Evaluating the Development and Potential Ecological Impact of Genetically Engineered

Taraxacum kok-saghyz

DISSERTATION

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Abstract

Natural rubber is a biopolymer with irreplaceable properties, necessary in tires, medical devices and many other applications. Nearly all natural rubber production is dependent on a single species, *Hevea brasiliensis*. *Hevea* has several disadvantages, including a long life cycle, epidemic diseases, and rising production costs which have led to interest in developing new sources of rubber with similar quality to *Hevea*. One species that meets this criterion is *Taraxacum kok-saghyz* (TK), a widely adapted species of dandelion that can produce substantial amounts of rubber in its roots in an annual growing period. Shortcomings of TK include an inability to compete with many weeds, resulting in poor establishment and yields. In addition, there is variability in the amount of rubber produced, plant vigor, and seed establishment. In order to address these shortcomings, genetic engineering or breeding may be used to introduce herbicide resistance and allocate more resources to rubber production.

We have demonstrated stable transformation in *Taraxacum* species using *Agrobacterium rhizogenes* to introduce genes of interest as well as hairy root phenotypes. Inoculated roots were subjected to selection by kanamycin and glufosinate and allowed to regenerate into plantlets without any hormonal treatments or additional manipulations. Transformed plants were reproductively viable and genes of interest
segregated independently. The natural ability of dandelions to regenerate entire plants from root fragments allows this method to be faster and simpler than methods applied to many commonly transformed species. This method was then leveraged to implement CRISPR/Cas genome editing to potentially improve rubber production.

The potential release of improved TK, with novel traits raises questions of potential gene flow into the ubiquitous weedy relative of TK, the Common Dandelion, *T. officinale* (TO). In order to evaluate this risk, genomic resources were developed to discriminate the nuclear and chloroplast genomes of TK and TO. Furthermore, assemblies of chloroplast genomes of TK and TO were generated. These resources were used to conduct gene flow studies and may also be used to improve and understand TK genetics.

In order to understand the potential for gene flow between TK and TO, Flow Cytometry Seed Screening (FCSS) was used to characterize the genome sizes of a global collection of TO. As diploid TO plants are outcrossing sexually reproducing and triploid TO plants are obligate apomicts, genome size estimates can be used to inform the potential for hybridization. All TO screened in North America proved to be apomictic triploids, while diploids where detected as a minority in regions of Central Europe, where they have previously been described. Diploid TO was receptive to TK pollen and readily produced viable, self-incompatible progeny. Triploid TO exhibited obligate apomixis, where clonal seed is produced regardless of pollination, and this phenomenon may preclude pollination by TK.
While apomixis may limit pollen mediated gene flow, TK may still be receptive to pollen produced by TO. In controlled crosses, TK pollinated by triploid TO produced low seed set; however, 23% of this seed was a result of hybridization. Some of these hybrids demonstrated the inheritance of apomixis and were able to produce viable seed, while those that were not apomictic were sterile. In fitness experiments hybrids produced seed more readily than TO, but had lower biomass after overwintering.

In outdoor seed production areas heavily contaminated with TO, seed produced was screened for hybrids over a three year period using phenotypic screening followed by genetic verification. Hybridization of TK by TO was only observed in these areas during one of these years, where bees were introduced, using bee hives, to augment pollination. The estimated frequency of hybridization during this period was 0.001%.

Overall, TK can be pollinated by triploid TO, which is the most abundant cytotype. This hybridization may be rare under field conditions due to factors such as interspecific pollen competition. Diploid TO can readily be pollinated by TK, indicating that the potential for gene flow and introgression will be greater in areas with diploid TO, such as Central Europe, than in areas with only triploid TO, such as North America.

While apomixis may greatly limit or entirely preclude pollen mediated gene flow between genetically engineered TK and apomictic TO, tools are needed in order to quantify this potential. Accordingly, TK germplasm possessing readily scorable transgenic traits was created for this purpose. Traits such as kanamycin resistance, glufosinate resistance and the expression of florescent proteins and the transcription
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Collectively, this research provides a toolkit to develop genetically engineered TK and also understand the potential ecological impact of biotech TK. While the potential for hybridization exists, the ability of novel traits to fully introgress within TO may be limited. However, more work is needed to fully understand the longevity of apomictic hybrid lineages in natural settings. While genetic engineering may contribute novel traits to *Taraxacum* species, new tools such as CRISPR/Cas offer the opportunity to accelerate the domestication of new crops, such as TK and may be used to further minimize ecological impact.
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Fields of Study

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Chapter 1: Introduction

1.1 Natural Rubber

Natural rubber is an important national resource, which is irreplaceable in most applications, such as the production of airplane and truck tires, as well as numerous medical devices (Mooibroek and Cornish, 2000). Virtually all natural rubber comes from the rubber tree (*Hevea brasiliensis*), which has a narrow growing range and is not amenable to cultivation in the United States. Furthermore, the expansion of rubber tree cultivation threatens protected forested areas and may negatively impact biodiversity (Warren-Thomas et al., 2015).

Rubber tree cultivation is also threatened by the fatal fungal disease, South American Leaf Blight (SALB), which has devastated commercial cultivation of rubber tree in its center of origin, South America (van Beilen and Poirier, 2007a). The threat of pathogens is compounded by the agronomic practices and narrow genetic diversity of *H. brasiliensis*, as rubber trees are clonally propagated and a small number of clones may represent a majority of the productive acreage at any one time. Rubber tree breeding stocks also possess extremely low genetic diversity as they are all derivative from a single collection from the Amazon region to Kew gardens, consisting of a few genotypes (Lieberei, 2007). *Hevea* also has long breeding and phenotyping cycles and commercial
varieties are only 2 to 3 generations removed from wild germplasm (Mantello et al., 2014). This long generation time hinders the ability of rubber tree growers to respond to pathogen threats through breeding or biotechnological means.

While disease resistance is a prime focus of Hevea breeding programs, new rubber tree cultivars may take 6 to 7 years to reach a rubber producing stage, which leaves opportunities for rubber supply crises (Lieberei, 2007). Additionally, the high inputs of manual labor to tap latex from Hevea plantations in order to produce rubber are incompatible with continued globalization and compel the exploration of additional means of producing natural rubber.

1.2 Taraxacum kok-saghyz

Taraxacum kok-saghyz (TK) is one of the most promising alternative sources of natural rubber, as it produces a high molecular weight rubber, comparable to that of H. brasiliensis (Schmidt et al., 2010). Rubber production is localized in the root, which can have dry weight rubber content as high as 18% (Krotkov, 1945). TK can be morphologically characterized by its thick, rounded blue-green leaves and pointed involucral bracts (Whaley and Bowen, 1947a). It is amenable to cultivation over a wide range of temperate areas and can be grown as an annual, facilitating rapid breeding and response to market demand (van Beilen and Poirier, 2007b). Historical efforts to cultivate TK have been limited by a lack of weed control, despite plantings on hundreds of acres in many locations (Whaley and Bowen, 1947a). Given recent volatility in rubber
prices, there has been renewed interest in TK. Furthermore, biotechnology is being considered as an avenue of crop improvement.

1.3 Potential hybridization between TK and wild TO

The large scale cultivation of improved TK raises the concern of gene flow to the wild, weedy common dandelion *Taraxacum officinale* (TO). TO is native to Eurasia, but it has spread, becoming a cosmopolitan weed (Richards, 1970). TO is well adapted to many agricultural landscapes, such as hay fields, pastures, vineyards and orchards; it is particularly prevalent in reduced tillage soy, corn and wheat cropping systems (Kapusta and Krausz, 1993; Stewart-Wade et al., 2002; Andersson and Milberg, 1998).

Both TK and TO have the same base chromosome number (x = 8) and share pollinators including bees, flies, butterflies and moths and have overlapping flowering times (Lyman and Ellstrand, 1984; van Dijk, 2003; Whaley and Bowen, 1947a). TO is present in all 50 states of the United States and every province of Canada. The cultivation of TK inevitably overlaps with the range of TO, the iconic, Common Dandelion. Accordingly, any effort to deploy TK with novel traits must be conscious of the potential for hybridization.

TO primarily exists as one of two cytotypes, diploid and triploid. The former is a self-incompatible sexual, while the latter is an obligate apomict. The majority of Common Dandelions are obligate apomicts; however, the presence of sexual diploids provides an avenue for novel clonal lineages to be created through hybridization between the diploid and triploid cytotypes, with the former acting at the maternal parent. This
breeding system allows TO to reap the benefits of both sexual recombination and apomixis, which contributes to its weedy character (van Dijk, 2003).

While TO is ubiquitous, the diploid cytotype has only been described in limited areas of Central Europe. It is thought that diploid populations may persist in highly variable environments, where meiosis allows adaptive genotypes to be continuously selected (van Dijk and van Damme, 2000; van Dijk, 2003). In North America, all Common Dandelions described by limited surveys have proven to be the triploid cytotype (Solbrig, 1971; Lyman and Ellstrand, 1984).

Triploid TO invariably exhibit obligate apomixis through meiotic diplospory and autonomous endosperm development, meaning that clonal seed is set by unreduced maternal gametes, without any need for pollination. While, in some Taraxacum species, a degree of sexual character is retained (facultative apomixis), TO has proven to be an obligate apomict. Pollen tube development has been observed in apomictic TO under low temperature treatments; however, further investigation has revealed that physical barriers were still present, such that fertilization could not occur (Baarlen et al., 2002).

As the majority of TO is of the triploid cytotype and all Common Dandelion described in North America has proven to be triploid, biotech risk assessment may be informed by hybridizations between triploid pollen donors and diploid dandelions. Hybridizations between disparate dandelion sections of differing cytotypes have been described (Morita et al., 1990). They found that a low amount of seed may be set by diploids, when pollinated by triploids. With molecular markers, they demonstrated that 87.5% of this seed was the result of selfing and not of true hybrid origin. While the
majority of diploid dandelions are self-incompatible, this incompatibility can be broken down by unbalanced interspecific pollen, a process termed the ‘mentor effect’. The mentor effect may serve to limit the number of unfit progeny resulting from hybridization with a triploid father; however, as dandelions are generally outcrossers, there may be a fitness cost associated with selfing. It is evident that broad triploid-diploid hybridizations can occur in *Taraxacum*; however, true hybridization may be a rare event. Similar research demonstrated that hybrid progeny can also be produced by triploid-diploid crosses of closely related *Taraxacum* species (Tas and van Dijk, 1999). In narrow crosses, the mentor effect still proved to be a strong factor as 90% of progeny were found to be the result of mentor effect selfing.

Pollen competition has also proven to be a potentially important factor in triploid-diploid *Taraxacum* hybridizations. In large scale hybridizations, diploid dandelions pollinated by triploid pollen produced many polyploid progeny, which were thought to be hybrids; however, when triploid pollen was mixed with conspecific diploid pollen, no polyploid progeny were observed, indicating that intraspecific diploid pollen may outcompete interspecific triploid pollen (Mártonfiová, 2006).

The hybridization of triploid Common Dandelion with the native diploid dandelion, *Taraxacum ceratophorum* has been described (Brock, 2004). The flowering phenologies of both species were found to strongly overlap. It was also observed that both species shared many pollinators, such as flies, bees, moths and butterflies. Hand pollination experiments showed that *T. ceratophorum* can set reduced seed when pollinated by the Common Dandelion. One third of this seed proved to be of true hybrid
origin and not the result of mentor effect selfing. While the fitness of the hybrids
generated in this study was not evaluated, many *Taraxacum* hybrids have been described
as vigorous (Brock, 2004; Morita et al., 1990; Shibaike et al., 2002).

Overall, hybridization between apomictic triploid and sexual diploid species is
common in *Taraxacum*. While the mentor effect serves as a barrier to hybridization, it
has not been shown to preclude hybridization entirely. Pollen competition may play an
important role in *Taraxacum* hybridizations; however, this phenomenon has not been
demonstrated under field conditions. Furthermore, chloroplast markers suggest that there
is commonly gene flow between many *Taraxacum* species (Wittzell, 1999).

