

Rapid and hormone-free *Agrobacterium rhizogenes*-mediated transformation in rubber producing dandelions *Taraxacum kok-saghyz* and *T. brevicorniculatum*



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ARTICLE INFO

Article history:

Received 14 August 2014

Received in revised form 4 December 2014

Accepted 8 December 2014

Available online 5 January 2015

Keywords:

Natural rubber

Taraxacum kok-saghyz

Taraxacum brevicorniculatum

Agrobacterium rhizogenes-mediated transformation

Hairy root

ABSTRACT

Taraxacum kok-saghyz (TK) and *Taraxacum brevicorniculatum* (TB) are rubber-producing dandelion species under development as potential crops and model systems of rubber biosynthesis. The former is of industrial interest, as it produces a high percentage of high quality rubber in its roots; the latter is an apomictic cousin of TK and is of interest as a model system for rubber biosynthesis and a source of vigor in breeding efforts. Accordingly, there is interest in developing genetic transformation protocols for applied research, such as metabolic engineering. A rapid and hormone-free transformation system was developed for these two species. Dandelions can naturally regenerate from root fragments, and can be vegetatively propagated using root cuttings. Here, we show that root fragments can regenerate entire plants on half strength Murashige and Skoog medium without iterative hormone treatments or manual manipulations. Regeneration efficiency was increased from 36.6% to 65.3% for TK and from 95.2% to 152.3% for TB by inoculation with *Agrobacterium rhizogenes* wild type strain K599. After root fragments were inoculated with *A. rhizogenes* harboring kanamycin resistance genes encoding neomycin phosphotransferase II (*nptII*), as well as green fluorescent protein or cyan fluorescent protein, non-composite transgenic plants were obtained within 8 weeks. A root diameter of at least 1 mm was required for efficient regeneration and transformation. Expression of fluorescent proteins in all cells was validated using confocal microscopy. On average, transformation efficiency (number of transgenic plants/number of root fragments) was 24.7% and 15.7% for TK and TB, respectively; about seven independent transgenic events were generated per starting plant for TK and four for TB. Overall, this high efficiency transformation method provides a rapid and simple system for these two dandelions to yield viable transgenic seeds in as little as 20 weeks. Protocols developed in this study allow introduction of genes of interest to facilitate the improvement of these rubber producing plants into domestic crops and provide an avenue to explore rubber biosynthesis and gene functions.

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Abbreviations: TK, *Taraxacum kok-saghyz*; TB, *Taraxacum brevicorniculatum*; NR, natural rubber; GFP, green fluorescent protein; CFP, cyan fluorescent protein; 1/2MS, half-strength Murashige and Skoog medium; MS, full-strength Murashige and Skoog medium; BAP, 6-benzylaminopurine; IAA, indole-3-acetic acid; PCR, polymerase chain reaction; RT-PCR, reverse transcription PCR; ANOVA, analysis of variance.

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1. Introduction

Natural rubber (NR, *cis*-1,4-polyisoprene) is a critical strategic resource for manufacturing at least 40,000 products, including tires, gloves, condoms, and medical devices (Cornish, 2001). NR production is dependent on the Brazilian or Para rubber tree (*Hevea brasiliensis* Muell. Arg.), which is cultivated mostly in South-east Asia (Mooibroek and Cornish, 2000). *H. brasiliensis* cultivation is threatened by South American Leaf Blight, a fungal disease caused by *Microcyclus ulei*, which devastated rubber production in South America and has precluded its reestablishment (Edathil, 1986). Moreover, *Hevea* rubber production must also contend with

rising labor costs and land competition with palm plantations (Mooibroek and Cornish, 2000; Van Beilen and Poirier, 2007). Accordingly, there is an imperative to develop alternative rubber resources and expedite their commercialization to meet market demands.

Among the 2500 plant species that are able to produce NR, very few can produce commercially-viable amounts of high quality rubber (Mooibroek and Cornish, 2000). Kazak dandelion (*Taraxacum kok-saghyz*, TK) and its vigorous apomictic cousin, *Taraxacum brevicorniculatum* (TB), are dandelion species of interest that produce high quality rubber in their roots. TK was discovered in Kazakhstan in 1931 and was cultivated over 1000 acres in the USA throughout World War II to alleviate NR shortages (Whaley and Bowen, 1947). TK is amenable to cultivation in large geographic areas with temperate climates and can be grown as an annual crop, in contrast to *H. brasiliensis*, which requires at least 6 years to reach a tappable rubber-producing stage (Lieberei, 2007). These characteristics may allow TK production to scale and adapt to meet changing market demands. TB is a vigorous apomictic rubber producing dandelion, which has been used as a model plant for rubber biosynthesis studies (Post et al., 2012). Since genetic engineering has been used to modify a large number of crops and potentially provides a fast and targeted tool to enhance traits, there is interest in using genetic engineering to improve agronomic and metabolic performance of TK and TB. The establishment of a high efficiency transformation method will allow the introduction of traits into these species within a short period of time.

Several studies have focused on the tissue culture and transformation of *Taraxacum*, and plants of various *Taraxacum* species have been regenerated from several tissue types, roots being the most favorable explants with the highest regeneration efficiency (Bowes, 1970, 1976; Lee et al., 2004; Bae et al., 2005). The high regeneration ability of roots is consistent with the well characterized ability of dandelion to vegetatively propagate from root fragments under natural conditions. However, the micro propagation of dandelions still reported different hormone treatments at multiple regeneration stages. Two approaches have been used in dandelion transformation. *Agrobacterium tumefaciens* has been used to transform *Taraxacum mongolicum*, *Taraxacum platycarpum* and TB (Song et al., 1991; Bae et al., 2005; Post et al., 2012), while *Agrobacterium rhizogenes* has been used to transform *T. platycarpum* (Lee et al., 2004). *A. rhizogenes* differs from *A. tumefaciens* in that it contains native bacterial *rol* genes, which are often co-transformed with genes of interest. These genes alter endogenous plant hormone concentrations, promoting rapid root growth and increasing the rate of regeneration (Pavli and Skaracis, 2010). We expect the acceleration of root growth to be most prevalent in transformed tissues, allowing transformed cells to persist and better compete for resources, resulting in a more rapid transformation system. Changes in root morphology and biomass followed by *A. rhizogenes*-mediated transformation had been observed in transgenic *T. platycarpum* (Lee et al., 2004). To date, while TK and TB have been transformed using leaf tissue as the explant, multiple steps, including callus induction, shoot elongation and root induction, were required during the regeneration stage (Post et al., 2012; Collins-Silva et al., 2012).

