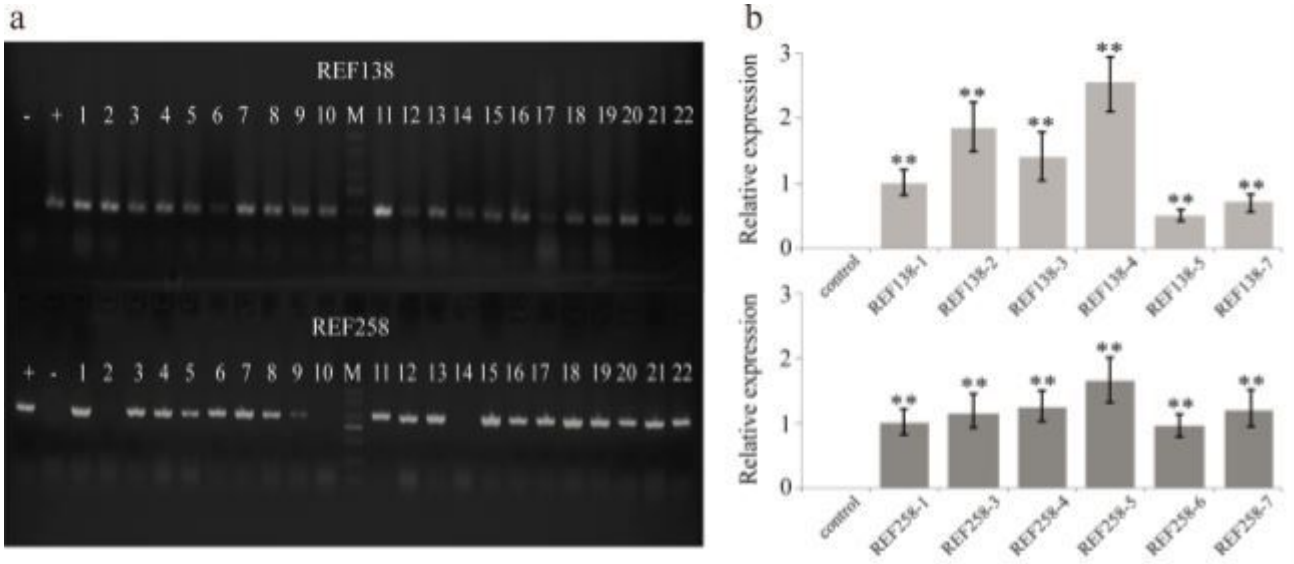

Figure 4

Molecular analysis of putative transgenic plants. a Genomic PCR analysis of putative transgenic plants. Twenty-two of the represented lines are shown. The expected bands for REF138 and REF258 were detected in most of the putative transgenic plantlets. +, Positive control of corresponding pHbREF138 and pHbREF258 plasmids; -, Negative control of wild-type 12-16; M, DL2000 DNA marker. b qPCR analysis of REF138 and REF258 heterogonous expression lines. Total RNA was extracted from control and six representative transgenic lines of REF138 and REF258. The actin gene in 12-16 was used as internal control. Data represent mean ± standard errors obtained from three repeats. **Indicates significant differences compared with the control at $P < 0.01$.