1.4 Research outline

Given the impetus to release novel TK germplasm to produce viable alternative
sources of natural rubber, methods of implementing biotechnology to improve TK are
needed. Furthermore, genomic resources are required both to facilitate gene flow
research, as well as new biotechnology strategies. In order to deploy genetically
engineered TK on a large scale, an understanding of the potential for hybridization is
necessary and new germplasm resources are needed to do so. In order to evaluate the
development and potential risks associated with the release of genetically engineered TK,
three aims detailed below were achieved:
Chapter 2: Development of efficient and accessible genetic engineering protocols for *T. kok-saghyz*

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

2.2 CRISPR/Cas9 genome editing of rubber producing dandelion *Taraxacum kok-saghyz*.

Chapter 3: Development of *T. kok-saghyz* genomic resources

Chapter 4: Ability of *T. kok-saghyz* to hybridize with *T. officinale* and produce fit hybrids

4.1 Evaluation of the hybridization potential between the rubber producing dandelion *Taraxacum kok-saghyz* and the common dandelion, *T. officinale*

4.2 The development of resources for rubber producing dandelions to track gene movement.
Chapter 2: Development of Efficient and Accessible Genetic Engineering Protocols for T. kok-saghyz

Genetic engineering offers the opportunity to rapidly introduce novel traits into plants. Traditional plant biotechnology utilizes Agrobacterium to insert DNA fragments into plant cells. Rare events where these fragments are integrated into the plant genome can then be selected for, and entire plants can be regenerated. Newer technology termed genome editing, can target specific areas of the genome and introduce fragments of DNA or mutagenize native DNA sequences. Here we aimed to establish techniques that utilize these technologies to facilitate the exploration of gene function and the development of improved TK. Accordingly the two specific aims for this chapter were:

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

2.2 CRISPR/Cas9 genome editing of rubber producing dandelion Taraxacum kok-saghyz.

This chapter is partly based on the following publication:

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This chapter is also partly based on the following manuscript which is in preparation to submit to the journal Plant Cell Reports:

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

We have developed a novel transformation system for TK and its close relative, *Taraxacum brevicorniculatum* (TB). By leveraging the natural ability of dandelions to regenerate plants from root fragments, stably transformed plants were obtained from roots inoculated with *Agrobacterium rhizogenes*, with no hormone treatments and minimal manipulations. Binary constructs harboring genes encoding kanamycin resistance and florescent proteins were able to stably transform root fragments of TK and TB at efficiencies of 25% and 15% respectively. Using this system, the timeline from cloning genes of interest to obtaining first generation, transgenic seed is as little as 120 days. A manuscript entitled “Rapid and hormone-free *Agrobacterium rhizogenes*-mediated transformation in rubber producing dandelions *Taraxacum kok-saghyz* and *T. brevicorniculatum*” has been published in Industrial Crops and Products and is included below (Zhang et al., 2015).

Role: Modified regeneration system, played an equal role with another student (Yingxiao Zhang) in transformation and validation on DNA and RNA levels, in transgenic plant acclimation and seed production.
Rapid and hormone-free Agrobacterium rhizogenes-mediated transformation in rubber producing dandelions Taraxacum kok-saghyz and T. brevicorniculatum

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Abbreviations: TK, Taraxacum kok-saghyz; TB, T. brevicorniculatum; NR, Natural rubber; GFP, Green Fluorescent Protein; CFP, Cyan Fluorescent Protein; 1/2 MS, 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.
Abstract

Taraxacum kok-saghyz (TK) and T. 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 florescent protein or cyan florescent 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 florescent 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.

*Keywords*: natural rubber, *Taraxacum kok-saghyz*, *Taraxacum brevicorniculatum*, *Agrobacterium rhizogenes*-mediated transformation, hairy root

**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 Southeast Asia (Mooibroek and Cornish, 2000). *H. brasiliensis* cultivation is threatened by South American Leaf Blight, a fungal disease caused by *Microcylus 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 (van Beilen and Poirier, 2007a). Accordingly, there is an imperative to develop alternative rubber resources and expedite their commercialization to meet market demands.
Among the 2,500 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 1,000 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; Bowes, 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 *T. mongoliam, T. platycarpum* and TB (Song et al., 1991; Bae et al., 2005; Post et al., 2012), while *A. 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.

**Materials and methods**

**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 (half 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.

**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 (Figure 2.1 A) and pEG-35S::CFP (Figure 2.1 B). 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 (5 g L\(^{-1}\) yeast extract, 10 g L\(^{-1}\) peptone, 1 g L\(^{-1}\) sucrose and 0.5 g L\(^{-1}\) MgSO\(_4\)·7H\(_2\)O), containing 100 mg L\(^{-1}\) kanamycin, shaken at 150 rpm at 28 °C. The Agrobacteria cultures were then pelleted and washed sequentially with liquid YEP medium and 1/2 MS medium containing 200 µM acethosyringone. Agrobacteria cultures finally were suspended in liquid 1/2 MS medium containing 200 µM acethosyringone with OD\(_{600}\) 0.6 for transformation.
**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-Benzylationopurine (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.

**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 minutes. Roots then were blotted dry on filter paper and transferred to co-culture medium (solid 1/2 MS medium with 200 µm 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<1mm and D≥1mm) 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.

**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 µL reaction containing 1X Standard Taq Reaction Buffer, 200 µM dNTPs, 0.2 µM forward and 0.2 µM 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’-AGAGGGTGAGATGATGCAA-3’ and vGFP_reverse: 5’-CCATGTGTAATCCAGCAGC-3’; the 650 bp region of CFP was amplified using primers vCFP_forward: 5’-TAAACGGCCACAAGTTCAGC-3’ and vCFP_reverse: 5’-CTTGTACAGCTCGTCCATGC-3’. PCR procedures used were 5 min initial denaturation at 95°C, 30s denaturation at 95°C, 30s annealing at 54°C, 60s elongation at 68°C for 35 cycles, followed by final extension at 68°C for 5 min. A total volume of 10 µL 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 DNasel 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 were 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'-CTTTGAAGAAGTCTGATGCCG-3'; RT-CFP_forward 5’-CACATGAAGCAGCACGACTT-3'; RT-CFP_reverse 5’-

TCCTTGAAGTCTGACGATCCCTT-3’. Endogenous gene β-actin (ACTB) was amplified using the same amount of cDNA and primers: ACTBforward: 5’-

AGCAACTGGGATGACATGGA-3'; ACTB_reverse: 5’-

CATACATGGCGGGACATTG-3’. A total volume of 10 µL 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.

**Florescent 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 Florescent software. GFP images were captured under excitation laser Argon-blue (488nm and 514nm) with excitation wavelengths 488 nm at 82% laser intensity. Images were collected from
497nm to 557nm with 865 smart gain and 50.1 μm pinhole. CFP were visualized under UV (405nm) laser with 77% laser intensity. Images were collected from 453nm to 531nm with 845 smart gain and 64.9 μm pinhole. Figures were created by Microsoft PowerPoint (version 14.0.7128.5000).

**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 μmol m\(^{-2}\) 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 T\(_1\) 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.

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 $P<0.05$.

Results and discussion

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 (Figure 2.2 A-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 (Figure 2.2 A-D). While 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.

**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±0.7% regeneration efficiency, which was 28.7% higher than the regeneration efficiency seen in non-inoculated plants (36.6±5.1%) (Figure 2.3 A). TB regeneration efficiency reached 152.3±8.2% (more than one shoot emerged from a single root fragment) with inoculation, significantly higher than the regeneration efficiency of 95.2±2.2% without inoculation (Figure 2.3 A), 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.

**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, (Figure 2.4 A-C and G-I). Compared to non-transgenic plants (Figure 2.4 D 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 (Figure 2.5 A and B) and transgene expression at the transcription level was confirmed by RT-PCR (Figure 2.6 A and B) using leaf tissue. At the tissue level, confocal microscopy of transgenic root and leaf tissues showed transformation of both tissue types (Figure 2.7 A-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).

**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 more mature roots $\geq 1$mm exhibited a
higher rate of regeneration (Figure 2.3 B). Interestingly, in contrast to TK, TB roots with diameters <1mm showed strong regenerative ability, although this was still lower than regeneration observed using larger roots (Figure 2.3 B). 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 ≥1mm 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 (W635172), 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.

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 tolerate hormonal imbalances potentially introduced by *rol*
genes of *A. rhizogenes* (Figure 2.4 E 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 (Figure
2.4 F and L, Figure 2.8 A-D). Both hairy root phenotypes and florescent protein genes
were heritable in the T₁ generation, with segregation (Figure 2.8 A-E).

The hairy root phenotypes observed in transformed plants persisted after the
transition to non-sterile growth in soil (Figure 2.4 E 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 secondary metabolism, they may have the potential to affect rubber
production or rubber molecular weight. If this growth habit proves to be undesirable,
genomes of interest can be segregated from native *A. rhizogenes* events in the T₁ 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.

**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.
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Figure 2.1 Binary vectors for green florescent protein (GFP) and cyan florescent 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 (→→).
Figure 2.2 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±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.
Figure 2.3 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≥1mm and D<1mm. Regeneration efficiency from root D≥1mm is indicated by the light gray bar (■) and regeneration efficiency from root D<1mm 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 $P<0.05$. 
Figure 2.4 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.
Figure 2.5 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.
Figure 2.6 Reverse transcription polymerase chain reaction (RT-PCR) analysis of green florescent protein (GFP) and cyan florescent 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 each independent transgenic event. Endogenous gene β-actin (ACTB) was used as endogenous gene control for each RT-PCR reaction.
Figure 2.7 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, B) and leaf tissue (C, D) of non-transgenic (WT) and transgenic (GFP) TK. (E) - (H), GFP expression in root tissue (E, F) and leaf tissue (G, H) of non-transgenic (WT) and transgenic (GFP) TB. (I) - (L), CFP expression in root tissue (I, J) and leaf tissue (K, L) of non-transgenic (WT) and transgenic (CFP) TK. (M) - (P), CFP expression in root tissue (M, N) and leaf tissue (O, 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 florescence intensity shown in figures is not quantitative.
Figure 2.8 Stable inheritance and segregation of hairy root phenotypes and florescent protein gene in *Taraxacum kok-saghyz* (TK) T₁ generation. (A) TK T₁ generation plant 6 weeks after germination with hairy root phenotypes. (B) TK T₁ generation plant 6 weeks after germination without hairy root phenotypes. (C) Three-month old TK T₁ generation plant grown under tissue culture conditions with hairy root phenotypes. (D) Three-month old TK T₁ generation plant grown under tissue culture conditions without hairy root phenotypes. (E) Polymerase chain reaction (PCR) analysis of Cyan Florescent Protein (CFP) of TK T₁ generation plant. L, 100 bp DNA ladder from New England Biolabs Inc. W, negative wild type non-transgenic plants control. P, positive plasmid control. T₁-1,2,3, TK T₁ generation plants. Size bars represent 2 cm.
2.2 CRISPR/Cas9 genome editing of rubber producing dandelion *Taraxacum kok-saghyz*

Genome editing techniques include: meganucleases, Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs) and the CRISPR/Cas system. All of these methods utilize proteins to recognize specific DNA sequences and introduce breaks, which can mutagenize target sequences and facilitate homologous recombination. These approaches vary in their specificity and efficiency, as well their monetary and intellectual cost of entry. The newest technology is the CRISPR/Cas system, which has demonstrated high efficiency genome editing in a growing number of species, with a simple implementation and potential for multiplexing. By adapting the previously described root inoculation and regeneration methods, a rapid pipeline to introduce CRISPR-type mutagenesis events, by causing double strand DNA breaks in genes of interest has been established for *Taraxacum* species. This research could facilitate agronomic and metabolic improvements of rubber-producing dandelions, as well as basic research on traits such as pappus development, apomixis and rubber biosynthesis.

Role: Conceived experiment, sourced plasmids, selected guide RNA target sequences, played an equal role with another student (Yingxiao Zhang) in transformation and validation of genome editing.
CRISPR/Cas9 genome editing of rubber producing dandelion *Taraxacum kok-saghyz*

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**Abstract**

CRISPR/Cas9 is a highly accessible genome editing tool. Here we demonstrate its potential for use in *Taraxacum* species. *Taraxacum kok-saghyz* (TK, Kazak Dandelion) is notable for its ability to produce high molecular weight rubber in its roots and is a potential alternative source of natural rubber. In order to accelerate the domestication of Kazak Dandelion, we have established a simple strategy to deploy CRISPR/Cas9 in this species. To knock out TK gene involved in inulin biosynthesis, the potential competing products of rubber, TK plantlets were inoculated with *Agrobacterium rhizogenes* harboring a plasmid encoding a Cas9 and sgRNA targeting TK *fructan:fructan 1-fructosyltransferase*, the enzyme catalyzing the polymerization of...
fructose to inulin. We were able to rapidly induce hairy roots harboring knockout alleles. Mutagenesis was affirmed by observing a loss of restriction sites within targeted genes, and by Sanger sequencing. Of 11 hairy root samples we tested, 10 showed the presence of gene sequence editing, suggesting a high editing efficiency induced by CRISPR/Cas9 system using *A. rhizogenes*-mediated transformation.

**Introduction**

The targeted modification of native genomic DNA in organisms and cells, termed genome editing, is a reverse genetics tool that allows basic and applied research. Previously developed genome editing approaches such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), rely on Protein-DNA interactions to determine specificity (Sander and Joung, 2014). Due to challenges in producing large, custom proteins and limited multiplexing capacity, the application of these approaches in plants has been limited (Bortesi and Fischer, 2015). However, a new technology, termed CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) has accelerated this field due to its simplicity and accessibility. Unlike ZFNs and TALENs, CRISPR relies on RNA-DNA complementary base pairing to determine specificity, which allows for quick construct design and simultaneous editing of multiple genes (Gao et al., 2015; Lowder et al., 2015; Xie et al., 2015). CRISPR/Cas9, which is derived from the type II CRISPR prokaryotic immune system, has been adapted to conduct gene knockouts and regulatory changes, as well as transgene insertions (Jinek et
al., 2012; Bortesi and Fischer, 2015). Specifically, genome editing through CRISPR/Cas9 provides a platform for research focusing on gene function, regulatory elements and genetic mechanisms underlying quantitative trait loci (QTLs). With the ability to introduce multiple targeted edits into plant genomes, CRISPR can also facilitate metabolic engineering, which usually requires gene manipulation of one or multiple pathways.