In the work described here, the strong regeneration capacity of dandelion roots under tissue culture conditions without the addition of plant hormones was used to generate previously undescribed protocols for *A. rhizogenes*-mediated transformation in *Taraxacum*. Using these methods, genes encoding green fluorescent protein (GFP) and cyan fluorescent protein (CFP) were transformed into TK and TB to yield non-composite transgenic lines in a short period of time. The methods described here offer a highly efficient and fast approach to generate transgenic plants without hormone treatments and without a callus stage.

2. Materials and methods

2.1. Plant materials

Seeds of TK from USDA accession KAZ08-017 (W6 35172) and an apomictic TB lineage donated by Peter van Dijk (Keygene, Wageningen, Netherlands), designated as Clone A, were used (Kirschner et al., 2013). Seeds were surface-sterilized with 70% ethanol for 2 min, followed by soaking in a 0.25% sodium hypochlorite solution with 0.5% sodium dodecyl sulfate for 10 min. Seeds then were rinsed with autoclaved water 5 times and germinated on solid half strength Murashige and Skoog (1/2 MS) medium (1/2 strength MS micro- and macro-salts (Caisson Laboratories, Inc., North Logan, Utah, USA) supplemented with full strength Gamborg's B5 vitamins, 10 g L⁻¹ sucrose and 8 g L⁻¹ agar (Sigma-Aldrich®, St. Louis, MO, USA)) (Murashige and Skoog, 1962; Gamborg et al., 1968). The plants were maintained at 23–27 °C under 16 h light/8 h dark photoperiod with a light intensity of 30 μmol m⁻² s⁻¹ using white-fluorescent tubes and grown for 12 weeks.

2.2. Binary vector and *Agrobacterium* strain

The pEarleyGate 100 series vector (Arabidopsis Biological Resource Center (ABRC) stock number: CD3-724) was amended by replacing the glufosinate resistance gene with the kanamycin resistance gene *neomycin phosphotransferase II* (*nptII*) as the selective marker (Earley et al., 2006). Kanamycin was used instead of glufosinate, as it is considered more ecologically innocuous and is more commonly used for selection in dicotyledonous plants (Nap et al., 1992; Miki and McHugh, 2004). Genes encoding GFP and CFP were amplified using high fidelity Platinum® Taq DNA Polymerase (Invitrogen™, Carlsbad, CA, USA) from pEarleyGate vectors sourced from Ohio State's Arabidopsis Biological Resource Center (ABRC stock number CD3-685 and CD3-684, respectively). Amplicons then were cloned into the modified pEarleyGate 100 vector, using the PCR8/GW/TOPO Cloning Kit and LR Clonase (Invitrogen™, Carlsbad, CA, USA) according to manufacturer's instructions, termed as pEG-35S::GFP (Fig. 1A) and pEG-35S::CFP (Fig. 1B). Expression vectors were introduced into *A. rhizogenes* K599 wild type (kindly provided by Prof. John Finer, The Ohio State University, OARDC, Wooster, OH, USA) by electroporation. *A. rhizogenes*, harboring expression constructs, was grown for 36 h in liquid YEP medium (10 g L⁻¹ yeast extract, 10 g L⁻¹ peptone, 5 g L⁻¹ NaCl), containing 100 mg L⁻¹ kanamycin, shaken at 150 rpm at 28 °C. The *Agrobacterium* cultures were then pelleted and washed sequentially with liquid YEP medium and 1/2 MS medium containing 200 μM acetosyringone. *Agrobacterium* cultures finally were suspended in liquid

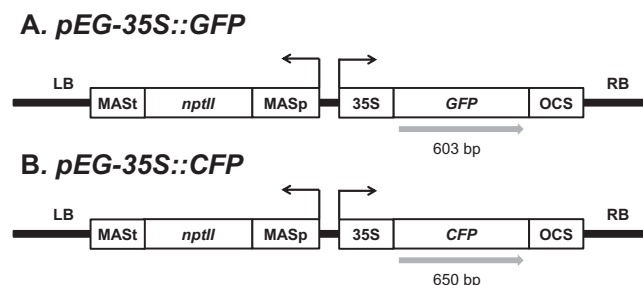


Fig. 1. Binary vectors for green fluorescent protein (GFP) and cyan fluorescent protein (CFP) expression. (A) Structure of pEG-35S::GFP construct. (B) Structure of pEG-35S::CFP construct. Kanamycin resistance gene *nptII* was controlled by Ti plasmid mannopine synthase (MAS) promoter and terminator. GFP and CFP were regulated by CaMV 35S promoter and octopine synthase (OCS) terminator. Black arrows (→) indicate the transcription direction of each gene. PCR amplified regions are shown as gray arrows (→).

1/2 MS medium containing 200 μ M acetosyringone with OD₆₀₀ 0.6 for transformation.

2.3. Optimization of explants and regeneration system

Different explants and regeneration media were used to optimize regeneration efficiency. Untransformed 1–2 cm root fragments and 1 cm² leaf discs of TK USDA line 17 and TB were grown on three different regeneration media, 1/2 strength MS medium (1/2 MS), full-strength MS medium (MS, full strength MS micro- and macro- salts with Gamborg's B5 vitamins, 20 g L⁻¹ sucrose and 8 g L⁻¹ agar) supplemented with 1 mg L⁻¹ 6-benzylaminopurine (BAP) (MS+BAP), MS medium supplemented with 1 mg L⁻¹ BAP and 0.2 mg L⁻¹ indole-3-acetic acid (IAA) (MS+BAP+IAA). The above BAP concentration in MS+BAP medium was selected as it has previously been reported to give the highest shoot formation efficiency from non-transformed and *A. rhizogenes* transformed *T. platycarpum* roots (Lee et al., 2004). Additionally, hormone concentrations in MS+BAP+IAA medium were selected based on reported shooting medium used for TK shoot regeneration (Collins-Silva et al., 2012). Approximately, 50 root fragments and 20 leaf discs were used for each replicate and three replicates were set for each medium. After 30 days regeneration, regenerated calli and shoot numbers were recorded to calculate regeneration efficiency.

