Chapter 6
Genetic Improvement of Guayule (*Parthenium argentatum* A. Gray): An Alternative Rubber Crop

Hussein Abdel-Haleem, Zinan Luo, and Dennis Ray

Abstract Among the more than 2000 plant species known to synthesize rubber, guayule, *Hevea* and *Taraxacum kok-saghyz* produce commercial grade rubber and latex. Guayule (*Parthenium argentatum* A. Gray), originates from the Southern Texas and Northern Mexico deserts, and is receiving wide attention as a natural rubber crop that could successfully grow in arid and semiarid regions. Continued improvement of guayule for higher biomass, rubber production and resistance to biotic and abiotic stresses, as well as maximizing agronomic practices are necessary to meet the increasing demand of the guayule rubber industry. Early domestication and commercialization efforts have all centered on using natural guayule stands and unimproved germplasm as a source of natural rubber. However, limited and sporadic breeding efforts have slowed down guayule's genetic gains compared to other crops. This chapter summarizes the most recent breeding progress, biotechnological advancements, and agronomic practices to increase guayule rubber and other industrial byproducts. This provides plant breeders an insight into the status of guayule improvement and possible directions to speed up the breeding progress.

Keyword Agronomy · Biotechnology · Genetic improvement · Genetic variability · Guayule · Natural rubber · Resin · Sustainability

6.1 Introduction

Guayule (*Parthenium argentatum* A. Gray), family Asteraceae (or Compositae), originates from north-central Mexico and southwest Texas in the USA (Hammond and Polhamus 1965). The potential supply shortage of natural rubber and unstable

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prices in rubber-importing industries make it imperative to exploit rubber production from alternative rubber-producing crops. Guayule is one of these resources, which could generate high molecular-weight and hypoallergenic natural rubber (Cornish 1996).

Most current breeding methods in guayule are restricted to sporadic single-plant selection, mass selection, recurrent selection and interspecific hybridization (Coffelt et al. 2015). No comprehensive and systemic molecular breeding efforts have yet been carried. Undeniably, conventional breeding studies have made some progresses in guayule, such as improved rubber yield, disease resistance and plant vigor; however, the nature of facultative apomixis and polyploidy, the narrow genetic variation in current germplasm, the challenges in agronomic practices, limited resources for sexual reproduction and unsustainable funding resources make it very difficult to continue guayule breeding efforts (Coffelt et al. 2015; Thompson and Ray 1988). To overcome these constraints, along with the conventional breeding, advanced biotechnological or molecular breeding methods need to be adopted. Recent advanced biotechnological tools such as tissue culture, genetic engineering, next generation sequencing (NGS) and flow cytometry have been used in guayule to accelerate the improvements of desired traits.

This chapter summarizes the most recent achievements in breeding progress, agronomic practices, biotechnology, genetics and genomics for guayule, and reports the applications of these advanced tools towards the improvement of rubber production, abiotic stresses and disease resistance. The chapter also discusses the problems and challenges facing guayule breeding, which will provide plant breeders with possible directions to speed up the breeding progress.

6.1.1 Origin and Distribution

Guayule (*Parthenium argentatum* A. Gray) is a perennial semiarid shrub native to the drylands of north-central Mexico in the states of Coahuila, Chihuahua, Durango, Zacatecas, San Luis Potosi and Nuevo Leon, and to the Trans Pecos area of Southwest Texas (Stockton Plateau and Big Bend Region) in the USA, covering some 34 million ha (Hammond and Polhamus 1965). Indigenous US guayule populations are scattered over a wide range of climatic conditions (Foster and Coffelt 2005), on plateaus of 1200–2100 m elevation, with an annual rainfall of 25–38 cm, but restricted to calcareous soils (Hammond and Polhamus 1965). Although guayule grows better in regions with sufficient rainfall, competition from other plants (e.g. grasses and shrubs) makes it hard to survive (Hammond and Polhamus 1965). Temperatures throughout its habitat ranges from 0–49 °C and the height of guayule plants barely exceeds 1 m in their native areas (Downes 1986; Goss 1991).