CRISPR/Cas9-induced genome editing has been demonstrated in multiple plant species, including model species, major food crops as well as fruit crops (Li et al., 2013; Gao et al., 2015; Nekrasov et al., 2013; Shan et al., 2013; Jiang et al., 2013; Ron et al., 2014; Wang et al., 2015; Jia and Wang, 2014). However, the application of CRISPR in industrial crops has not been reported. Kazak dandelion (Taraxacum kok-saghyz, TK) is a rubber producing dandelion species being developed as a potential source of natural rubber and model system for rubber biosynthesis. Virtually all natural rubber comes from the Brazilian rubber tree (Hevea brasiliensis Muell. Arg.), which has a narrow growing range, little genetic diversity and requires manual tapping to extract latex (Mooibroek and Cornish, 2000). TK does not suffer from these shortcomings; however, it is largely undomesticated, competes poorly with weeds and has highly variable rubber production.

To accelerate the domestication of TK as a rubber producing crop, CRISPR may provide an avenue for editing the TK genome to increase rubber yield and introduce desirable agronomic traits. Moreover, the application of CRISPR on TK will facilitate the investigation of rubber biosynthesis mechanisms, which have not been fully
understood (Cornish and Xie, 2012). Recently, a high efficiency Agrobacterium rhizogenes-mediated transformation system has been developed for TK (Zhang et al., 2015), providing a fast approach to introduce CRISPR/Cas9 elements.

In TK, natural rubber is stored in roots as an end product. However, a greater amount of carbon is typically stored as inulin, which is metabolized to overwinter and rapidly produce shoots after overwintering. It has been shown that cold induction decreases inulin and increases rubber in large TK root systems (Cornish et al., 2013), suggesting antagonism between these major carbon sinks. In this study, we intend to disrupt inulin biosynthesis by knocking out fructan:fructan 1-fructosyltransferase (1-FFT), the most important enzyme involved in inulin biosynthesis. This could potentially allocate more carbon to rubber biosynthesis, while also reducing weediness.

In the research described here, we demonstrate that CRISPR/Cas9-induced targeted genome editing can rapidly be achieved in TK through A. rhizogenes-mediated transformation, which will expedite research in TK and potentially other dandelion species.

Materials and methods

Plant material

Plants from TK USDA accession KAZ08-017 (W6 35172) were used for CRISPR/Cas9-induced mutagenesis. Sterilized plants were obtained according to Zhang et al., (2015). Plantlets used for transformation were obtained by culturing root fragments
on solid, half strength Murashige and Skoog (1/2 MS) medium for one month (Murashige and Skoog, 1962; Gamborg et al., 1968; Zhang et al., 2015).

Construction of expression vectors

Two components are required for CRISPR/Cas9 mutagenesis: a guide RNA (sgRNA) targeting the gene to be knocked out, and a nuclease, Cas9. Plasmid pICH86966::AtU6p::sgRNA_PDS which expresses sgRNA was a gift from Sophien Kamoun, (Addgene plasmid # 46966; Nekrasov et al., 2013). The sgRNA was modified to specifically target the second predicted exon (exon2) of TK fructan fructan 1-fructosyltransferase (1-FFT) using Q5® Site-Directed Mutagenesis Kit (New England Biolabs Inc., Ipswich, MA, USA). Primers FFT2_Ins _foward: 5’- CGCACCAATTGTTTTAGAGCTAGAAATAGCAAG-3’ and FFT2_Ins _reverse: 5’-TACGGGTTGTAATCGCTATGTCGACTCTATC-3’ were used to amplify plasmid pICH86966::AtU6p::sgRNA_PDS to replace the original sgRNA targeting sequence by ACAACCCGTACGCACC_AATT (The primers were designed using NEBaseChanger™; restriction enzyme cutting sites overlapping with Cas9 cutting sites are underlined). Amplification products were then treated by Kinase-Ligase-DpnI enzyme mix provided by the mutagenesis kit and transformed into Escherichia coli, according to the manufacturer’s instructions. The sgRNA expression cassette was amplified using primers sgRNA_cl_forward: 5’-ACTAGATCGACGCTGCAAGA-3’ and sgRNA_cl_reverse: 5’-TTGTACAAGAAAGCTGGGTCT-3’ and digested using EcoRI and XbaI. Cas9 expression vector pFGC-pcoCas9 (a gift from Jen Sheen, Addgene plasmid # 52256) was
linearized with the same enzymes mentioned above. The sgRNA expression cassette and pFGC-pcoCas9 were combined using T4 ligase. The final construct, named as pFGC-pcoCas9_FFT_exon2, was introduced into *A. rhizogenes* K599 wild type by electroporation, and further used for plant transformation.

**A. rhizogenese-mediated transformation of TK**

Roots of plantlets at the four leaf stage were removed and the sectioned surface of the plantlets was dipped into *A. rhizogenes* cell suspensions for about 10 seconds. *A. rhizogenes* cell suspensions were prepared according to Zhang et al., (2015). Plantlets were then placed on 1/2 MS medium with 200µM acetosyringone. After 3-days of co-culture and a 7-day recovery period using protocols described previously (Zhang et al., 2015), plantlets were transferred to 1/2 MS medium with 400mg L\(^{-1}\) Timentin.

**Detection of genome editing**

DNA was extracted from hairy roots by grinding in liquid nitrogen to a powder, which was then suspended in preheated (65°C) lysis buffer containing 2% (w/v) CTAB (cetyl trimethyl ammonium bromide), 100mM Tris-HCl (pH 7.5), 1.4M NaCl, 20mM EDTA, 2% (w/v) PVP (polyvinylpyrrolidone) and 0.2% (w/v) β-mercaptoethanol (Kabelka et al., 2002). DNA was purified by treating with phenol:chloroform followed by chloroform. DNA was precipitated by adding 1/10 volume of 3 M sodium acetate and 0.1 µg µl\(^{-1}\) (final concentration) glycogen, followed by an addition of 2.2 volumes of 100% ethanol. Extracted DNA was used for mutagenesis detection by the restriction enzyme site loss method (Parry et al., 1990). Genomic regions flanking the 20 bp target sequence
within \(1-FFT\) exon2 were amplified by FFT2\_v\_forward: 5’-
ATTGTTCCACATGGGTTGGT-3’ and FFT2\_v\_reverse: 5’-
GCCTTCGATGTCGTACCATT-3’. Polymerase chain reaction (PCR) products were
digested with the restriction enzyme \textit{MluCl} to detect the loss of the cut site targeted by
CRISPR/Cas9. The PCR products from mutagenized samples were further cloned into a
plasmid vector using The TOPO® TA Cloning® Kit (Thermo Fisher Scientific Inc.,
Waltham, MA, USA) so that InDels could be resolved from each other and wild-type
sequences by Sanger sequencing.

\section*{Results}

\textit{Agrobacterium rhizogenes}-mediated transformation of \textit{TK}

Hairy roots were produced in three weeks on medium with 400 mg L\(^{-1}\) Timentin.
Compared to non-transformed plants, \textit{A. rhizogenes} inoculated plantlets showed
plagiotropic and high density root growth (Figure 2.9).

\textit{CRISPR/Cas9 genome editing in TK}

Among hairy root populations tested, 10 of 11 were found to contain digestion-
resistant PCR products for Exon 2. The presence of a digestion-resistant band induced by
CRISPR/Cas9 is shown in Figure 2.10. Sequencing of targeted regions in samples
exhibiting digestion-resistant PCR products revealed introduced InDels (Insertions and
deletions). Alignments showing types of mutations introduced within two CRISPR/Cas9
treated root populations are shown in Figure 2.11. InDels, which are not multiples of three, cause frame shifts, resulting in a protein that is not likely to be functional.

**Discussion**

This research has demonstrated editing of the TK genome using a transient CRISPR/Cas9 approach. By using hairy root phenotypes induced by wild type *A. rhizogenes* K599 to enrich for mutagenized tissue, instead of selection with herbicides or antibiotics, novel alleles can be quickly generated. The method also has the potential for minimizing off-target effects, by limiting the exposure of a genome to CRISPR elements. The heterogeneous band pattern shown in the restriction enzyme site loss analysis, suggests that one allele was mutagenized (Figure 2.10). However, the efficiency of mutagenesis would likely be increased by including a selection step. This strategy is augmented by the ability of *Taraxacum* species to rapidly produce plantlets from root fragments (Zhang et al., 2015; Lee et al., 2004).

By using simple cloning strategies, such as those described here, genes of interest in *Taraxacum* genomes may be rapidly knocked out and changes in phenotype can be observed. This approach may lead to the accelerated domestication of rubber producing dandelions such as *T. kok-saghyz* and *T. brevicorniculatum* (TB), by minimizing metabolites that compete with rubber for energy and eliminating traits such as seed shattering and overwintering. In order to deploy CRISPR/Cas9 biotech traits, hairy root genes and CRISPR elements would need to be removed. As TK is an outcrossing species this could be achieved by crossing with wild-type TK and selecting for segregating
mutant alleles. Deploying traits in TB would be more complex as it producing clonal seed through apomixis. Complete knockouts in TB could be obtained by pollinating mutagenized TK with mutagenized TB and then selecting apomictic segregants lacking hairy root phenotypes or other transgenic elements. These methods of genome editing in Taraxacum may also allow for genes potentially implicated in important traits, such as rubber biosynthesis and apomixis, to be rapidly screened.

Overall, we have demonstrated a simple, rapid method to deploy CRISPR/Cas9 in Taraxacum species. Enriching for mutagenized tissue by subculturing hairy roots, permits genome edited plantlets to be rapidly generated. Plantlets regenerated from Taraxacum hairy roots have been shown to be reproductively viable (Zhang et al., 2015). This will allow A. rhizogenes T-DNA insertions to be removed and populations possessing novel alleles to be generated and evaluated.
Figure 2.9 Hairy root induction of *Taraxacum kok-saghyz* transformed by *Agrobacterium rhizogenes* harboring a sgRNA and Cas9 expression cassette. (A) An uninoculated plant. (B) A plant inoculated by *A. rhizogenes* harboring a sgRNA and Cas9 expression cassette.
Figure 2.10 Detection of Exon2 targeted genome editing by the restriction enzyme site loss method. L, NEB 100bp ladder; Control, plants transformed using sgRNA and Cas9 not targeting *Taraxacum kok-saghyz* gene 1-FFT Exon 2; Exon2, plants transformed using Exon2 targeted sgRNA and Cas9; uncut, undigested PCR products; cut, PCR products digested by *MluCI*. 
Figure 2.11 Alignment of reads with CRISPR/Cas9-induced InDels in *Taraxacum kok-saghyz* gene 1-FFT Exon2. The wild-type sequence is shown at the top. The targeted sequence is shown in red, while mutations are shown in blue. PAM denotes the Protospacer Adjacent Motif. The sample names are shown on the left. The sizes of the InDels introduced are shown in purple. The numbers of colonies where a given mutation was observed are shown in the parenthesis.

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<th>WT</th>
<th>FFT–Exon2</th>
<th>MluCI</th>
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<tr>
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<td>CTACCAATACAACCGGTACGCCCA- ATT TGGGGCAA</td>
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<td>-3 (x2)</td>
<td>CTACCAATACAACCGGTACGCCCAATT TGGGGCAA</td>
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<td>1</td>
<td>+1 (x6)</td>
<td>CTACCAATACAACCGGTACGCCCAATT TGGGGCAA</td>
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Chapter 3: Development of *T. kok-saghyz* genomic resources

When exposed to interspecific pollen, TK may produce seed by hybridization or mentor effect selfing, which is induced by unbalanced pollen. In order to resolve true hybrid from selfed progeny, species specific molecular markers are needed. By using publically available Expressed Sequence Tag resources, a complement of gel-based molecular markers was created, which can differentiate TK and TO. Furthermore, we have assembled whole chloroplast genome sequences of TK, TO and TB, and developed gel-based markers to distinguish the chloroplasts of TK and TO. Developing chloroplast markers is advantageous as chloroplast sequences are highly conserved and are more likely to be applicable to field studies. Furthermore, chloroplast markers are strictly maternally inherited and can be used to detect maternal gene flow, even after many generations. Nuclear markers are beneficial as they can detect seed mediated or pollen mediated gene flow. In Chapter 3, we describe the discovery of nuclear and chloroplast molecular markers in *Taraxacum* species and detail chloroplast genome assembly and annotation.