2.4. Inoculation, co-culture and selection

Root fragments of TK and TB were cut from 12-week-old plants and inoculated with *A. rhizogenes* harboring GFP and CFP expression vectors by mixing on a shaker at 100 rpm for 15 min. Roots then were blotted dry on filter paper and transferred to co-culture medium (solid 1/2 MS medium with 200 μ M acetosyringone). After 3 days of co-culture with agrobacteria, root fragments were washed sequentially with water and liquid 1/2 MS medium with 400 mg L⁻¹ Timentin, and then transferred to solid 1/2 MS medium with 400 mg L⁻¹ Timentin. After 1 week of recovery, TK root fragments were washed with liquid 1/2 MS medium with 400 mg L⁻¹ Timentin and 5 mg L⁻¹ kanamycin and then transferred to plates with 1/2 MS medium with 400 mg L⁻¹ Timentin and 5 mg L⁻¹ kanamycin. After 1 week of recovery, TB root fragments were washed with liquid 1/2 MS medium with 400 mg L⁻¹ Timentin and 15 mg L⁻¹ kanamycin and then transferred to plates with 1/2 MS medium with 400 mg L⁻¹ Timentin and 15 mg L⁻¹ kanamycin. Roots were separated into two groups by diameter ($D < 1$ mm and $D \geq 1$ mm) and grown on 1/2 MS medium with selection for about 4 weeks. Regenerated plantlets with hairy root phenotypes were transferred to solid 1/2 MS medium with 400 mg L⁻¹ Timentin and 10 mg L⁻¹ kanamycin for TK while 20 mg L⁻¹ kanamycin was used for TB. After 3 weeks further selection, transgene events were validated in selected plants. Root fragments of TK and TB were also inoculated with *A. rhizogenes* K599 wild type using the same method. After recovery, root fragments were transferred to 1/2 MS medium with 400 mg L⁻¹ Timentin for regeneration. Approximately 30 root fragments were used for each replicate and three replicates were used for each treatment.

2.5. PCR and reverse transcription PCR

Putative transgenic plants were validated by polymerase chain reaction (PCR) of GFP or CFP. Total genomic DNA was extracted from leaves of plants transformed with K599 harboring fluorescent protein expression vectors as well as leaves of non-transgenic plants as negative controls. A 2% CTAB method was scaled to a 96 well format using the GenoGrinder platform (SPEX, Metuchen, NJ, USA) for DNA extraction (Kabelka et al., 2002). PCR was performed in a 15 μ L reaction containing 1X Standard Taq Reaction Buffer, 200 μ M

dNTPs, 0.2 μ M forward and 0.2 μ M reverse primers, 0.4 U Taq DNA Polymerase and 10 ng DNA. Primers used to amplify 603 bp region of GFP were vGFP_forward: 5'-AGAGGGTGAAGGTGATGCAA-3' and vGFP_reverse: 5'-CCATGTGTAATCCCAGCAGC-3'; the 650 bp region of CFP was amplified using primers vCFP_forward: 5'-TAAAC-GGCCACAAGTTCAGC-3' and vCFP_reverse: 5'-CTTGACAGCTCG-TCCATGC-3'. PCR procedures used were 5 min initial denaturation at 95 °C, 30 s denaturation at 95 °C, 30 s annealing at 54 °C, 60 s elongation at 68 °C for 35 cycles, followed by final extension at 68 °C for 5 min. A total volume of 10 μ L PCR products was loaded on 2% agarose gels (w/v) with ethidium bromide for electrophoresis. All the reagents were obtained from New England Biolabs Inc., Ipswich, MA, USA.

Total RNA was extracted from leaves of plants transformed with K599 harboring fluorescent protein expression vectors, as well as leaves of non-transgenic plants as negative controls, following the method described by Chomczynski and Sacchi (2006). RNA from each sample were treated by DNase I using TURBO DNA-free™ Kit to remove DNA (Invitrogen™, Carlsbad, CA, USA). First-strand cDNA was synthesized using SuperScript™ II Reverse Transcriptase (Invitrogen™, Carlsbad, CA, USA). The amount of 50 ng cDNA was used for reverse transcription PCR (RT-PCR) using reactions and procedures described above for GFP and CFP transformants, as well as the following primers: RT-GFP_forward: 5'-AGAGGGTGAAGGTGATGCAA-3'; RT-GFP_reverse: 5'-CTCTTGAA-GAAGTCGTGCCG-3'; RT-CFP_forward: 5'-CACATGAAGCAGCAGC-GACTT-3'; RT-CFP_reverse: 5'-TCCTTGAAGTCGATGCCCTT-3'. Endogenous gene β -actin (ACTB) was amplified using the same amount of cDNA and primers: ACTB_forward: 5'-AGCAAC-TGGGATGACATGGA-3'; ACTB_reverse: 5'-CATACATGGCGGG-GACATTG-3'. A total volume of 10 μ L PCR products were loaded on 2% agarose gels (w/v) with ethidium bromide for electrophoresis. All the reagents which were not specifically mentioned above were obtained from New England Biolabs Inc., Ipswich, MA, USA.

2.6. Fluorescent protein visualization

Fluorescent protein functional expression was confirmed for both leaf and root tissue using a confocal scanning microscope (Molecular and Cellular Imaging Center, The Ohio State University, OARDC, Wooster, OH, USA). After 8 weeks of selection, root and leaf samples from non-transgenic plants, as well as from PCR and RT-PCR confirmed transgenic plants, were placed in glass bottom dishes. Samples were covered with glass cover slips and water was added between the bottom of dishes and the glass cover. Samples were placed under a Leica TCS SP5 confocal scanning microscope and images were captured using Leica Application Suite Advanced Fluorescent software. GFP images were captured under excitation laser Argon-blue (488 nm and 514 nm) with excitation wavelengths 488 nm at 82% laser intensity. Images were collected from 497 nm to 557 nm with 865 smart gain and 50.1 μ m pinhole. CFP were visualized under UV (405 nm) laser with 77% laser intensity. Images were collected from 453 nm to 531 nm with 845 smart gain and 64.9 μ m pinhole. Figures were created by Microsoft PowerPoint (version 14.0.7128.5000).

2.7. Subculture of validated plants and analysis of transgene inheritance

Hairy roots that were greater than 1 cm long, with a diameter greater than 1 mm, from transformed plants validated by PCR, RT-PCR and microscopy, were placed on 1/2 MS medium with 400 mg L⁻¹ Timentin and 10 mg L⁻¹ kanamycin for TK and 20 mg L⁻¹ kanamycin for TB. At least 2 new plantlets were generated for each event before transitioning the transgenic event to non-sterile conditions.