6.1.2 Botanical Classification

Guayule is a flowering shrub belonging to the Asteraceae, which is a large family with 1911 genera and 32,913 accepted species (List 2013). The Asteraceae are easily distinguished from other plant families mainly due to their characteristic inflorescence, which appears to be a single flower but is actually a cluster (composite) of smaller flowers (Judd et al. 2007). Several taxa in this family, such as *Taraxacum kok-saghyz* (rubber dandelion) and *Solidago speciosa* (goldenrod), can also produce considerable amounts of natural rubber and are potential natural rubber resources (Goss 1991). However, of the 17 species of *Parthenium*, only guayule produces appreciable amounts of rubber (Coffelt et al. 2015).

The genus *Parthenium* belongs to the subtribe Ambrosiinae, which includes the *Ambrosia, Parthenice, Dicoria, Iva, Hymenoclea* and *Xanthium* genera. However, *Parthenium* can be distinguished from the other genera because it is insect pollinated. For instance, the very closely related genus, *Parthenice*, does not produce nectar and can only be pollinated by wind. Other closely related genera such as *Dicoria* and *Iva*, even with a similar capitula, are also wind pollinated. The majority of species in the subtribe Ambrosiinae accumulate large amounts of leaf sesquiterpene lactones, which are also found in *Parthenium* (West et al. 1991).

The 17 species of Parthenium range morphologically from small trees (e.g. *P. tomentosum* var. *stramonium*) to small shrubs (e.g. *P. incanum*) (Rollins 1950) (Fig. 6.1). *Parthenium hysterophorus* is a weedy species in many countries and its

Fig. 6.1 Guayule and related species. *Parthenium tomentosum, P. argentatum* and *P. incanum* grown at the Maricopa Agricultural Center, Maricopa, Arizona. (Photo courtesy of Greg Leake, USDA Agricultural Research Service)

sesquiterpene compounds can cause contact dermatitis (Rodriguez et al. 1976). However, the sesquiterpene generated in guayule has not yet been found to cause contact dermatitis (Downes 1986; Rodriguez et al. 1981). Trichome morphology of the desert species of *Parthenium* is also specialized. Unlike the prominent uniseriate conical trichome observed in tree species such as *P. tomentosum*, *P. schottii* and *P. fruticosum*, and the very long uniseriate, whiplike trichomes in *P. incanum* and *P. rollinsianum*, guayule is covered with a layer of unique T-shaped trichomes (West et al. 1991).

The general morphology of guayule is summarized by Goss (1991) and Hammond and Polhamus (1965). The guayule taproot system may lose its prominence and give way to intricate system of fibrous laterals and their branches, which mainly spread in the upper 15 cm of soil but can extend up to 3 m or more to facilitate soil moisture utilization under conditions of very shallow water penetration (Hammond and Polhamus 1965; Muller 1946). Another factor that helps guayule survive damage caused by meager rainfall, erosion or harvesting is the formation of adventitious shoots (*retoños* in Mexico). On shallow exposed roots the basal portion of the retoños will then develop and extend the root system (Hammond and Polhamus 1965). Guayule leaves are up to 10 cm long, which is three times their width (Downes 1986; Fangmeier et al. 1984). Leaf size and shape vary among guayule accessions and are usually used as a morphological characteristic (Fig. 6.2).

Leaf size and shape are largely affected by water supply (Downes 1986; Fangmeier et al. 1984). Insufficient water supply can lead to shriveling and shedding of older leaves, but younger leaves can survive when water supply is recovered (Downes 1986; Fangmeier et al. 1984). The T-shaped trichomes in the leaf surfaces produce the light silvery gray sheen color characteristic of the guayule plant (Downes 1986; Hammond and Polhamus 1965). The primary stem terminates with the formation of the first inflorescence, but further growth will occur at the uppermost buds by developing new branches. Elongation of the uppermost lateral buds results in a symmetrical and closely-branched shrub (Lloyd 1911). Flowers are borne in heads with 5 fertile ray-florets, each with 2 subjacent sterile disk-florets, which contain an abortive pistil and fertile stamens (Downes 1986; Hammond and Polhamus 1965).