This chapter is partly based on the following manuscript which is in preparation to submit to the journal Molecular Ecology Resources:

Role: Contributed to design of MiSeq sequencing experiments and marker development, planted and maintained validation populations, prepared DNA for PCR.
Chloroplast genome sequence diversity in *Taraxacum* sp. complements EST diversity in species differentiation of dandelions produced for rubber and their weedy relatives

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**Abstract**

Kazak dandelion (*Taraxacum kok-saghyz*, TK) is being developed as a domestic source of natural rubber to meet increasing global demand. However, the domestication of TK is complicated by two additional dandelion species, *Taraxacum brevicorniculatum* (TB) and the common dandelion (*Taraxacum officinale*, TO). TB is often present as a seed contaminant within TK accessions, while TO is a ubiquitous weed that may hybridize with TK harboring novel traits. To discriminate these three species at the molecular level, and facilitate gene flow studies between potential rubber crop TK and its ubiquitous weedy relative TO, we generated genomic and marker resources for the three dandelion species. Complete chloroplast genome sequences of TK (151,338 bp), TO...
(151,434 bp) and TB (151,281 bp) were obtained using the Illumina GAII and MiSeq platforms. By sequencing multiple genotypes for each species and testing variants using gel-based methods, four chloroplast Single Nucleotide Polymorphism (SNP) variants were found to be fixed between TK and TO in large populations. Additionally, Expressed Sequence Tag (EST) resources were developed for TO and TK. These sequence resources permitted the identification of six nuclear species-specific SNPs for use as markers. The availability of chloroplast genomes of these three dandelion species, as well as chloroplast and nuclear molecular markers, will provide a powerful genetic resource for germplasm differentiation and purification, and the study of potential gene flow among *Taraxacum* species.

**Introduction**

Kazak dandelion (*Taraxacum kok-saghyz* Rodin, TK) is being developed as an alternative natural rubber source in response to increasing global demand and instability of current sources. Natural rubber production is fragile due to its reliance on a single source, the Brazilian or Para rubber tree (*Hevea brasiliensis* Muell. Arg.), which is cultivated mostly in Southeast Asia (van Beilen and Poirier, 2007a). This production could be easily disrupted by the accidental introduction of South American Leaf Blight, a fatal fungal disease caused by *Microcylus ulei* (Edathil, 1986), which is currently controlled by quarantine measures. Moreover, *Hevea* rubber production is also threatened by high labor costs and land competition with palm plantations (van Beilen and Poirier,
To establish a more sustainable natural rubber production system, TK has been explored in many temperate countries as a potential domestic rubber-producing crop (Kirschner et al., 2013). TK, which originated in southeastern Kazakhstan (Krotkov, 1945), is a diploid (2x = 16) outcrossing, self-incompatible species. The wide environmental adaptation and fast generation time make TK one of the most promising potential rubber producing plants.

The potential for domestication of TK is complicated by two additional dandelion species, *Taraxacum brevicorniculatum* Koroleva (TB) and *Taraxacum officinale* F.H. Wigg. (TO, common dandelion). TK, TB and TO are sympatric species, and germplasm collections are potentially mixed (Kirschner et al., 2013). TB has often been misidentified as TK in *ex situ* germplasm collections, though recent work to discriminate them using taxonomy and Amplified Fragment Length Polymorphisms (AFLPs) has helped classify accessions (Kirschner et al., 2013). TB also produces natural rubber in its roots, albeit to a lesser extent than TK (approximately 2-3% of the dry weight in TB, compared to as high as 30% in TK) (Janina Post et al., 2012; Whaley and Bowen, 1947a). However, TB is a more vigorous species with a high accumulation of biomass, and could also potentially be used as a rubber producing plant. TB and TK share the same geographical origin and have been co-introduced into North America and Europe, where TB is often an unintentional seed contaminant. TB is a triploid (3x = 24), which exhibits obligate apomixis, where clonal seeds are produced without pollination. TB has been used to investigate functions of genes related to rubber biosynthesis in previous studies (Janina Post et al., 2012; Schmidt et al., 2009; van Deenen et al., 2012). However, TB has often
been misidentified as TK in many ex situ germplasm collections until TB and TK were discriminated using morphological, taxonomic and Amplified Fragment Length Polymorphism (AFLP) analyses (Kirschner et al., 2013).

Another dandelion species relevant to TK is TO, the ubiquitous weedy dandelion. TO is distributed worldwide and has been identified in all areas in North America. TO has virtually no rubber production, but it is a vigorous, highly successful weed. TO is a perennial and is most successful as an agricultural weed in no-till systems. All TO reported in North America are obligate apomictic triploids (3x=24) (Lyman and Ellstrand, 1984; Solbrig, 1971). However, sexual, diploid TO (2x=16) have been identified in Europe (van Dijk and van Damme, 2000).

TK domestication would involve large plantings and possibly the introduction of genetic modifications to improve agronomic performance and rubber yield. The potential for TK and TO to hybridize, raises concerns about gene flow between species. There are two potential pathways of gene flow: pollen-mediated gene flow and seed-mediated gene flow. In pollen-mediated gene flow, transgenes contained in TK pollen could be potentially introduced into TO and produce hybrid progeny. Alternatively, TK could potentially serve as the pollen acceptor and be fertilized by TO pollen to produce hybrid progeny. In the case of seed-mediated gene flow, progeny produced by TK could be either from hybridization or through the “mentor effect”, where self-incompatibility is broken down by the introduction of polyploid pollen (Morita et al., 1990; Tas and Van Dijk, 1999). Similarly, in the case of pollen mediated gene flow, apomictically produced
TO seed may represent the majority, or the entirety of seed produced in controlled crosses. In order to understand the potential for gene flow between TK and TO, species specific molecular markers are needed to differentiate hybrids from apomictically produced TO and self-pollinated TK.

Considering the differences of rubber content and reproduction system among the aforementioned three species, as well as the potential production of TK/TO hybrids, it is important to distinguish them in studies related to molecular genetics, genomics, plant breeding and gene flow risk assessment. Since the fecundity of weedy dandelions (TB and TO) has been reported to be 40 times higher than TK, once seed from weedy dandelion is introduced into TK seed, the contamination can be magnified significantly through one generation (Whaley and Bowen, 1947). Information which can be used to resolve three dandelion species as well as their potential hybrids includes data on morphology and ploidy. However, morphology data may vary through developmental stages and is highly reliant on the experience of the observers. Ploidy detection using flow cytometry cannot be easily multiplexed. Moreover, genome size of a potential hybrid may overlap with the genome size of the three dandelion species. Therefore, it is necessary to develop molecular markers to provide an accurate and high throughput approach for species and hybrid differentiation.

One source of sequence diversity that can be used to differentiate species is the chloroplast genome. Due to the slower evolution of chloroplast genomes compared to nuclear genomes, chloroplast sequences have often been used for phylogenetic studies
and species identification (Scarcelli et al., 2011; Shibaike et al., 2002). Moreover, the genetic information in angiosperm chloroplasts is inherited maternally in most cases, making the chloroplast genome a good indicator to track maternal ancestry (Wittzell, 1999). Therefore, the development of chloroplast markers will provide an accurate molecular tool to differentiate *Taraxacum* species. Furthermore, the maternal parent could be easily identified in putative hybrid progeny when the parental information is absent, regardless of when hybridization occurred. However, no chloroplast sequences of these three species have been published.

To identify hybrids, chloroplast markers alone are insufficient, as they are dominant and only indicate maternal ancestry; however, chloroplast markers may be complemented with markers from the nuclear genomes of these species. To date only limited genomic resources are available for TK and TO. 16,441 expressed sequence tags (ESTs) can be found on the National Center for Biotechnology Information (NCBI), which were sequenced from TK root RNA (Collins et al., 2009, unpublished). More EST data obtained from whole plants (41278 ESTs, 16858 unigenes) are available for TO (Barker et al., 2008; Lai et al., 2012). No TB sequence data has been reported.

In this study, chloroplast genomes have been sequenced for TK, TB and TO and chloroplast markers have been developed and validated. At the same time, nuclear markers were developed using previously published ESTs. The genomic and marker resources described in this paper will not only provide a molecular toolkit for germplasm
identification and purification, but also allow or accurate gene flow studies between TK and TO.

Materials and methods

Chloroplast genome and nuclear fragments sequencing

To generate a complete TK chloroplast genome sequence, chloroplast DNA was extracted from a mixture of genetically distinct TK plants. To reduce the polysaccharide content, which interferes with DNA extraction, young leaves were harvested from 1-2 month-old greenhouse grown TK plants subjected to a two day dark treatment before harvesting. About 20g leaf tissue were ground in liquid nitrogen and suspended in 400 ml grinding buffer (0.35 M sorbitol, 50 mM HEPES/KOH, pH 7.5, 2 mM EDTA, 1 mM MgCl$_2$, 1 mM MnCl$_2$ and 4.4 mM sodium ascorbate (added just before use) (modified from Schuler and Zielinski, 1989; Palmer, 1988). After filtering the tissue through four layers of grinding buffer moistened miracloth, the filtrate was collected by centrifuging at 4,500 x g for 20 min. The re-suspended pellets were placed on the top of the 30-50% sucrose gradient and centrifuged for 45 min at 10,000 x g, 4°C, in a swinging bucket rotor. The intact chloroplasts formed a layer between the 30% and 50% sucrose and were separated from the broken chloroplast remnants (Figure 3.1A). Isolated chloroplasts were treated by DNase using Ambion® TURBO DNA-free™ Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) to remove the nuclear DNA. Chloroplast DNA was extracted using GenElute™ Plant Genomic DNA Miniprep kit (Sigma-Aldrich®, St. Louis, MO, USA) and enriched using the REPI-g® Mini Kit (Qiagen, Inc., Hilden, Germany). DNA
quality was initially checked and quantified using a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). Distinctive individual band patterns shown after DNA digestion by restriction enzyme *EcoRI* indicated the high percentage of chloroplast DNA (Figure 3.1B). DNA was submitted to The Molecular and Cellular Imaging Center (MCIC) (OARDC) for additional quality control and sequencing using the Illumina GAII sequencing platform.

To generate TK chloroplast genomes from multiple genotypes as well as complete TO and TB chloroplast genomes, three species were sequenced in a single MiSeq run. A total of 24 genotypes were selected for TK, including 19 USDA lines, three mixed genotypes from USDA lines and a single cytoplastic male sterile line (Table 3.1). Twenty four TO genotypes from a global collection of TO seed (Dr. John Cardina, the Ohio State University, OARDC, Wooster, OH, USA), including seed collected from North America, Europe and China, were used for sequencing (Table 3.2). An TB “Clone A” donated by Peter van Dijk (Keygene, Wageningen, Netherlands), which originally came from Botanical garden, Marburg, Germany, as well as 11 genotypes descended from plants collected from Kazakhstan and distributed broadly by Dr. Anvar Buranov were used for TB chloroplast sequencing (Table 3.3) (Kirschner et al., 2013). The total DNA from 60 leaf samples was extracted using a 2% cetyl trimethylammonium bromide (CTAB) DNA extraction protocol (Kabelka et al., 2002). DNA amount was normalized to 1ng µL⁻¹ and used for entire chloroplast genome amplification by Long Range Polymerase Chain Reaction (PCR) using Q5® High-Fidelity DNA Polymerase (New England Biolabs Inc., Ipswich, MA, USA). Primers were designed on the conserved
regions of the draft TK chloroplast sequence generated by the Illumina GAII data (Table 3.4). Amplified fragments were normalized within each species to have the same molarity and submitted for MiSeq sequencing.

**Chloroplast genome assembly and annotation**

More than 25 million paired-end reads were generated by the Illumina GAII sequencing platform for TK, while more than 10, 12 and 6 million reads were generated by MiSeq sequencing for multiple genotypes of TK, TO and TB, respectively. Quality control was conducted using the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/index.html by Hannon Lab). For TK GAII data, the quality cutoff score used was 40 (-q). A quality score of 20 was used for all Miseq data. By using program Velvet (version 1.2.10), with parameters, kmer=35, -cov_cutoff = 20 (Zerbino and Birney, 2008), a complete TK chloroplast genome sequence was generated from high quality GAII short reads. TO and TB chloroplast genome were generated after aligning assembled contigs to the TK chloroplast genome as a reference by BLASTn (Madden, 2003).

Complete chloroplast genomes of TK, TO and TB were annotated using the Dual Organellar GenoMe Annotator (DOGMA, Wyman et al., 2004). Annotation errors were manually corrected. An annotation map was generated using OrganellarGenomeDRAW (OGDRAW, Lohse et al., 2013).
**Comparative analysis and phylogenetic analysis in Asteraceae family**

Phylogenetic analysis was conducted using the Rubisco (Ribulose-1, 5-bisphosphate carboxylase/oxygenase) large subunit gene *rbcL* from all species in the Asteraceae family with available chloroplast genome sequences. Multiple sequence alignment was carried out using ClustalW, followed by phylogenetic tree generation using MEGA v.5.05 (Tamura et al., 2011).

**Chloroplast species-specific marker discovery**

To develop chloroplast species-specific markers, TK, TO and TB short reads were mapped to the chloroplast genome sequences of all these three species using Bowtie 2 (Langmead and Salzberg, 2012). Fixed variants among these three species are detected by Freebayes and considered as candidate species-specific markers.