Validated transformed plants were transferred into sterile peat pellets soaked with liquid 1/2 MS medium with 400 mg L⁻¹ Timentin. After two weeks, the media in the peat pellets was replaced with water and the transgenic plants with peat pellets were transferred to micro propagation trays, where the humidity was lowered over a period of 1 week. Transformed plants in peat pellets then were transferred into 3.8 L pots filled with Pro-mix and then moved into a growth chamber with a 12 h light/12 h dark photoperiod, light intensity of 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$, at 22 °C, and relative humidity of 80%. After 1 month, transgenic TK plants were reciprocally crossed with at least three different genotypes of non-transgenic TK to obtain T₁ populations. Seeds were collected 15 days after pollination. These seeds were germinated in Pro-mix and leaves were collected 20 days after germination for DNA extraction. DNA was extracted and CFP amplified using the methods described previously.

2.8. Statistical analysis

Regeneration efficiency was calculated using the number of regenerated shoots, calli or plants over the number of starting leaf discs or root fragments. Treatment effects were detected using one-way analysis of variance (ANOVA) and Tukey's HSD multiple comparison of mean test by R (R Core Team, 2013). Influence of

root size on regeneration efficiency was analyzed using vectors as a random factor. Significant differences were claimed at $P < 0.05$.

3. Results and discussion

3.1. Selection of regeneration media and explants

To investigate the optimal medium and explant for TK and TB to achieve highest regeneration efficiency, three different media treatments were used to determine their ability to mediate the regeneration plants from leaf discs and root fragments. These three media had different effects on both TK and TB regeneration efficiency from leaf discs. The MS + BAP and MS + BAP + IAA media induced calli from leaf edges whereas 1/2 MS medium did not induce calli, shoots, or roots from leaf discs. When using roots as explants, MS + BAP and MS + BAP + IAA induced callus production as well, with few shoots appearing on calli. Fragments regenerated on 1/2 MS medium with no addition of hormones were able to generate plantlets in a period of 14 days (Fig. 2A–D). Direct shooting from explants was considered an ideal approach for plant regeneration, as it both shortens the regeneration cycle and limits the introduction of undesired somaclonal variation, which can occur in the callus phase (Nwauzoma and Jaja, 2013). Plantlets regenerated from root fragments on 1/2 MS medium were able to develop more quickly and vigorously than other methods (Fig. 2A–D). While

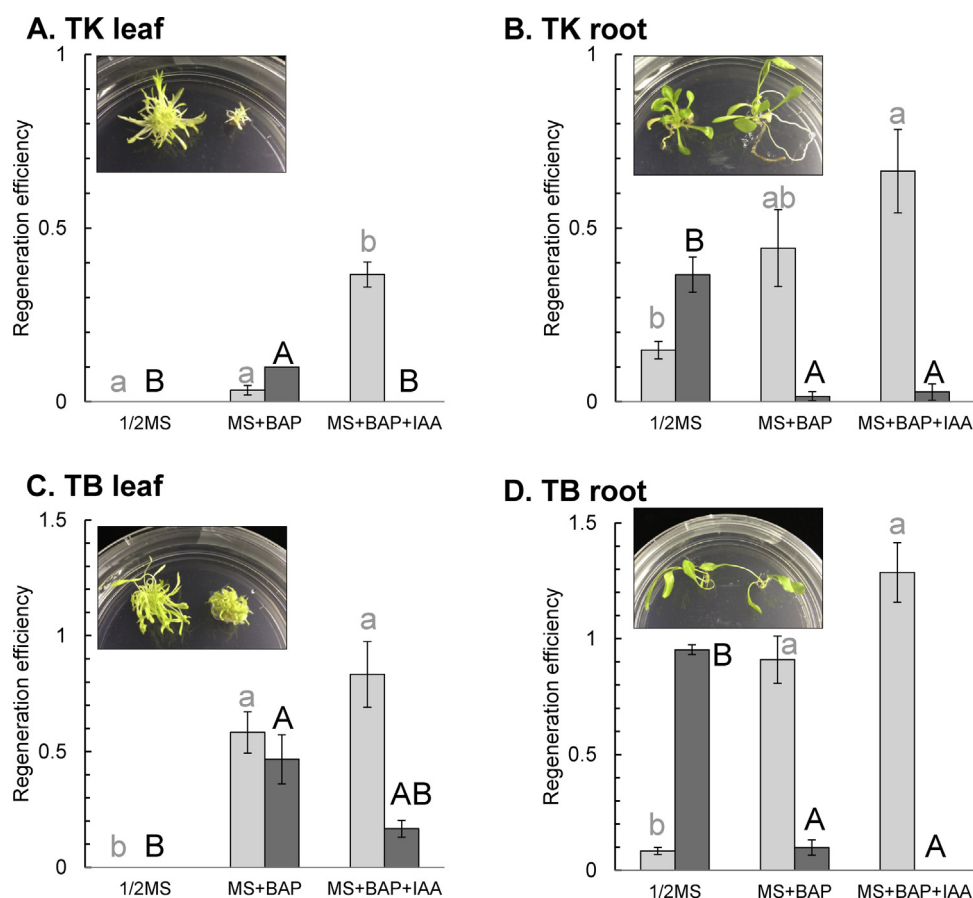


Fig. 2. Effects of different explants (leaf disc and root) and three media (1/2 MS, MS + BAP and MS + BAP + IAA) on *Taraxacum kok-saghyz* (TK) and *T. brevicorniculatum* (TB) regeneration efficiency. (A) Regeneration efficiency of TK from leaf discs. Inserted photograph shows the regenerated shoots. (B) Regeneration efficiency of TK from root fragments. Inserted photograph shows the regenerated shoots using 1/2 MS medium. (C) Regeneration efficiency of TB from leaf discs. Inserted photograph shows the regenerated shoots. (D) Regeneration efficiency of TB from root fragments. Inserted photograph shows the regenerated shoots using 1/2 MS medium. Regeneration efficiency was calculated by dividing the number of regenerated calli or shoots by the number of starting leaf discs or root fragments. Callus regeneration efficiency is indicated by the light gray bar (□) and shoots regeneration efficiency is indicated by the dark gray bar (■). Vertical bars indicate standard errors (SE). Statistical analysis was carried out using one-way ANOVA with the medium as the treatment. Comparison was conducted with same explants and within species. Mean \pm SE followed by same lower or upper case letters are not significantly different for their respective data set according to Tukey's HSD at $P < 0.05$.