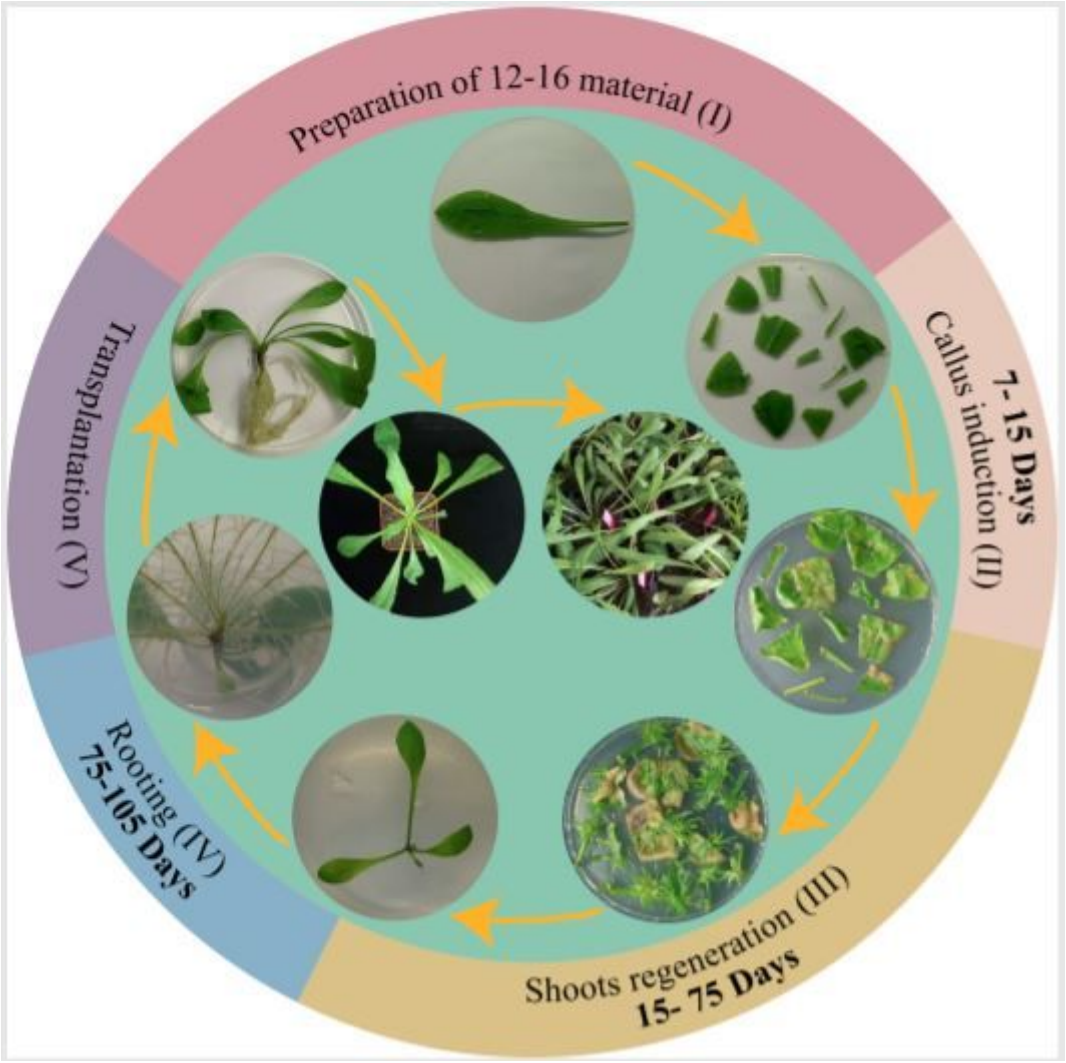

Figure 5

Different developmental stages of 12-16 leaf somatic embryogenesis cycle. (I) Preparation of 12-16 material; (II) Callus induction; (III) Shoots regeneration; (IV) Rooting; (V) Transplantation.

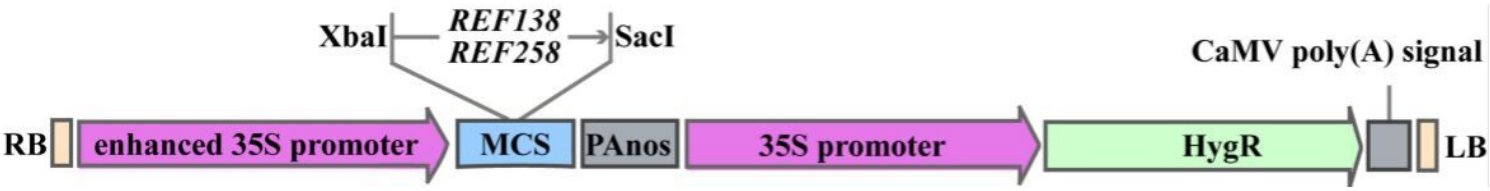

Figure 6

The T-DNA region structure of the binary expression vector pSuper1300. The T-DNA of the pSuper1300 contains the hygromycin phosphotransferase (Hpt) gene for hygromycin resistance (HygR) with expression driven by the cauliflower mosaic virus (CaMV) 35S promoter. Exogenous genes encoding REF138 or REF258 were inserted into multiple cloning site (MCS) with expression driven by the enhanced 35S promoter. LB, left border; RB, right border; PAnos, nopaline synthase terminator.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS2.doc
- TableS1.doc