**Nuclear species-specific marker discovery**

To develop nuclear species-specific markers using available Expressed Sequence Tag (EST) resources, 41,301 ESTs of TO and 16,441 ESTs of TK were obtained from NCBI. Using the pipeline described by Kozik (2007) ("CDS Assembly and SNP Discovery."Cgpdb.ucdavis.edu/SNP_Discovery_CDS. N.p., Aug. 2007.), ESTs were assembled into contigs and filtered. Alignments containing redundantly detected SNPs and EST reads from both TK and TO were selected for marker validation. Interspecific variants with conserved flanking regions were selected manually from these alignments.
Species-specific marker validation

Markers were validated through gel based assays. Primers were designed by Primer 3 to validate Cleaved Amplified Polymorphisms (CAPs) which were identified by CAPS Designer (Fernandez-Pozo et al., 2014), using the following PCR procedure: 5 min initial denaturation at 95°C, followed by 35 cycles of 40s denaturation at 95°C, 60s annealing at 54°C, 60s elongation at 68°C, as well as a final extension step at 68°C for 5 min. Tetra-primer ARMS-PCR was also carried out to detect Single Nucleotide Polymorphism (SNP) using the similar PCR procedure with a 58 °C annealing temperature (Ye et al., 2001). All the PCR reactions were conducted using reagents obtained from New England Biolabs (Inc., Ipswich, MA, USA) in a 10 µL reaction, following the manufactures instructions.

Results

Chloroplast genome generation, characterization and annotation

The complete chloroplast genome sequences of TK, TO and TB were obtained. The genome sizes of these three species are similar with other species in the Asteraceae family. The chloroplast genome can be divided into four regions, which are one Large Single Copy (LSC) region, one Small Single Copy (SSC) region and two Inverted Repeat (IR) regions. The genome size, and regions, as well as GC content of each species are listed in Table 6. The annotated chloroplast genomes of these three species are represented in one circular map (Figure 3.2). A total of 135 genes have been identified for each of the three species, including 80 protein-coding genes, 8 rRNA genes, 36 tRNA
genes as well as 11 pseudogenes and Open Reading Frames (ORFs). There are 59 protein-coding genes, 21 tRNA genes and 2 pseudogenes and ORFs located in the LSC region; while 11 protein-coding genes, 1 tRNA genes and 3 pseudogenes and ORFs located in the LSC region. All the rRNA genes are located in the IR regions, along with 5 protein-coding genes, 7 tRNA genes and 3 pseudogenes and ORFs. Genes located in the IR regions are duplicated.

**Phylogenetic analysis in Asteraceae family**

Sequence alignment showed that TK, TB and TO chloroplast genomes are highly homologous with other members in Asteraceae family. Phylogenetic analysis showed that TK has a closer genetic distance to TB than TO. The *Taraxacum* species group is most closely related to lettuce (Figure 3.3) out of the species analyzed.

**Chloroplast species-specific marker development**

Variant calling revealed 229 intraspecific variants within 24 genotypes of TK, including 176 SNPs, while only 31 intraspecific variants within 24 genotypes of TO were detected, including 12 SNPs. A total of 248 variants were identified as fixed between TK and TO, among which three CAP markers and one Tetra-primer ARMS-PCR marker were developed as species-specific markers. The primers and restriction enzymes used for marker detection are included in Table 3.6. These four markers were further validated in a large TO population, including 24 genotypes used for MiSeq and other 59 genotypes from the TO seed world collection, as well as a TK population, including multiple genotypes from the USDA TK collection and TK populations from current OSU breeding
programs (Table 3.1 and 3.2). All four markers showed band patterns within these populations, which can be used to differentiate TK and TO.

**Nuclear species-specific marker development**

A total of 6,200 contigs were assembled from existing TO and TK EST resources, totaling 4.2 Megabases (MB), containing 16,900 redundantly detected variants. Of these contigs, 1,372 contained redundant variants and reads from TO and TK. A total of 23 redundant, putatively species specific SNPs were tested as CAPs in TK and TO populations mentioned above. Of these, 2 (9%) did not exhibit any diversity, 16 (69%) were polymorphic within both species and 5 (22%) were fixed between TK and TO (Table 3.7).

**Discussion**

It is expected that the most likely avenue of hybridization between TK and TO is the pollination of TK by TO, as the majority of TO are obligate apomicts (Lyman and Ellstrand, 1984; Solbrig, 1971). Therefore, a maternally inherited, chloroplast marker would allow a TK maternal background to be detected within dandelions that are phenotypically similar to TO. This may be exceptionally useful for detecting maternal ancestry under field conditions, as they are not eroded by recombination. However, in controlled crosses, chloroplast markers alone are insufficient to identify hybrids, as all progeny produced by a mother plant will possess the same chloroplast genome regardless of whether hybridization has occurred. To provide tools to address this, nuclear markers were developed to allow the detection of true hybrids.
The complete annotated chloroplast sequences for TK, TO and TB, allows the development of chloroplast engineering within *Taraxacum*. The availability of the native chloroplast sequences of an organism can allow constructs to more readily achieve homologous recombination (Verma et al., 2008). Chloroplast engineering is a powerful tool that provides a high level of transgene expression because of the polyploid nature of chloroplast genomes and the large number of chloroplasts present in a single plant cell (Bendich, 1987). Furthermore, chloroplast engineering should prevent the escape of transgenes via pollen, as chloroplasts are maternally inherited (Daniell, 2002; Scott and Wilkinson, 1999). Chloroplast engineering also allows multigene transformation and chloroplast gene manipulation (Daniell *et al*., 2005). This research may enable chloroplast engineering in TK to divert additional assimilate to rubber production.

In comparing the chloroplast sequences of TK, TO and TB it became apparent that TK and TB were highly similar. Moreover, all four gel-based markers which could discriminate TK and TO could not discern TK and TB. These results suggest that TB and TK share a maternal ancestor. This study may enable additional research on *Taraxacum* chloroplast diversity, by demonstrating a complement of primers which can amplify entire chloroplast genomes. Furthermore, it may inform chloroplast sequencing efforts to resolve *Taraxacum* phylogenies, by informing which regions may have higher interspecific diversity.
Conclusions

In this study, both interspecific and intraspecific chloroplast and nuclear markers have been discovered. Interspecific markers may assist in species identification and gene flow studies. Intraspecific markers may have uses in population genetics to test correlations between genetic information carried by chloroplast and nuclear genomes and geographic or environmental data. Intraspecific markers can be used to characterize population structures, revealing information about local adaptation, important evolutionary events and genetic communication frequencies. Additionally, intraspecific nuclear markers are also be used to validate hybrids controlled crosses, develop genetic maps and conduct marker assisted breeding.
Figure 3.1 *Taraxacum kok-saghyz* chloroplast DNA isolation. (A) *T. kok-saghyz* chloroplast isolation using sucrose gradient. (B) Chloroplast DNA quality analysis by restriction enzyme digestion. L, 1kb Ladder (Promega); 1, Chloroplast DNA without amplification; 2, Chloroplast DNA after amplification; 3, Amplified chloroplast DNA after digestion with *EcoRI*. 
Figure 3.2 Chloroplast genome annotation map for *Taraxacum kok-saghyz*, *T. brevicorniculatum* and *T. officinale*. Chloroplast genome map represents all three species since their gene number, order and names are the same. Genes on the outside are transcribed in the counterclockwise direction while genes on the inside are transcribed in the clockwise direction, as shown by the arrows.
Figure 3.3 Phylogenetic analysis using rbcL gene from all species with available chloroplast sequences in the Asteraceae family.
Table 3.1 *Taraxacum kok-saghyz* genotypes for sequencing and marker validation

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Table 3.2 continued

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* Source NO. indicates the collector of *Taraxacum officinale* seed.
Table 3.3 *Taraxacum brevicorniculatum* genotypes for sequencing and marker validation

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Table 3.4 Primers used for chloroplast genome amplification by Long Range PCR

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Table 3.5 Chloroplast genomes of *Taraxacum kok-saghyz*, *T. brevicorniculatum* and *T. officinale*

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Table 3.6 Chloroplast *Taraxacum kok-saghyz* and *T. officinale* species-specific markers

<table>
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<th>Annotation</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Length</th>
<th>Ta</th>
<th>Enzyme</th>
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<tbody>
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<td>C1</td>
<td>inter space between <em>rbcL</em> (ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit) and <em>accD</em> (Acetyl-CoA carboxylase carboxyltransferase beta subunit)</td>
<td>5'-ACTCTTTCCACCCATCTGT-3'</td>
<td>5'-TGAACCACCATCTTTTCATAGAG-3'</td>
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<td><em>TaqI</em></td>
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<td>C2</td>
<td><em>accD</em></td>
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<td>5'-CGCGATCGGGGTCTTACTA-3'</td>
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<td><em>NcoI</em></td>
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<td>C3</td>
<td><em>ycf1</em> (Hypothetical chloroplast RF1)</td>
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Note: C1-3 are CAP markers and C4 is a Tetra-primer ARMS-PCR marker.
Table 3.7 Nuclear species-specific and intraspecific markers

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<th>Reverse Primer</th>
<th>Length</th>
<th>Ta</th>
<th>Enzyme</th>
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</tr>
<tr>
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<td>5'-CTTGAGCCATGCCTGAGTT-3'</td>
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<td>54</td>
<td>DpnII</td>
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<tr>
<td>N6</td>
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<td>5'-TGAGATTTATTGACGACACC-3'</td>
<td>5'-CCGCACCTATGCCCCCTT-3'</td>
<td>358</td>
<td>56</td>
<td>AluI</td>
</tr>
<tr>
<td>N7</td>
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<td>5'-AGGGTCTTGATCTGTTGCT-3'</td>
<td>5'-CTTGAGCCATGCCTGAGTT-3'</td>
<td>323</td>
<td>54</td>
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<tr>
<td>N8</td>
<td>enoyl reductase</td>
<td>5'-ACTACTCGGAGCGGAAGAGA-3'</td>
<td>5'-AATCACCCCAACCCCTAACC-3'</td>
<td>606</td>
<td>54</td>
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</tr>
<tr>
<td>N9</td>
<td>cinnamyl alcohol dehydrogenase 5</td>
<td>5'-TGATGTTTACACCAGGGTA-3'</td>
<td>5'-AGCATGAGAGAGAGGGAGAC-3'</td>
<td>504</td>
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<tr>
<td>N10</td>
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<td>5'-AGCATGAGAGAGAGGGAGAC-3'</td>
<td>504</td>
<td>54</td>
<td>HaeIII</td>
</tr>
<tr>
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<td>5'-TGATGTTTACACCAGGGTA-3'</td>
<td>5'-AGCATGAGAGAGAGGGAGAC-3'</td>
<td>504</td>
<td>54</td>
<td>HaeIII</td>
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<tr>
<td>N12</td>
<td>cinnamyl alcohol dehydrogenase 5</td>
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<td>5'-AGCATGAGAGAGAGGGAGAC-3'</td>
<td>504</td>
<td>54</td>
<td>HaeIII</td>
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<tr>
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<td>HaeIII</td>
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<td>N18</td>
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<tr>
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<td>aquaporin tip2-2</td>
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<td>5'-CCGCACCTATGCCCCCTT-3'</td>
<td>358</td>
<td>56</td>
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<tr>
<td>N21</td>
<td>aquaporin tip2-2</td>
<td>5'-TGAGATTTATTGACGACACC-3'</td>
<td>5'-CCGCACCTATGCCCCCTT-3'</td>
<td>358</td>
<td>56</td>
<td>SerII</td>
</tr>
</tbody>
</table>

Note: N1-5 are TO/TG species-specific markers and N6-21 are intraspecific markers.
Chapter 4: Ability of \textit{T. kok-saghyz} to Hybridize with \textit{T. officinale} and Produce Fit Hybrids

4.1 Evaluation of the hybridization potential between the rubber producing dandelion \textit{Taraxacum kok-saghyz} and the common dandelion, \textit{T. officinale}.

4.2 The development of resources for rubber producing dandelions to track gene movement.

This chapter is partly based on the following manuscript which is in preparation to publish in the journal Molecular Ecology:

4.1 Evaluation of the hybridization potential between the rubber producing dandelion *Taraxacum kok-saghyz* and the common dandelion, *T. officinale*. 
Evaluation of the hybridization potential between the rubber producing dandelion

*Taraxacum kok-saghyz* and the common dandelion, *T. officinale*

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**Abstract**

*Taraxacum kok-saghyz* (TK) is a species of dandelion which can produce high quality rubber in its roots; however, TK competes poorly with weeds. In order to overcome this shortcoming, there is interest in developing genetically engineered TK germplasm. The potential release of such germplasm raises the question of gene introgression into the ubiquitous weedy relative of TK, the Common Dandelion, *T. officinale* (TO). The potential for introgression may be influenced by the reproductive biology of TO, which can exist as a diploid sexual or polyploid obligate apomict. In order to inform risk, we have conducted a global survey of TO genome size consisting of 636 accessions, where we exclusively found triploid, obligate apomicts in North America, but found rare sexual diploids in central Europe. As weedy TO types exhibit
obligate apomixis, they are expected to be unreceptive to TK pollen; however, it may still be possible for them to pollinate TK. To this end, unidirectional crosses were conducted and progeny were evaluated with molecular markers. These crosses produced low seed set and seeds with a low germination rate. However, 23% of progeny proved to be the result of true hybridization. Outdoor seed production areas heavily contaminated with TO were also screened for naturally occurring hybridization during a three year period using a combined strategy of both phenotyping and genotyping. Hybrids were only detected during one of these years, at a rate of one in 100,000, when pollination was augmented with bee hives. Hybrids from controlled crosses exhibited TO characteristics, such as lacerate leaves and apomixis. Some apomictic hybrids were able to produce viable seed, while non-apomicts were sterile. Seed produced by apomictic hybrids demonstrated the ability to establish and produce subsequent apomictic progeny when in competition with perennial rye grass. While, the prevalence of apomixis in TO may limit subsequent pollen mediated gene flow and introgression, more work is needed to understand the longevity of apomictic hybrids under natural conditions.