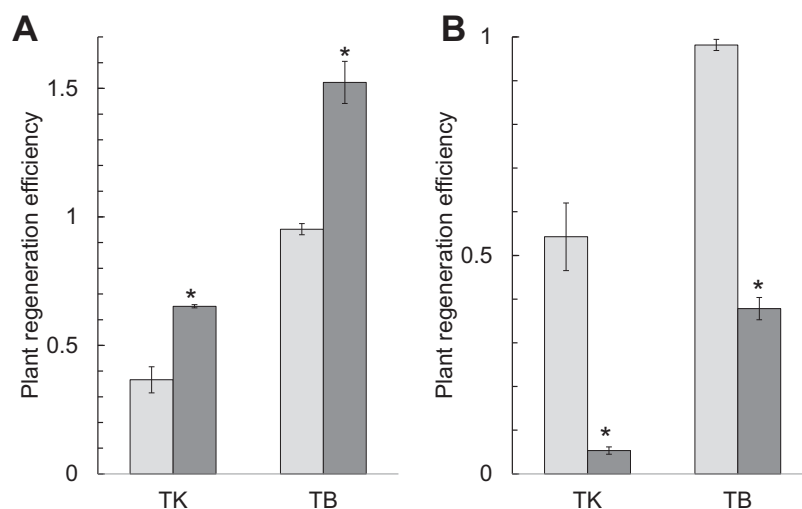


Fig. 3. Effects of inoculation and root size on *Taraxacum kok-saghyz* (TK) and *T. brevicorniculatum* (TB) regeneration efficiency. (A) Plant regeneration efficiency of TK and TB from root fragments without and with inoculation. Regeneration efficiency without inoculation is indicated by the light gray bar (□) and regeneration efficiency with inoculation is indicated by the dark gray bar (■). (B) Plant regeneration efficiency of TK and TB from root fragments with diameter $D \geq 1$ mm and $D < 1$ mm. Regeneration efficiency from root $D \geq 1$ mm is indicated by the light gray bar (□) and regeneration efficiency from root $D < 1$ mm is indicated by the dark gray bar (■). Plant regeneration efficiency was calculated by dividing the number of regenerated plants by the number of starting root fragments. Vertical bars indicate standard errors. Stars indicate the significant differences between treatments within species according to Tukey's HSD at $P < 0.05$.

shoots were induced on other media using either roots or leaf discs, under these conditions a short callus stage was observed prior to the appearance of shoots. Additionally, these shoots were much smaller than shoots induced from roots on 1/2 MS medium and were not able to develop to plantlets without using rooting media. Therefore, the use of root tissues and 1/2 MS medium were selected as optimal recovery conditions for downstream generation of transformed plants. While the hormone-free transformation method here provides several advantages, it must be noted that our studies only incorporated phytohormones (IAA and BAP) at the concentrations described above. Since, callus tissues do exhibit sensitivity to gradients in hormone concentrations, it is entirely possible that the inclusion of either IAA or BAP at lower or higher concentrations might provide additional advantages in the regeneration of transgenic tissues (i.e., increased growth or the production of additional root mass) which would not have been revealed in our assays. As the hormone-free regeneration method provided a rapid and simple approach for TK and TB regeneration, however, this method was selected as a focus for the current study. One advantage to this method is that it circumvents iterative hormone treatments requiring transfer to several different media accompanied by manual manipulations. Additionally, this method also maintained a high regeneration efficiency by reducing the number of steps required and the potential losses and costs associated with them.

3.2. Regeneration capability of transgenic and non-transgenic roots

Plant root fragments were first transformed using *A. rhizogenes* wild type strain K599. Shoot emergence was observed 10 days after transformation, followed by the formation of hairy roots. Within one month, TK plantlets were obtained with $65.3 \pm 0.7\%$ regeneration efficiency, which was 28.7% higher than the regeneration efficiency seen in non-inoculated plants ($36.6 \pm 5.1\%$) (Fig. 3A). TB regeneration efficiency reached $152.3 \pm 8.2\%$ (more than one shoot emerged from a single root fragment) with inoculation, significantly higher than the regeneration efficiency of $95.2 \pm 2.2\%$ without inoculation (Fig. 3A), a phenomenon we suspect is due primarily to both the strong regenerative ability of TB and the rapid growth and differentiation induced by hairy root transformation.

3.3. Selection and regeneration of GFP and CFP transgenic plants

To achieve efficient selection for transgenic plantlets, we tested a range of concentrations and identified 10 mg L^{-1} and 20 mg L^{-1} as kanamycin concentrations effective at eliminating non-transgenic TK or TB, respectively (data not shown). Plants able to survive under 10 mg L^{-1} (TK) or 20 mg L^{-1} (TB) kanamycin could be obtained within 8 weeks after selection, (Fig. 4A,–C and G–I). Compared to non-transgenic plants (Fig. 4D and J), transformed plants exhibited hairy root phenotypes, including wrinkled and high density leaves as well as plagiotropic and extensively branched roots. Transgene presence was validated by PCR analysis (Fig. 5A and B) and transgene expression at the transcription level was confirmed by RT-PCR (Fig. 6A and B) using leaf tissue. At the tissue level, confocal microscopy of transgenic root and leaf tissues showed transformation of both tissue types (Fig. 7A–P). GFP and CFP were shown to express stably in the protoplasm and nuclei of root and leaf tissues. It is important to note, however, that due to slight differences in organ morphology between TK and TB, the fluorescence intensity in the images is not quantitative; i.e., the increased fluorescence intensity observed in TB vs. TK roots may not indicate higher GFP or CFP expression. Collectively, transgenes were present and functionally expressed in both leaf and root tissue, suggesting that the kanamycin concentrations used for selection were sufficient to produce non-composite plants. However, composite plants may be useful in basic research to evaluate transport phenomena between roots and shoots (Ko et al., 2014). Moreover, large scale production of secondary metabolites could be achieved using hairy roots from composite plants, particularly for lethal transgene events or species with poor regeneration ability (Benabdoun et al., 2011).