Flowering in guayule is largely stimulated by favorable water conditions. Under irrigation, flowering is possible from early spring to late fall (Backhaus

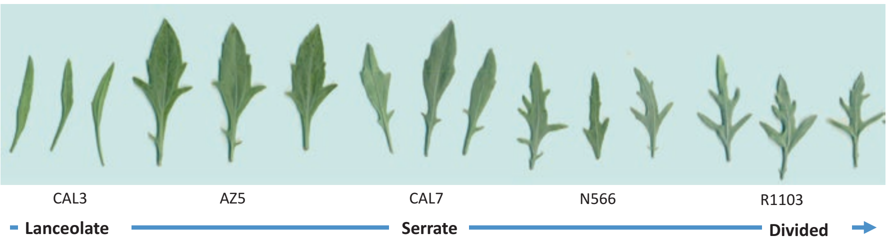

Fig. 6.2 Guayule leaf shape and size from different accessions

et al. 1979; Downes 1986). As mentioned, guayule is both wind and insect pollinated. Ladybird beetles, lygus bugs, cucumber beetles and honeybees are all effective carriers of guayule pollen. The mature fruit contains an embryo enclosed in two seed coats, which inhibits or delays germination (Downes 1986; Hammond and Polhamus 1965).

Rubber particles in guayule are mainly located in the cortical parenchyma cells in the stems and roots (Coffelt et al. 2015). In mature guayule plants, rubber is found in vascular rays of the phloem and xylem while in young plants, rubber is mainly stored in the cortex, pith and vascular rays and resin canals (Benedict et al. 2010; Downes 1986). The rubber content is proportional to the amount of bark parenchyma tissue (Artschwager 1943), and usually reaches its lowest point in July and increases to a peak in January in 2-year-old guayule plants (Benedict et al. 2010). Reportedly, low temperature in fall/winter can stimulate formation of rubber-producing cortical parenchyma and the rubber particles in it (Benedict et al. 2009, 2010).

6.1.3 Domestication, Selection and Early Improvements

Guayule domestication and commercialization has experienced four stages, during which sporadic selection and breeding efforts occurred (Coffelt et al. 2015). The initial commercialization attempt began at the beginning of the twentieth century due to the high price of imported *Hevea* rubber from the Amazon Region (Bonner 1991). At that time, wild guayule stands were harvested in Mexico and several extraction methods were evaluated (Ray et al. 2005). Natural rubber production from guayule in Mexico reached a peak in 1910, during which 24% of the total natural rubber imported into the USA was guayule rubber from Mexico (Bonner 1991). However, production by the Continental Rubber Company ceased in 1912 due to the Mexican Revolution. Commercialization efforts then moved across the border to Arizona and California (Ray 1993), where agronomic and breeding efforts of collecting and selecting plants and their seeds from wild stands continued for 17 years, but halted again in 1929, due to the Great Depression (Bonner 1991; Ray 1993).

The second domestication and commercialization effort occurred with the Emergency Rubber Project (ERP) during World War II. This effort led to the planting of over 13,000 ha of guayule at 13 different locations, but ceased with the end of the war, and the renewed availability of natural rubber from Southeast Asia, as well as the development of synthetic rubber (Huang 1991). The four-year ERP was successful, realizing the production of some 1400 mt of guayule rubber, and provided significant knowledge regarding basic biology, agronomy, and was the origin of the guayule germplasm upon which current breeding programs are based (Hammond and Polhamus 1965; Thompson and Ray 1988).