**Introduction**

Natural rubber is an essential resource that is produced by the Brazilian rubber tree (*Hevea brasiliensis*). Rubber tree cultivation is restricted to tropical regions, requires labor intensive tapping and is threatened by the fatal fungal disease South American Leaf Blight (Edathil, 1986). Potential alternative sources of natural rubber include the desert shrub, guayule (*Parthenium argentatum*) and Kazak dandelion (*Taraxacum kok-saghyz*,
TK). The use of TK as a rubber producing crop is speculated to be advantageous, as it can be grown as an annual in temperate environments and is possibly amenable to mechanized agriculture. However, TK is largely undomesticated and competes poorly with weeds. TK may be improved to overcome this shortcoming by breeding or biotechnology. The development of TK with novel traits, such as herbicide resistance, raises the concern of gene flow to wild *Taraxacum* species, specifically the common dandelion (*Taraxacum officinale*, TO).

TO is generally a polyploid obligate apomict, while a minority of TO in regions of Central Europe have been described as diploid (van Dijk and van Damme, 2000). Limited characterizations of TO in North America have not detected sexual biotypes (Lyman and Ellstrand, 1984; Solbrig, 1971). As apomictic TO is considered unreceptive to pollen, gene flow via pollen may be precluded (van Baarlen et al., 2002), although large scale experiments to fully detect residual maternal, sexual reproduction of apomictic TO have not been conducted. However, the fertilization of diploid dandelions by interspecific polyploids has been demonstrated in both broad and narrow crosses, where crosses produce a mixture of seed as a result of true hybridization and selfing induced by polyploid pollen (mentor effect) (Morita et al., 1990; Tas and Van Dijk, 1999). This result suggests that seed mediated gene flow, where diploid TK is pollinated by polyploid TO, may be the most likely avenue of hybridization and potentially transgene escape.
Given the aforementioned phenomena in *Taraxacum*, there is potential for transgene introgression from TK into TO, as the latter is ubiquitous and overlaps in extent with virtually all potential cultivation sites. Additionally, both dandelions share pollination syndromes, base chromosome number (x = 8) and overlap in flowering time.

In order to evaluate the potential for hybridization between TK and TO, a new global collection of TO was characterized by flow cytometry. Controlled crosses were conducted, with apomictic TO pollen donors and TK pollen recipients, as well as TK pollen donors and rare diploid TO. To determine the extent of hybridization under realistic conditions, seed lots were screened for contamination by hybrids over a three year period. Furthermore, lifecycle characteristics of hybrids were evaluated in order to inform risk.

**Methods**

*Flow Cytometry Seed Screening*

*Taraxacum* seed with TO phenotypes were collected from North American, South American, Europe and China for genome size analyses. A total of 636 *Taraxacum* accessions were used for flow cytometry. Among the accessions, 582 were collected from the USA, 16 were from Canada and 5 were from Chile. A total of 28 accessions were collected from Europe, including 9 from the Netherland, 3 from Germany, 5 from Italy, 8 from Norway, 2 from Austria and 1 from Slovenia. Additionally, 5 accessions were collected from northeast China.
Ten seed from each *Taraxacum* accession were chopped in 1.4 ml of Galbraith’s buffer amended with 1% (w/v) polyvinylpyrrolidone (PVP) m.w. 10K (Galbraith et al., 1983). Seed fragments from radish (*Raphanus sativus*) were included as an internal standard, estimated at 1.05 GigaBases GB (Kitashiba et al., 2014). The solution was then passed through a 30 micron nylon mesh filter and brought to a final volume of 1.5 ml, by adding suspensions of propidium iodide and RNase to final concentrations of 50 µg ml⁻¹ each. Samples were gently mixed and incubated in the dark for 20 minutes at 20 °C. Samples were run on a Partec Cyflow PA equipped with a green (532 nm) laser, at speed of 0.3 µl s⁻¹ and a lower limit of 0.73 until 100 µl had been sampled. Gains were adjusted such that the control exhibited a fluorescence value of 50.

**Emasculation**

Emasculations were conducted to detect apomxis using flower buds from stalks of at least 5 cm. Cuts were made roughly 0.5 cm above where bracts emerged using a sterilized scissor. Three buds were sampled for each genotypes tested. Buds were allowed to mature for three weeks after emasculation before seed set was scored.

**Controlled Crosses and genotyping**

TK flower buds were covered with 3 x 3 cm pollen exclusion bags custom made from PQ218 DelNet bags (DelStar Technologies, Middletown, Delaware, USA) using a heat sealer. After anthesis, bags were removed and an entire TO flower was used as a pollen donor, by brushing two flowers together in a circular motion for ten seconds.
After crossing, TO flowers were discarded and TK flowers were resealed and allowed to mature for 3 weeks. After collection, seeds were allowed an additional 5 weeks in cold storage (4 °C) before evaluation. A total of six TO clonal genotypes were used to pollinate a diverse family of TK consisting of 75 genotypes. Clonal TO genotypes used for this experiment included three accessions from Pennsylvania, two from Iowa and one from Ohio. DNA was extracted using a 2% CTAB method adapted to a 96 well plate format in the Genogrinder platform (SPEX, Metuchen, NJ, USA) (Kabelka et al., 2002; Zhang et al., 2012). Plants were evaluated with a complement of five Cut Amplified Polymorphism (CAP) markers, designated as N1, N2, N3, N8 and N9, which were able to discriminate between parental TO and TK populations (Zhang et al., unpublished).

**Germination rate**

Pappi were removed and seeds were subjected to a 2 minute 5% bleach treatment followed by three water rinses. Seed were germinated in petri dishes on distilled water-wetted filter paper (Whatman No. 1) in three replications of 50 seed each. Seed were allowed to germinate for two weeks at room temperature under ambient light conditions, before scoring. All plants that emerged from seed were counted as germinants, while only emerged plants with healthy green tissue were counted as viable.

**Ability to establish**

A total of seven hybrid lines, from three different full sibling families were grown in direct competition with their parental species. Seed were germinated in petri dishes
and seedlings were placed 2 cm from the corners of 10 x 10 x 34 cm tree pots filled with Pro-mix. Hybrids were grown in competition with TK and in competition with TO.

Controls included all three dandelion types grown in competition with themselves. Two replications were conducted, each made up of 28 experimental units, and a randomized complete block design was used, with the left and right side of a growth chamber each being a complete block. The growth chamber was set with 300 umol of light, a relative humidity of 40% and a temperature of 22 °C. Plants experienced a 12 hour light / 12 hour dark photoperiod. No fertilizer was used. Each experimental unit was photographed every 2 weeks after planting; plants were harvested and weighed on the sixth week.

*Ability to establish and overwinter in outdoor setting*

Four week old seedlings of TK, TO and hybrids were selected at the four leaf stage and transplanted into outdoor boxes with established perennial ryegrass, four weeks after seeding at a rate of 39 g m$^{-2}$. A single TK seed lot, a composite TO seed lot of TO collected from Wooster, Ohio and 4 apomictic hybrid genotypes, were used in a randomized complete block design replicated 3 times total in planting boxes. Each experimental unit contained 18 transplants of its respective dandelion type. Each experimental unit measured 45.7 by 17.3 cm, a total area of 791 cm$^2$, with a spacer of 10.2 cm separating experimental units on all four sides.

Establishment and seed collection from each experimental were measured 2 weeks after the transplantation. Experimental units were observed from Fall 2014 to Spring 2015. Flower heads and seed were collected from August 2014 to November
2014. At the beginning of May 2015, plants were harvested and fresh and dry plant weight, root weight and shoot weight were measured. The numbers of surviving plants for each genotype as well as the number of buds and flowers of each plant were counted. Survivorship was calculated by dividing the number of surviving plants by the starting number of 18 plants. Seed number was estimated by multiplying seed head number with the average seed number of three full seed head for each month. Data were analyzed using one-way ANOVA and comparison of means was done using a Turkey test in R (R Core Team, 2013).

Results and Discussion

Genome size characterization of TO

A histogram showing the genome sizes of a global collection of 738 TO is shown in Figure 4.1. Of these accessions, the majority (90%) were from North America. Two diploid cytotypes with total genome sizes of 1.61 and 1.80 GB were detected in accessions from Munich, Germany and Durnstein, Austria, respectively. These diploid accessions contained seeds with both triploid and diploid cytotypes, which demonstrates how genetic information is exchanged between sexual and apomictic TO. When grown and emasculated, diploid accessions demonstrated a lack of apomixis, while 96 selected triploid TO accessions invariably exhibited apomixis after emasculation.
**Controlled crosses**

Of the 360 controlled crosses conducted between apomictic TO pollen donors and TK, 40% set seed and produced a total of 362 filled seed. These seed had a germination rate of 30%, resulting in 109 putative hybrids.

Phenotyping and genotyping of these individuals confirmed the formation of 25 true hybrid progeny among 109 viable plantlets, to yield a hybridization rate of 23%. All hybrids were derived from 10 specific crosses, involving multiple parental genotypes. Hybrids were heterozygous for all five CAP markers and exhibited vigorous growth habits and lacerate leaves characteristic of TO. Phenotypes of TK, TO and hybrids are displayed in Figure 4.2, as A, B and C, respectively. Furthermore, diploid TO accessions were found to readily produce viable hybrid seed when pollinated by TK.

**Screening of seed production areas**

In order to sample the large number of seed produced in outdoor seed production areas, which were often adjacent to flowering TO populations (Figure 4.3), phenotyping screening was used to select dandelions with weedy phenotypes characteristic of TO and TO-TK hybrids produced in controlled crosses. Individuals with such phenotypes were then subjected to characterization with species-specific, chloroplast CAP markers in order to distinguish TO seed contamination from TO-TK hybrids, as the latter are expected to possess chloroplast sequences derived from TK. Plants were sampled four to six weeks after emergence. Results from three years of sampling seed production areas are shown in Table 4.1.
Characterization of hybrids

Of the 25 hybrids detected in Objective 2.2, 15 of 25 were found to set seed after emasculation, indicating the inheritance of apomixis. The remaining 10 hybrids did not set seed regardless of if they were emasculated, suggesting that they lack apomixis and self-compatibility. These results suggest that the majority of hybrids may be able to reproduce clonally; however, additional sexual recombination must overcome apomixis and self-incompatibility.

Hybrids typically exhibited larger total genome sizes than either TK or TO and ranged from 2,700 to 3,800 megabases (MB). This suggests that they were created from polyploid TO pollen. Seed from 15 apomictic hybrid lines, as well as composite samples of TK and TO were germinated on filter paper. The majority of hybrids were able to produce viable seed (Figure 4.4).

The growth of hybrids varied based on their competitive environment. Analysis of Variance (ANOVA) was conducted based on the category of competition experienced by the hybrids (e.g. self-competition, competition with TK or competition with TO), the hybrid genotype and a potential interaction between the category of competition and the hybrid genotype. The categories of competition and hybrid genotype were found to be significant (p < 0.05). This suggests that the type of competition hybrids experience influences their biomass (Figure 4.6); additionally, some hybrid clones may perform better than others under growth chamber conditions.
These results demonstrate that when hybrids are grown in the presence of TK, they are able to achieve higher per plant biomasses than when grown in self-competition or with TO (Figure 4.6). The average plant fresh weight for hybrids grown with TK was 26.22 g as opposed to hybrids grown in self-competition which had an average of 15.62 g. This suggests that hybrids may be able to outcompete TK and gain additional resources. However, when grown in competition with their parental TO genotypes, hybrids performed similarly to when in self-competition. These results show that under controlled conditions, hybrids have the potential to outcompete TK. If hybrids were formed with herbicide resistant TK germplasm, they may have the potential to outcompete TK in the presence of herbicide and minimize the utility of the transgenic trait. Additionally, these results demonstrate that under idealized conditions, hybrids may perform similarly to TO.