3.4. Influence of root size on regeneration efficiency and transformation efficiency

To investigate the influence of root size on regeneration efficiency and transformation efficiency, two size categories of root fragments were used for GFP and CFP transformation. We found that root diameter significantly impacted ($P < 0.05$) the recovery of transformants. In TK, young adventitious roots with diameters < 1 mm were generally unable to regenerate plantlets, while

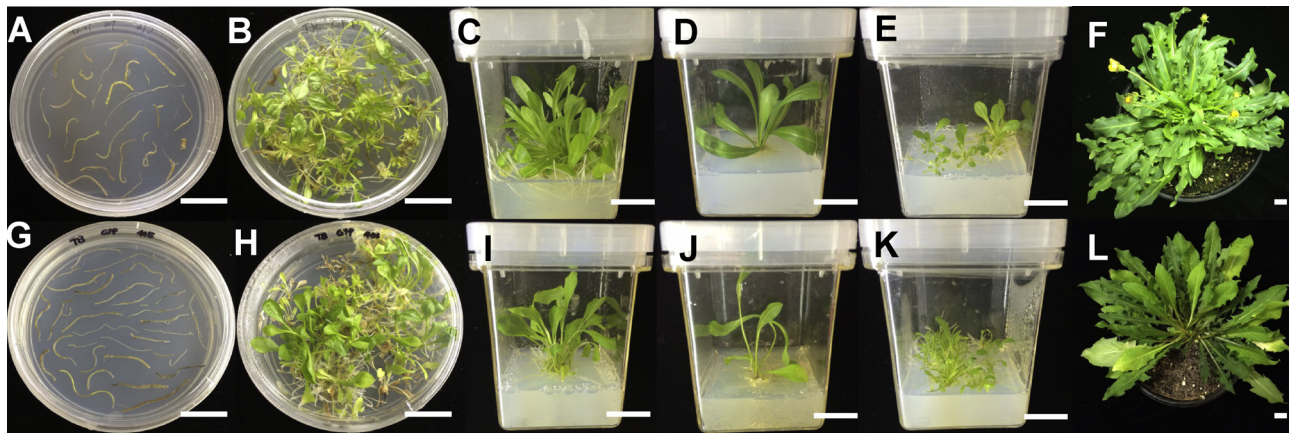


Fig. 4. The *A. rhizogenes*-mediated transformation of *Taraxacum kok-saghyz* (TK) and *T. brevicorniculatum* (TB) using root fragments as explants. (A) TK root fragments explants. (B) Complete TK putative transgenic plants, including leaves and hairy roots, were regenerated on 1/2 MS medium without hormone addition under kanamycin selection. (C) A transgenic TK plant after 2 months of selection with hairy root phenotypes. (D) A 2-month-old non-transgenic TK plant. (E) Transgenic TK plants regenerated from transgenic hairy roots. (F) A transgenic TK plant established in soil with hairy root phenotypes and flowers. (G) TB root fragments explants. (H) Complete TB putative transgenic plants including leaves and hairy roots were regenerated. (I) A transgenic TB plant after 2 months of selection with hairy root phenotypes. (J) A 2-month-old non-transgenic TB plant. (K) Transgenic TB plants regenerated from transgenic hairy roots. (L) A transgenic TB plant established in soil with hairy root phenotypes. Size bars represent 2 cm.

more mature roots ≥ 1 mm exhibited a higher rate of regeneration (Fig. 3B). Interestingly, in contrast to TK, TB roots with diameters <1 mm showed strong regenerative ability, although this was still lower than regeneration observed using larger roots (Fig. 3B). We have observed that larger root systems can be obtained by adding hormones such as indole-3-butyric acid to growth media. We expect that root fragments taken from such plants would have similarly favorable regenerative abilities. On average, transformation efficiency (number of transgenic plants/number of root fragments) of roots with diameters ≥ 1 mm was 24.7% and 15.7% for TK and TB, respectively; about seven independent transgenic events were generated per starting plant for TK and four for TB.

The TK germplasm selected for this research, USDA accession KAZ08-017 (W6 35172), exhibited average regeneration abilities from both shoots and roots (data not shown) comparable to those observed in other KAZ accessions. While the transformation of other TK accessions was not tested in this research, given the

average regeneration rate of KAZ08-017, the methods described here are likely to be successful when applied to other TK accessions.

3.5. Subculture, acclimation and inheritance analysis of validated transgenic plants

Taraxacum plants were initially subcultured from leaves, which required multiple steps over a 12 week period (data not shown). An alternative, simpler subculture method from roots was developed. Hairy roots induced by *A. rhizogenes* infection were excised and moved to 1/2 MS medium without hormones. After 30 days, plantlets had regenerated and showed hairy root phenotypes, suggesting that the strong regenerative capability of roots was able to

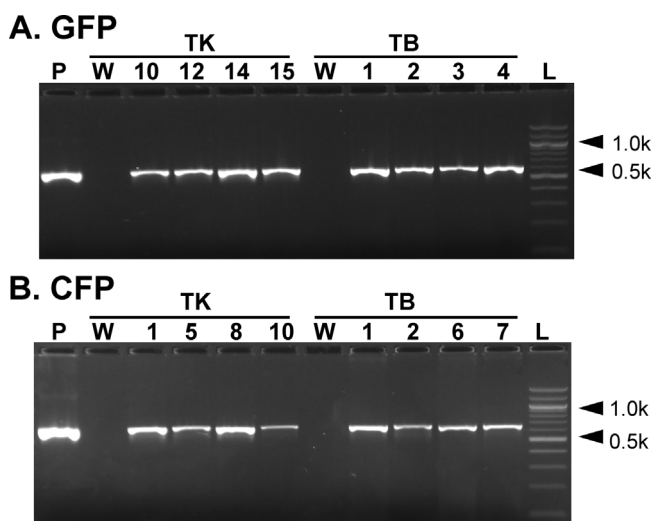


Fig. 5. Polymerase chain reaction (PCR) analysis of green fluorescent protein (GFP) and cyan fluorescent protein (CFP) in transgenic *Taraxacum kok-saghyz* (TK) and *T. brevicorniculatum* (TB) plants. (A) PCR analysis of GFP in four independent transformants of each species. (B) PCR analysis of CFP in four independent transformants of each species. Leaf tissue was used for PCR analysis. L, 100 bp DNA ladder from New England Biolabs Inc. P, positive plasmid control. W, negative wild type non-transgenic plants control. Each number indicates an independent transgenic event.