The third major effort to commercialize guayule started in the late 1970s because of the quadrupling of crude oil price (Ray et al. 2005), which raised the fear of a potential natural rubber shortage due to either natural disaster or political unrest in Southeast Asia. This fear induced the enactment of two laws by the US Congress: the Native Latex Commercialization and Economic Development Act of 1978 and the Critical Agricultural Materials Act of 1984 (Laws 95–592 & 98–284), providing for the support of guayule projects for about 12 years (Huang 1991; Ray et al. 2005). A tremendous amount of work, although not as concentrated as the second effort, was accomplished during this third effort, resulting in significant yield increases and refined cultural practices to fit modern mechanized agriculture (Foster and Coffelt 2005; Ray 1993; Ray et al. 2005; Whitworth and Whitehead 1991). This third effort also demonstrated that guayule could be planted, cultivated, harvested and processed as an alternative natural rubber resource to replace *Hevea* in high temperature applications, especially tires (Ray et al. 2005). However, as the political climate changed, this effort was also terminated.

With the occurrence of widespread life-threatening latex allergy from *Hevea* rubber products (Ownby et al. 1996), it was realized that guayule latex was a perfect potential replacement due to its hypoallergenic feature and similar rubber quality (Cornish 1996; Siler et al. 1996). This opened the fourth and present commercialization effort in the 1990s and made it closer to reality when Yulex Corporation (www.Yulex.com) was granted the exclusive license to US Patent No. 5580942 (Cornish 1996) and to US Patent No. 5717050 (Cornish 1998) on guayule latex processing and products. During this process, a business institution was established, a financial base developed, seed collections of promising lines increased, a latex extraction processing plant constructed and large areas of plantings were in progress to support industry demand (Coffelt et al. 2015). Recently, the National Institute of Food and Agriculture (NIFA) funded a USD 6.9 million grant to a research consortium led by the Cooper Tires, and an Agriculture and Food Research Initiative (AFRI) grant for USD 15 million to a group led by the University of Arizona. Both grants include collaboration among industry, academic and governmental entities. In addition, other companies, such as Bridgestone Americas (www.bridgestone.com), Cooper Tires (http://us.coopertire.com/), and Versalis S.P.A. (https://versalis.eni.com/irj/portal/anonymous?guest_user=anon_en) are investing in research activities to develop new and/or improved germplasm and production practices, and rubber quality for tire manufacturers. Unlike previous commercialization efforts, this most recent one is mainly driven by commercial companies instead of government institutions. The interest by these commercial companies is not only for guayule natural rubber and/or latex products, but also due to a predicted increase in natural rubber demand and the potential shortage in the *Hevea* rubber supply resulted from plant diseases and/or political instability in the rubber-producing countries in Southeast Asia. Moreover, other byproducts from guayule such as termite-resistant wood products (Nakayama et al. 2001), perfumes (Battistel et al. 2018), and bioenergy products (Kuester 1991), make guayule an attractive crop in arid and semiarid regions.

6.2 Guayule Cultivation Strategies

For guayule, as in any crop, increased yield will come from continuous improvement through genetic enhancement and cultivar development strategies, and, at the same time, optimization of agronomic practices for maximum biomass and rubber production (Foster and Coffelt 2005; Ray et al. 2005). Guayule agronomy and agronomic practices are described below.

6.2.1 Planting Method and Plant Establishment

Transplanting seedlings is a reliable means to establish guayule stands, although direct seeding, using seed conditioning techniques and precision planting, has been successful on an experimental scale in Texas, New Mexico and Arizona (Foster and Coffelt 2005). Conditioning facilitates seed emergence and to overcome dormancy, resulting in stands with a more rapid and uniform manner (Chandra and Bucks 1986; Foster and Coffelt 2005). The survival rate of transplants can reach 95% at El Paso, Texas and in Arizona, when frequent irrigations are applied (Bucks et al. 1984; Foster and Coffelt 2005; Miyamoto and Bucks 1985). Although direct seeding has advantages over transplanting in terms of cost, improvements are needed before applying this technique on a commercial scale. Direct seeding has been successful in establishing the crop, but can be improved by conditioning seeds with polyethylene glycol (PEG), gibberellic acid and light; by precision planting to accurately plant seeds on the soil surface; by precise irrigation strategies and by enhancement of seed viability and germination rate through breeding (Foster and Coffelt 2005).