**Ability of hybrids to establish and produce seed**

While growth chamber experiments suggest that TK-TO hybrids may outcompete TK and have comparable performance to their paternal TO genotypes, such experiments may not translate to realistic conditions. To address this, TK, TO and hybrids were transplanted into outdoor raised beds seeded with perennial rye grass (*Lolium perrenne*) and allowed to flower and overwinter. Under these conditions, TK was unable to establish while TO and hybrids were able to establish and flower. TO and hybrids seemed to exhibit different reproductive strategies, as hybrids flowered early and prolifically, while TO began flowering much later (Figure 4.7 and Figure 4.8). After
transplanting in July, hybrids generally exhibited greater flower and seed production than TO prior to winter dormancy (Figure 4.9 and 4.10).

TO and hybrids were able to overwinter; however, the perennial ryegrass did not, as planting boxes were intentionally left uncovered to test dandelion survivorship (Figure 4.11). After overwintering, in the absence of perennial rye grass, TO and hybrids were able to continue flower production (Figure 4.12). In general, the biomasses (including fresh and dry plant weight, root weight and shoot weight) of TO were higher than those of hybrids after overwintering (Figure 4.13 and Figure 4.14). The seed produced by TO had a lower germination rate compared to hybrids, which may reflect a greater degree of seed dormancy (Figure 4.15). While hybrids allocated more resources to early flowering, TO allocated more resources to biomass, which may allow them to flower earlier and better compete for resources after overwintering. The impact of this disparity in this experiment may have been minimized by a loss of the perennial rye grass, which was did not provide competition during spring emergence.

Conclusions

The potential for the exchange of genetic material between TK and TO may be informed by the genome size of TO, as triploid TO cytotypes are considered obligate apomicts, which are not likely to be receptive to pollen. We have screened a large number of TO accessions and only found the triploid cytotype in North America, while diploids were rarely found in Central Europe. Such diploids were able to be pollinated by TK to produce viable hybrids. This suggests that the potential for pollen mediated gene flow between TK and TO may be greater in regions with diploid TO, such as Central
Europe. However, more work to understand the degree of protection provided by apomixis needs to be conducted.

As the triploid, apomictic TO cytotype was primary, and often the exclusive type detected, pollination of TO by TK is not likely, and TK pollination by TO is the more likely avenue of hybridization. Controlled crosses demonstrated that such hybridizations are possible. Furthermore, sampling of seed production areas demonstrated that hybridization may also rarely occur under more natural conditions, particularly when pollination is augmented by additional pollinators (1 in 100,000).

Hybrids produced were often apomictic and able to produce viable, clonal seed. The inheritance of apomixis was stable over generations and may allow hybrids to proliferate; however, it may also prevent further sexual reproduction and introgression. While more research is needed to understand the long term viability of hybrids in a wild setting, the disparity in life strategies between hybrids and TO suggest that they may be maladapted. It is likely that hybrids may outcompete current versions of TK. This may result in the need for additional management practices in order to minimize the presence of hybrids in TK fields, particularly if herbicides are continuously applied.
Figure 4.1 A histogram of the genome sizes observed in a global collection of *Taraxacum officinale*. Outliers, expected to be diploid sexuals are indicated by arrows.
Figure 4.2 Phenotypes of *Taraxacum kok-saghyz* (A) *T. officinale* (B) and a hybrid (C).
Figure 4.3 Outdoor *Taraxacum kok-saghyz* seed production areas adjacent to flowering *T. officinale* populations.
Figure 4.4 Seed characteristics of *Taraxacum officinale* (TO), *T. kok-saghyz* (TK) and hybrids (H8, 11-19, 21, 22, 39, 103, 156). Data represents three replicates, each consisting of 50 seed.
Figure 4.5 Size of *Taraxacum kok-saghyz* (TK) and hybrid (H22) transplants after establishment at different time points in controlled environment. Hybrids are in the top left and bottom right positions of the pots in the center column, while TK is at the top right and bottom left.
Figure 4.6 A boxplot showing the fresh weight biomass acquired by hybrids (H) in different competitive categories. Categories with the same letters showed no significant differences (Adjusted P>0.05). Adjusted P values for Tukey means comparisons between H and H vs TK (*Taraxacum kok-saghyz*), H and H vs TO (*T. officinale*), H vs TO and H vs TK are 0.001, 0.998 and 0.001, respectively.
Figure 4.7 Monthly and cumulative seed head production of *Taraxacum kok-saghyz*, *T. officinale* and hybrids (H11, H12, H14 and H21) in outdoor raised beds in competition with perennial rye grass (*Lolium perenne*).
Figure 4.8 Estimated monthly and cumulative seed production of *Taraxacum kok-saghyz*, *T. officinale* and hybrids (H11, H12, H14 and H21) in outdoor raised beds in competition with perennial rye grass (*Lolium perenne*).
Figure 4.9 Total seed head production of *Taraxacum kok-saghyz*, *T. officinale* and hybrids (H11, H12, H14 and H21) in outdoor raised beds in competition with perennial rye grass (*Lolium perrenne*). Genotypes with the same letters were not significantly different (P>0.05).
Figure 4.10 Estimated total seed production of *Taraxacum kok-saghyz*, *T. officinale* and hybrids (H11, H12, H14 and H21) in outdoor raised beds in competition with perennial rye grass (*Lolium perenne*). Genotypes with the same letters were not significantly different (P>0.05).
Figure 4.11 Overwintering survivorship of *Taraxacum kok-saghyz*, *T. officinale* and hybrids (H11, H12, H14 and H21) in outdoor raised beds in competition with perennial rye grass (*Lolium perenne*). Genotypes with the same letters were not significantly different (P>0.05).
Figure 4.12 Flower production of *Taraxacum kok-saghyz*, *T. officinale* and hybrids (H11, H12, H14 and H21) in outdoor raised beds after overwintering. Genotypes with the same letters were not significantly different (P>0.05).
Figure 4.13 Biomass of *Taraxacum kok-saghyz*, *T. officinale* and hybrids (H11, H12, H14 and H21) harvesting in May of 2015, after being transplanted in July of 2014. Genotypes with the same letters were not significantly different (P>0.05).
Figure 4.14 Overwintering biomass of *Taraxacum officinale* and average performance hybrid H14 in outdoor raised beds as examples of harvested experimental units in the hybrids characterization study.
Figure 4.15 Germination rates of seed produced by *Taraxacum officinale* and hybrids (H11, H12, H14 and H21) in outdoor raised beds in competition with perennial rye grass (*Lolium perren*). Genotypes with the same letters were not significantly different (P>0.05).
Table 4.1 Hybrid screening of seed production areas

<table>
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<th>Year</th>
<th>Estimated Number of Plantlets Screened</th>
<th>Number of Plants with <em>Taraxacum officinale</em>/Hybrid Phenotype</th>
<th>Validated Hybrids</th>
</tr>
</thead>
<tbody>
<tr>
<td>2013</td>
<td>1,700,000</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>2014</td>
<td>900,000</td>
<td>140</td>
<td>11</td>
</tr>
<tr>
<td>2015</td>
<td>750,000</td>
<td>39</td>
<td>0</td>
</tr>
</tbody>
</table>
4.2 The development of resources for rubber producing dandelions to track gene movement.

Abstract

The potential for gene flow between the rubber producing dandelion, *Taraxacum kok-saghyz* (TK) and the ubiquitous weedy dandelion *T. officinale* (TO) is complicated by apomixis and polyploidy. Apomixis may greatly limit or entirely preclude fertilization by pollen, while mixed ploidy levels between species may further limit the amount of hybridization which can be observed. It is important to understand whether such rare hybridization events can occur in order to estimate the potential impact of genetically engineered dandelions. Here we have developed TK germplasm that may be used to detect pollen mediated hybridization between TK and TO on a large scale. This germplasm includes plants expressing various florescent proteins, kanamycin resistance, glufosinate resistance, and the transcription factor *IbMYB*, which gives tissue a purple color by upregulating anthocyanin biosynthesis. These resources may provide a tool kit to understand the potential for gene flow within the complex reproductive biology of *Taraxacum*.

Introduction

Plants that have not been widely cultivated are emerging as new sources of industrial products, driven by the need for critical industrial resources and the unsustainability of petroleum-based alternatives. Many emerging crops are semi-
domesticated, exhibit weedy characteristics, and may have a propensity to hybridize with weedy relatives. One such species is *Taraxacum kok-saghyz* (TK), which is further complimented by wild relatives that vary in ploidy level and reproductive biology, such as the common dandelion (*T. officinale*, TO).

TO most commonly exists as an obligate apomictic triploid, meaning all seed produced is an asexual clone of the mother plant. However, some apomictic species exhibit facultative apomixis, where some seed may still be produced sexually. In order to affirm that obligate apomixis in TO is, in fact, obligate, we need to be able to screen large amounts of seed produced by many TO accessions under different environmental conditions. Additionally, sexual cytotypes of TO could be present in some locations, where TO diversity has not yet been characterized.

In order to assess the potential of facultative apomixis and the presence of sexual *Taraxacum* species adjacent to growing areas, a collection of germplasm resources have been developed to allow the movement of genes to be easily monitored between species in greenhouse or field experiments. Such traits include the expression of fluorescent proteins, kanamycin and glufosinate resistance genes, and the transcription factor *IbMYB*, which leads to anthocyanin accumulation, coloring tissue purple (Mano et al., 2007; Zhang et al., 2015). Here we detail the generation of tracking germplasm and methods for rubber producing dandelions.
4.2.1 Development of tracking resources using florescent proteins and kanamycin resistance

Methods

Generation of germplasm

TK from USDA accession KAZ08-017 (W6 35172) and a single TB genotype (Clone A) were transformed with cassettes encoding *neomycin phosphotransferase II* (*nptII*), which results in kanamycin resistance, as well as four florescent proteins (Table 4.2, Shaner et al., 2005) using the methods described in Zhang et al., (2015). Genes encoding mCherry and TdTomato were cloned from plasmid pRsetB-His7tag-Peredox-mCherry (a gift from Gary Yellen, Addgene plasmid # 32382, Hung et al., 2011) and plasmid pCAG-TAG (a gift from Shankar Srinivas, Addgene plamid # 26771, Trichas et al., 2008), respectively, and inserted into a modified pEarleyGate 100 series vector (Arabidopsis Biological Resource Center, stock number: CD3-724) harboring *nptII* as the plant selective marker, using the PCR8/GW/TOPO Cloning Kit and LR Clonase (Invitrogen™, Carlsbad, CA, USA). Transformed plants were generated from TK and TB roots using the methods previously described (Zhang et al., 2015).

Validation of screening methods

A foliar kanamycin spray approach (Xiang et al., 1999) was modified from and applied to *Taraxacum* species. T₁ seed were produced by crossing transgenic TK harboring kanamycin resistance genes with non-transgenic TK, while transgenic TB seed were produced apomictically. Non-transgenic TK, TB and TO populations, as well as TK
and TB T₁ generations, were used for kanamycin spray selection. Seed were sown in soil with three replications for each treatment. After the emergence of the first two true leaves (2 weeks after germination), plants were sprayed with 100 mg L⁻¹ kanamycin solution with 0.1% Triton X-100 once a day for three days, followed by a similar solution with 200 mg L⁻¹ kanamycin for another three days. Ten days later, plants were sprayed with a 500 mg L⁻¹ kanamycin solution. Control plants were sprayed with a 0.1% Triton X-100 solution. Survivorship was counted, and pictures were taken, 45 days after seed sowing.

Results

Kanamycin resistance gene and genes encoding four florescent proteins were successfully introduced into TK and TB. Genes were detected using PCR on the DNA level. After kanamycin treatment, all non-transgenic TK, TB and TO plants were killed, suggesting that kanamycin resistance was a novel trait among TK, TB and TO (Figure 4.16 and 4.17). On average, 42.8% of the TK T₁ generation plants survived kanamycin selection, which agreed with the expected 1:1 ratio of segregation for a single insertion (Figure 4.16 and 4.17). On average, 58% of TB T₁ generation plants survived kanamycin selection (Figure 4.16 and 4.17). As TB is apomictic, seed produced are expected to be clones of the mother plant, thus 100% of the progeny are expected to be transgenic. This disparity could be caused by residual sexual activity (facultative apomixis), or chimerism of the mother plant. However, as all seedlings produced by transgenic TB plants tested positive for genes of interest, this deviation from expectations is best explained by gene silencing.
4.2.2 Development of tracking resources using \textit{IbMYB}

Another approach to track gene movement is to use reporter genes whose expression can be visualized by human eye. The transcriptional factor \textit{IbMYB} has been cloned from sweet potato and used as a visible marker. Both transient and stable expression of this gene resulted in the accumulation of anthocyanin pigment, thus making plants exhibit purple color (Mano et al., 2007). Here gene \textit{IbMYB} was transformed into TK as a report marker to track potential gene movement from TK to TO through pollen.

\textbf{Methods}

\textit{Glufosinate killing dose}

To find out the minimum glufosinate killing concentration needed to use \textit{bar} (bialaphos resistance) gene as the selective marker, root fragments were placed on a series of 1/2 MS medium supplemented with different concentrations of glufosinate, namely 0, 1, 2, 3, 5 and 10 mg L\(^{-1}\). Three replications were used for each treatment. After one month, plantlets generated from root fragments were counted and the glufosinate killing dose was selected.