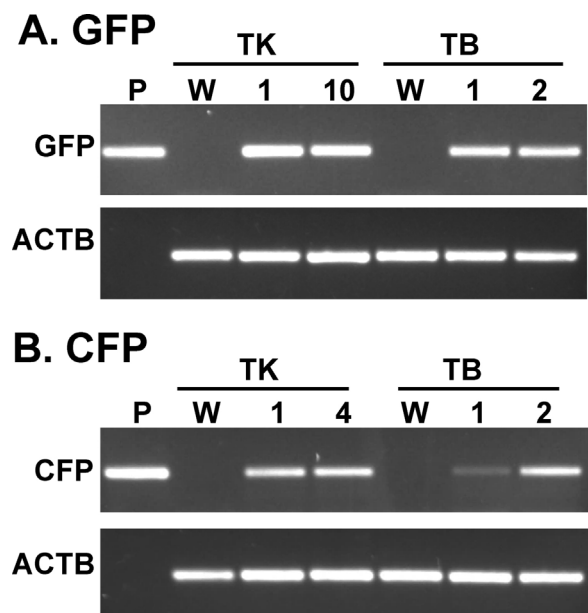


Fig. 6. Reverse transcription polymerase chain reaction (RT-PCR) analysis of green fluorescent protein (GFP) and cyan fluorescent protein (CFP) expression. (A) RT-PCR analysis of GFP in two independent transformants of each species. (B) RT-PCR analysis of CFP in two independent transformants of each species. Leaf tissue was used for RT-PCR analysis. P, positive plasmid control. W, negative wild type non-transgenic plant control. Each number stands for an independent transgenic event. Endogenous gene β -actin (ACTB) was used as endogenous gene control for each RT-PCR reaction.

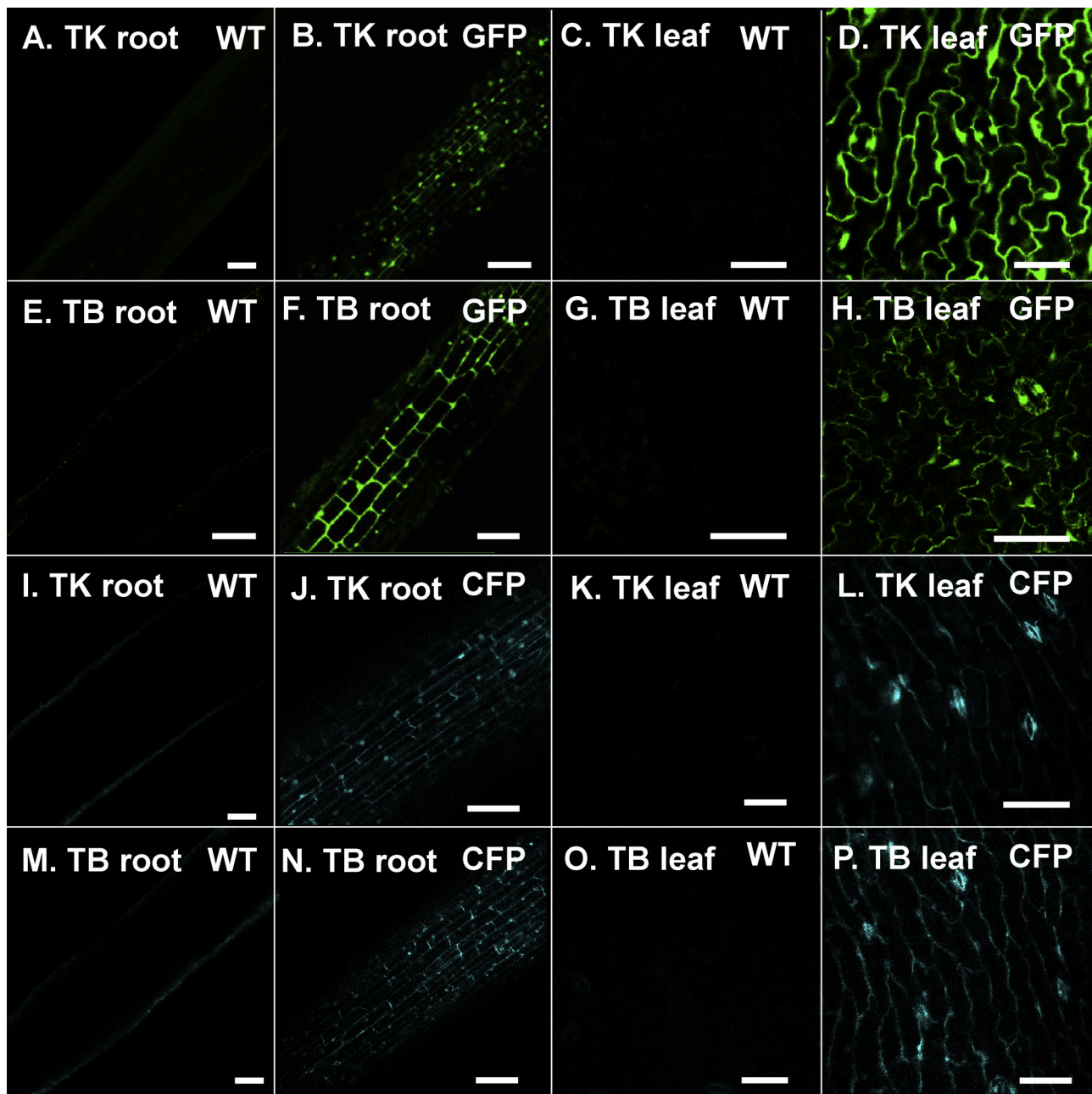


Fig. 7. Stable green fluorescent protein (GFP) and cyan fluorescent protein (CFP) expression in transgenic *Taraxacum kok-saghyz* (TK) and *T. brevicorniculatum* (TB) under a Leica TCS SP5 Confocal Microscope. (A)–(D), GFP expression in root tissue (A and B) and leaf tissue (C and D) of non-transgenic (WT) and transgenic (GFP) TK. (E–H), GFP expression in root tissue (E and F) and leaf tissue (G and H) of non-transgenic (WT) and transgenic (GFP) TB. (I)–(L), CFP expression in root tissue (I and J) and leaf tissue (K and L) of non-transgenic (WT) and transgenic (CFP) TK. (M)–(P), CFP expression in root tissue (M and N) and leaf tissue (O and P) of non-transgenic (WT) and transgenic (CFP) TB. Size bars represent 50 μm . Leaf and root tissue used for microscopy was obtained from plants after 8 weeks of selection. The fluorescence intensity shown in figures is not quantitative.

tolerate hormonal imbalances potentially introduced by *rol* genes of *A. rhizogenes* (Fig. 4E and K). As hairy root transformants generally produce many hairy roots, this system allows for rapid duplication of transgene events.