6.2.2 Irrigation

Even though guayule is desert shrub, it is an inefficient water user, requiring 1000–1300 mm of water per year for maximum rubber production (Nakayama et al. 1991). Rubber yields in transplants are positively correlated to irrigation (Hunsaker and Elshikha 2017). Therefore, the quantity of optimal irrigation depends both on growing conditions and production practices (Foster and Coffelt 2005; Maas et al. 1988; Miyamoto et al. 1984a,b; Retzer and Mogen 1946). Water quality and water stress are two limiting factors affecting guayule production and rubber yield. It has been reported that water salinity exceeding 1.0 ds/m results in the failure of plant establishment and that above 4.5 ds/m leads to mortality during early plant growth (Maas et al. 1988; Miyamoto et al. 1984a,b; Retzer and Mogen 1946). However, salt accumulation can be minimized when off-centered or double-row planting is coupled with alternate row watering (Foster and Coffelt 2005). Water stress can increase rubber content (percent rubber), but decreases shrub biomass (Hunter and Kelley

1946). In order to clearly define the interaction between water stress and rubber yield, the crop water stress index (CWSI) was applied to guayule (Nakayama and Bucks 1983). This index uses shrub canopy temperature and atmospheric vapor pressure deficits as they relate to plant water and soil water stresses. Based on this, several studies have reported that an inverse correlation was found between rubber yield and CWSI (Nakayama and Bucks 1984). Although the relationship varied during different guayule growing stages (Bucks et al. 1985a) and in varieties with different levels of drought tolerance, supplemental water is still needed to increase rubber yield and to shorten the growth cycle (Foster and Coffelt 2005).

6.2.3 Clipping

Guayule harvest includes digging whole plants or clipping the above ground biomass, as first suggested by Lloyd (1911). Initially clipping above ground, followed by digging the entire plant, can increase rubber productivity per unit area by allowing for early investment return to growers and distribute the cost of stand establishment across several years, as well as reduce the labor load of digging (Ray et al. 1986). Foster and Coffelt (2005) summarized clipping as the preferred harvesting method because rubber yield was greater in clipped plants than whole plants. Sequential clipping could also increase rubber yield compared to whole plant harvesting over the lifespan of a field. When clipping 2-year-old plants there were no yield differences between direct-seeded plants and transplants.

6.2.4 Fertilization

Guayule is reportedly a low user of soil nutrients; fertilizer requirements depend on soil fertility and plant-growing conditions (Foster and Coffelt 2005). The application of nitrogen fertilizer can improve plant growth and rubber productivity in both transplants and direct-seeded plants, more than other major nutrients. For example, plant height and width in transplants were significantly increased under nitrogen treatments compared to non-fertilized controls (Cannell and Youngner 1983). Seed biomass was also increased by applying nitrogen (112 kg/ha) in guayule production fields at Marana, AZ (Rubis 1983). However, to achieve maximum production, both sufficient irrigation and nitrogen applications were required in 2-year-old shrubs (Bucks et al. 1985b). Likewise, in direct-seeded guayule, nitrogen fertilizer application of 280 kg/ha (Hammond and Polhamus 1965) was shown to be beneficial in California, provided that heavy seeding rates were applied (Kelley et al. 1946). Briefly, the combination of sufficient irrigation, fertilizer supplements, sowing density, soil and climate conditions, all potentially affect the ultimate plant growth and rubber yield in guayule.

6.2.5 Weed Control

Hand weeding and post-emergence oil sprays were two major weed control strategies used in the 1950s. More recent weed control has focused on exploring different modern herbicides. A number of common herbicides and preplant incorporated herbicides were tested in the USA in California, Arizona, New Mexico and Texas, and in Australia (Foster and Coffelt 2005). Trifluralin was reported to efficiently control broadleaf and grass weeds in Arizona and California during the pre-emergence stage in guayule transplants (Elder et al. 1983; Siddiqui et al. 1982) and fluridone is a broad-spectrum herbicide that gave 91% weed control without injuring guayule plants (Foster and Coffelt 2005). DCPA (9.0 kg a.i./ha), bensulide (3.4 kg a.i./ha) and pendimethalin (0.6–1.1 kg a.i./ha) were shown to be effective in direct-seeded plants in Maricopa, AZ (Foster et al. 2002). Pendimethalin obtained a Special Local Needs registration for pre-emergence control of most annual grasses and broadleaf in Arizona (Agriculture 2003). However, no post-emergence treatments have been found to successfully control weeds in both transplants and direct-seeded plants, except during dormant periods (Ferraris 1986; Foster and Coffelt 2005; Siddiqui et al. 1982).