\textit{TK transformation}

\textit{Agrobacterium rhizogenes} wild type strain K599 harboring a plasmid expressing \textit{IbMYB} (kindly provided by Prof. Christopher Taylor, The Ohio State University, OARDC, Wooster, OH, USA) was prepared according to Zhang et al., (2015) and used for TK transformation. Rooted TK plantlets at the four leaf stage were wounded and dipped into \textit{Agrobacterium} liquid culture. Plantlets were then transferred to 1/2 MS
medium with 200µM acetosyringone for 3 days of co-culture. Plantlets were then washed and allowed to recover for 7 days on 1/2 MS medium with 400 mg L\(^{-1}\) timentin. Plantlets were transferred to 1/2 MS medium with 400 mg L\(^{-1}\) timentin and 1.2 mg L\(^{-1}\) glufosinate. To regenerate transgenic plants, hairy roots were removed from plants after an additional 3 weeks, cut into 2 cm fragments, and placed on 1/2 MS medium with 400 mg L\(^{-1}\) timentin and 5 mg L\(^{-1}\) glufosinate.

**Results**

*Determiniation of glufosinate killing dose*

The regeneration of shoots from TK root fragments was completely inhibited by glufosinate concentrations of 2 mg L\(^{-1}\) or higher. Small shoots were able to emerge on 1/2 MS medium supplemented with 1 mg L\(^{-1}\) glufosinate (Figure 4.18). Therefore, 1.2 mg L\(^{-1}\) was selected as an initial treatment to enrich for transformed hairy roots.

*TK transformation*

Purple hairy roots were observed on TK plantlets grown under selection for four weeks after inoculation. These purple roots were excised and moved to new media with 5 mg L\(^{-1}\) glufosinate. After one month, small plantlets were regenerated from purple hairy roots, which showed a purple leaf phenotype, as well as purple hairy roots (Figure 4.19).

**Discussion**

This research has generated a collection of germplasm, which can be used to detect the movement of genetic material from rubber producing dandelions TK and TB to
other *Taraxacum* species. Additionally, this research has further validated the heritability of transgenic traits introduced by *Agrobacterium rhizogenes*-mediated transformation of rubber producing dandelions. Kanamycin spray selection may be used to detect transgenic, kanamycin resistant progeny among populations containing multiple species within 45 days. The generation of germplasm containing different fluorescent proteins may allow for multiple containment strategies and management practices to be tested simultaneously and scored using microscopy. Plants indicating the presence of a transgene by herbicide or antibiotic resistance could then be classed into different treatment categories based on their florescence. Furthermore, the movement of transgenic pollen could be monitored by microscopy or flow cytometry.

The generation of purple TK by the introduction of *IbMYB* through glufosinate selection provides two additional traits that can be used to monitor gene flow. A visual marker is an ideal trait to quickly track gene flow on a large scale. However, more work is needed to affirm the stability of this trait. Similar to kanamycin resistance, glufosinate resistance may also be adapted to a spray selection to indicate the presence of transgene. Furthermore, the use of a root-based TK glufosinate gradient to generate transgenic TK provides an additional resource for TK transformations, allowing for the use of more plasmids and the selection of multiple cassettes into a single genotype.

**Conclusions**

The amount of reproductive isolation resulting from apomixis in TO is important to understand in order to make informed decisions about the large scale cultivation of
*Taraxacum* species possessing novel traits. In order to resolve this, germplasm resources were generated, which are conducive to large scale screening. These resources will also allow the movement of transgenic germplasm, pollen and seed to be monitored.
Figure 4.16 Kanamycin spray selection on non-transgenic and transgenic seed families of *Taraxacum kok-saghyz* (A), *T. brevicorniculatum* (B) and non-transgenic *T. officinale* (C).
Figure 4.17 Survivorship of transgenic *Taraxacum kok-saghyz* (TK), *T. brevicorniculatum* (TB) and controls (non-transgenic TK, TB and *T. officinale* (TO)) after kanamycin treatment. Each bar represents the mean ±se of each treatment.
Figure 4.18 Generation of shoots from *Taraxacum kok-saghyz* root fragments on 1/2 MS medium supplemented with different concentrations of glufosinate.
Figure 4.19 Hairy root transformations of *Taraxacum kok-saghyz* (TK). (A) Hairy roots produced by TK transformed with *Agrobacterium rhizogenes* wild type strain K599 (control); (B) Enlarged hairy roots from Figure 2A; (C) A plantlet regenerated from hairy roots in Figure 2A; (D) Purple hairy roots produced by TK transformed with *IbMYB*; (E) Enlarged purple hairy roots from Figure 2D; (F) A plantlet regenerated from purple hairy roots in Figure 2D.
Table 4.2 Characteristics of fluorescent proteins stably transformed into *Taraxacum kok-saghyz* and *T. brevicorniculatum*

<table>
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<tr>
<th>Color</th>
<th>Protein</th>
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<th>Emission (nm)</th>
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<td>587</td>
<td>610</td>
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<td>Monomer</td>
</tr>
<tr>
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<td>TdTomato</td>
<td>554</td>
<td>581</td>
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*(Shaner et al., 2005)*
Summary and outlook

Natural rubber is a critical resource that is largely produced by a single species, *Hevea brasiliensis*, which has narrow genetic diversity and long breeding cycles (Lieberei, 2007). These limitations amplify the potential impact of diseases, such as South American Leaf Blight (SALB), which has greatly limited South American rubber production (van Beilen and Poirier, 2007a). *Taraxacum kok-saghyz* (TK) is an outcrossing rubber producing dandelion species, with a short breeding cycle. Additionally, TK is adapted to grow in a wide range of temperate areas, as opposed to the rubber tree, which is limited to certain tropical regions. However, TK is not domesticated and exhibits variable rubber production and demonstrates little ability to establish in the presence of weeds. This has prevented the adoption of TK as a rubber producing crop plant.

One strategy to overcome these shortcomings is biotechnology, which has become commonplace in agricultural landscapes. However, transformation methods for TK had not been well described. By leveraging the ability of *Taraxacum* to regenerate plants from root fragments, we developed an efficient, simplified method to produce transgenic TK. Furthermore, this method was adapted to conduct genome editing using a CRISPR/Cas approach. These methods may allow rubber producing dandelions such as...
TK and TB to be improved, while also allowing basic research on Taraxacum genetics to be conducted.

The large scale introduction of new germplasm harboring novel traits raises concerns of gene flow with wild weedy relatives, whether such traits were introduced by breeding or biotechnology. Furthermore, while newer genetic engineering methods, such as the biolistic introduction of genes lacking plant pest elements and genome editing, may not currently be adequately regulated, the potential for ecological impact is the same.

In order to address the question of gene flow between TK and the ubiquitous weed, TO, we have developed a set of molecular markers to differentiate the chloroplast and nuclear genomes of these species. These markers were then used to validate hybrids in controlled crosses and open seed production areas. Hybridizations, where apomictic, triploid TO pollinated TK, were observed in these experiments. Many of these progeny exhibited the inheritance of apomixis and were able to produce clonal seed, which could establish, produce additional clonal progeny, and overwinter. However, hybrids that did not demonstrate the inheritance were unable to produce seed. While such hybridizations may occur, the potential for introgression between TK and apomictic TO may be limited due to apomixis and unbalanced gametes.

Although the majority of TO are apomictic triploids, sexual diploids have been described in regions of Central Europe (van Dijk and van Damme, 2000). In screening a global collection of TO seed, this was affirmed, as sexual, diploid cytotypes were found in Austria and Germany. Diploid TO from these accessions were readily pollinated by TK and produced viable, non-sterile hybrids. Despite this sampling being focused on
North America, including nearly 600 North American accessions, no diploid TO were found in this region. This suggests that North America may have a lower risk of transgene introgression from TK into TO than regions of Central Europe.

While polyploidy and obligate apomixis may provide strong barriers to introgression between TK and TO, better tools were needed in order to understand this potential. In order to address this, transgenic germplasm was generated for TK and TB. These transgenes can be scored using spray selection or visual screening to indicate gene flow. These strategies allow for large scale experiments that are impractical using molecular markers.

In addition to allowing for large scale tracking of gene movement, biotechnology can also reduce the weediness of new crops by introducing domestication traits through CRISPR/Cas genome editing. In *Taraxacum*, inulin production may be implicated in weediness, as it is a long term storage carbohydrate, which allows dandelions to tolerate stress and overwinter. A lack of inulin may eliminate the ability of engineered dandelions to overwinter and naturalize, resulting in a more controlled introduction of germplasm. Furthermore, by knocking out inulin synthesis, dandelions may divert extra assimilate into rubber production. Another trait of interest in *Taraxacum* is the development of the pappus, which allows seed to be dispersed by the wind (Tackenberg et al., 2003). As seed mediated hybridization, where TK is pollinated by TO, is expected to be the most likely avenue of geneflow, controlling the movement of seed may minimize the proliferation of hybrids. Additionally, such a trait may allow of easier harvesting of seed in a field setting and lower levels of volunteerism in adjacent areas.
While editing out genes with CRISPR/Cas may be a way to rapidly introduce domestication traits into a TK crop, once hybridization has occurred, these deleted alleles would likely be complimented by wild TO alleles. One solution to this may be to include CRISPR/Cas elements targeting TO genes within TK. By introducing CRISPR/Cas elements that cause mutations in, or down regulate the expression of TO genes, rubber producing dandelions could potentially be introduced, which eliminate weedy traits from wild dandelions populations if hybridization occurs.

The ploidy of TK can also be manipulated in order to minimize the potential for hybridization. Methods of tetraploid induction by colchicine treatment have previously been described for TK (Warmke, 1945). By treating TK seed with colchicine according to these methods, tetraploid TK have been generated. When crossed with diploid TK, triploid seed were readily and exclusively produced and yielded viable plants. Triploids that lack apomixis are expected to be sterile; therefore, the production of triploid seed may limit the potential for hybridization. This may be applicable to TK, as roots are the harvestable unit, rather than seeds. However, the large scale production of triploid seed to be used in direct seeding efforts may pose challenges.

Apomixis is a trait already present in *Taraxacum* which has utility in producing advanced germplasm and in minimizing geneflow. No major crops exhibit apomixis, but the ability to deploy apomixis in crops would have a profound impact on plant breeding, by permanently fixing desirable gene combinations. Despite the strong incentive to use apomixis, the genetics underlying this phenomenon have not been resolved, as it is not amenable to traditional gene identification strategies. This is because apomixis genes
prevent recombination in extensive genomic regions, which accumulate mutations (Ozias-Akins and van Dijk, 2007). Traditional gene identification strategies rely on sexual recombination to resolve which genomic regions are associated with a trait. This lack of recombination in very large, highly diverse regions has greatly limited the exploration of apomixis genetics. However, with new tools, such as Next Generation Sequencing and CRISPR/Cas genome editing, it may be possible to identify genetic elements implicated in apomixis.

CRISPR/Cas technology is rapidly evolving and currently several genes can be targeted simultaneously (Xie et al., 2015). By using genome editing, genes implicated in apomixis by RNA-seq data can be rapidly tested by eliminating their function and observing changes in reproductive biology. Dandelions are an excellent system in which to study apomixis as they have a rapid life cycle and a growing number of genomic resources many other apomictic genera lack, including transcriptome and whole genome assemblies (Luo, unpublished, Zhuang, unpublished). Most importantly, a rapid and simple method of genetic manipulation in dandelion species has been realized (Zhang et al., 2015).

The use of apomixis in a rubber producing dandelion may provide genetic isolation between cultivated and wild dandelions, by preventing the sexual reproduction of seed. *Taraxacum brevicorniculatum* (TB) is a rubber producing dandelion which possesses apomixis. While TB is more vigorous than TK, it has limited rubber production. The improvement of TB by breeding is limited by the inheritance of apomixis and its polyploid genome. However, the ability of TB to produce rubber may
be improved by biotechnology, which may be more practical than introducing a complex trait such as “vigor” into TK. As apomictic dandelions can produce seed without any need for pollination, pollen sterility could be introduced into them without impacting seed production. Male sterility could be introduced by biotech means, or Cytoplasmic Male Sterility (CMS), which has been characterized in *Taraxacum* and observed within existing TK germplasm (van der Hulst et al., 2004; Hodgson-Kratky et al., 2015).

Overall, the unsustainable use of fossil fuels compels the exploration of new crops as sources of bioproducts. Such crops may be weedy, relatively undomesticated and have potential to hybridize with related weedy species. These crops may be rapidly improved by new breeding and biotechnological methods. However, the large scale cultivation of novel, improved germplasm raises new questions of potential gene flow. New tools such as CRISPR/Cas offer the potential to introduce domestication traits, which may minimize the potential ecological impact of new crops. TK is a prime example of such a crop. While there is potential for hybridization between cultivated TK and wild TO, in the absence of diploid TO, introgression between these species will be limited. However, we have developed tools to further explore this question. The development of TK as a new crop provides a unique opportunity to consider ecological impact during the domestication process. This body of work may help inform risk-reward decision making to determine if new dandelions rubber crops should be released and how they should be managed.
References


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