Transgenic plants can be transferred from tissue culture to growth chambers or greenhouses within 21 days. The survival rates of transgenic plants were 95% and 100% for TK and TB, respectively. After recovery and growth in soil for 30 days, transgenic TK plants were able to flower and produce viable progeny in reciprocal crosses (Fig. 4F and L, Fig. 8A–D). Both hairy root phenotypes and fluorescent protein genes were heritable in the T_1 generation, with segregation (Fig. 8A–E).

The hairy root phenotypes observed in transformed plants persisted after the transition to non-sterile growth in soil (Fig. 4E and J). This growth habit was reported to increase root to shoot biomass ratio and increase the production of secondary metabolites, including both alkaloids and terpenoids (of particular interest, since increases in terpenoid production could potentially increase rubber yields from TK or TB) in several species (Cai et al., 1995; Kim et al., 2002; Srivastava and Srivastava, 2007). While the generation of numerous adventitious roots, instead of a few tap roots, may allow for better competitiveness and utilization of soil nutrients, it also could result in roots that are too fragile to be harvested. Additionally, while the hairy root phenotypes generally increase

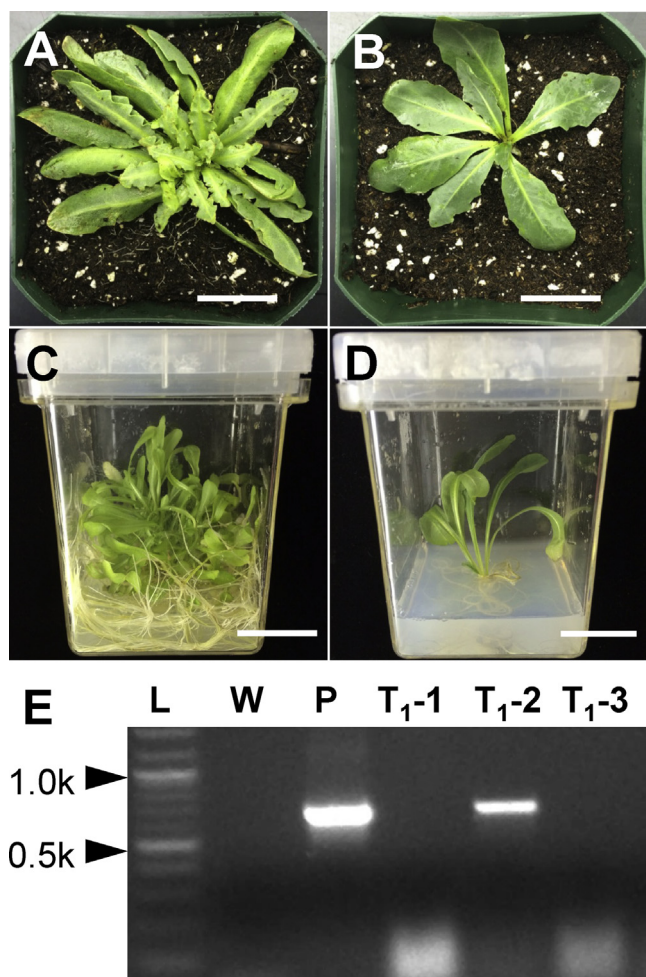


Fig. 8. Stable inheritance and segregation of hairy root phenotypes and fluorescent protein gene in *Taraxacum kok-saghyz* (TK) T_1 generation. (A) TK T_1 generation plant 6 weeks after germination with hairy root phenotypes. (B) TK T_1 generation plant 6 weeks after germination without hairy root phenotypes. (C) 3-month old TK T_1 generation plant grown under tissue culture conditions with hairy root phenotypes. (D) 3-month old TK T_1 generation plant grown under tissue culture conditions without hairy root phenotypes. (E) Polymerase chain reaction (PCR) analysis of cyan fluorescent protein (CFP) of TK T_1 generation plant. L, 100 bp DNA ladder from New England Biolabs Inc., W, negative wild type non-transgenic plants control. P, positive plasmid control. T_1 -1,2,3, TK T_1 generation plants. Size bars represent 2 cm.

secondary metabolism, they may have the potential to affect rubber production or rubber molecular weight. If this growth habit proves to be undesirable, genes of interest can be segregated from native *A. rhizogenes* events in the T_1 generation. As the integration of native *A. rhizogenes* genes is independent of the integration of genes of interest, they will generally be inserted in different regions of the genome and will not be linked to each other. Alternatively, *A. tumefaciens*-mediated transformation method may be achieved using the high efficiency regeneration system described here. The potential implications of a hairy root growth habit and metabolic modification of TK and TB will be evaluated in future work.

4. Conclusions

We present here data detailing the development of a novel plant transformation system using *A. rhizogenes* to transform root tissue efficiently and leveraging the ability of *Taraxacum* species to regenerate entire plants from root fragments to create a rapid pipeline for the generation of transgenic dandelion lines. The regeneration of plants from root fragments in tissue culture without

hormone treatment has not previously been reported in *Taraxacum*. The method presented here could be used to increase accessibility, reproducibility, and throughput in transformation efforts. Progeny of crosses between TB and TK segregate TK phenotypes, suggesting that transgene events could be moved between species and that TB can serve as a clonal courier of transgene events, where its vigorous growth rate and polyploidy could facilitate challenging transformations. Collectively, these results provide a platform for future transgene events in rubber producing dandelion species that may be used to investigate components of rubber biosynthesis and improve rubber yield as well as agronomic traits.

Acknowledgements

We are grateful for financial support from PanAridus, LLC, the OARDC SEED grant program, the Institute of Materials Research at the Ohio State University, and the Ohio Third Frontier. This work is also supported by the USDA National Institute of Food and Agriculture, Hatch project 230837. We thank Prof. John Finer's lab for providing *A. rhizogenes* K599, Nikita Amstutz for tissue culture support, Prof. Tea Meulia and Muhammad Akbar Abdul Ghaffar for help with confocal microscopy, and Prof. John Cardina and Prof. David Francis for their valued input in writing this manuscript.

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