6.3 Germplasm Biodiversity and Conservation

Native people in Mexico discovered rubber in guayule centuries before its first commercial use, chewing guayule bark to produce rubber balls used in sports games. Commercial use began at the end of nineteenth century, when companies collected large quantities of guayule shrubs from natural stands and extracted rubber at industrial plants in Mexico and Texas. This led to a rapid depletion of natural stands and the guayule germplasm resources in these areas. With an interest in increasing the genetic diversity in the available guayule germplasm, several germplasm collections were made during the twentieth century, collecting guayule and related *Parthenium* species from their native habitats (Ray et al. 2005). Unfortunately, much of this recently-collected genetic material has been lost due to a reduction in research funds, the termination of guayule breeding programs, and/or reduced seed viability and low germination rates of collected seeds. The result being that few improved germplasm lines have come from these collections. Nevertheless, there has been improvement for rubber and resin concentrations, faster growth, higher biomass and disease resistance (Estilai 1985,1986; Ray et al. 1999; Tysdal et al. 1983), and these lines have been preserved.

Guayule and *Parthenium* accessions are curated at the USDA-ARS National Arid Land Plant Genetics Resources Unit (NALPGRU), Parlier, CA. The current USDA guayule collection includes 110 accessions of guayule (*P. argentatum*), 1 of *P. confertum*, 15 of *P. incanum*, 2 of *P. schottii*, 2 of *P. tomentosum var. stramonium* and 5 accessions of an unknown *Parthenium* hybrid. Not all of these accessions are available upon public request due to low seed numbers and/or poor seed viability. To address the reduction in guayule genetic resources two points were addressed by the guayule research community; first, to explore and collect more guayule genetic material from wild populations and, second, to characterize the present USDA guayule collections. In 2005 the National Plant Germplasm System (NPGS) sponsored an exploration trip made by Terry Coffelt, Michael Foster and David Stout to collect new genetic materials from areas in Texas where guayule was previously reported. They were able to collect 16 accessions including 3 of guayule, 1 of *P. confertum*, 1 of *P. hysterophorus* and 10 of *P. incanum*. The NPGS is also sponsoring Hussein Abdel-Haleem and Claire Heinitz (USDA, guayule curator) to conduct an exploratory field trip around the Bend National Park in Texas during 2019, with the hope of collecting new genetic material.

There are efforts to characterize the present guayule collection both genetically and phenotypically. Recent studies indicate that the USDA guayule accessions have a wide range of ploidy levels including diploid (2n = 36), triploid (3n = 54), tetraploid (4n = 72) and pentaploid (5n = 90) (Gore et al. 2011; Ilut et al. 2015, 2017), with the improved germplasm mostly tetraploid. Using high throughput genotyping technology, genotyping by sequencing (GBS), Ilut et al. (2015, 2017) developed 50 K single-nucleotide polymorphism (SNP) genetic markers and used them to genotype 69 accessions of the USDA guayule collection and *P. incanum* to study genetic diversity and correlations within the collection.

As the available USDA guayule collection is now genotypically characterized, we initiated efforts to phenotypically characterize them under field conditions (Fig. 6.3), including variations in plant height (Fig. 6.4), biomass, and other agronomic traits. These populations will also be evaluated for rubber and resin production under field and stress conditions. These data will serve as a phenotypic atlas to better understand the collection and to help select parental material for future breeding programs (Fig. 6.5).

6.4 Traditional Breeding

Guayule has experienced intermittent research efforts since the first large-scale rubber extraction in 1888, but thus far no commercial fields have been developed anywhere in the world. Guayule yields were first improved by large-scale cultivation techniques instead of breeding. This is normal in new crops since plant breeding programs usually take many years to achieve genetic gains and many new crops lack sustainable funding support to continue plant breeding programs.

In addition, guayule is a perennial shrub, which usually takes more than 2 years for the first harvest. Other factors also hampered its breeding progress, including: the lack of nondestructive rubber/latex quantification techniques, narrow genetic variation among the currently available germplasm and asexual reproduction by facultative apomixis. In general, there are three continuous steps for guayule genetic

Fig. 6.3 An aerial image of USDA guayule collections planted at fields at Maricopa agricultural Center, Maricopa, AZ (Photo by Hussein Abdel-Haleem, USDA Agricultural Research Service)

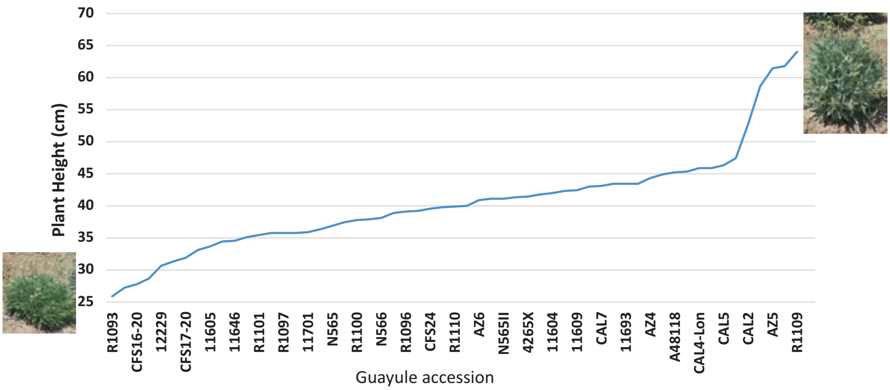

Fig. 6.4 Variations in plant heights of transplanted 1-year old guayule accessions planted under field growth conditions

improvement that begin with creating new genetic materials from different resources including commercial guayule varieties, improved breeding lines, old varieties, wild landraces and related wild species. This step is followed by crossing and selection, then testing the selected germplasm with desired trait(s) in common variety trials (Fig. 6.5).

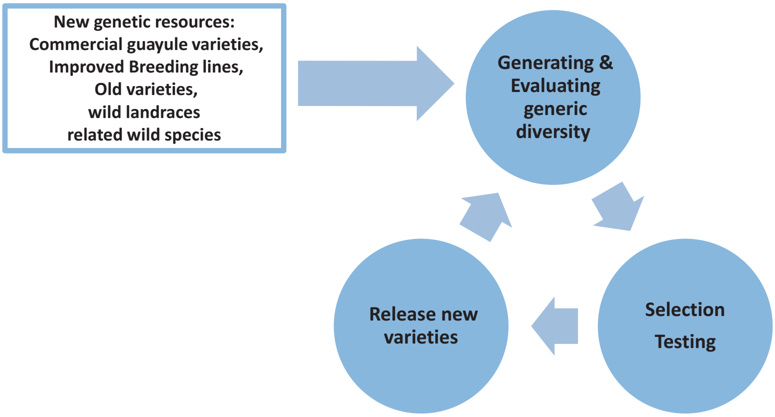

Fig. 6.5 Genetic improvement steps in a guayule breeding program

6.4.1 Guayule Genome Size and Reproduction System

6.4.1.1 Polyploidy

Due to the superiority of diploids in terms of biomass, vigor and disease resistance, it is not surprising that most of today's guayule germplasm collections consists of polyploids ranging from triploids to pentaploids (Estilai and Ray 1991; Gore et al. 2011) with the majority being apomictically reproducing triploids and tetraploids. Previous research suggested that apomictic polyploids are self-compatible, but do not always produce viable seeds. This is not a problem if a selected variety is grown just for rubber production and there is no need to maintain the plants as a seed source (Coffelt et al. 2015). However, it will become a problem if the breeding programs aim to maintain the selected variety by producing seeds.

6.4.1.2 Sexual Reproduction

Diploid guayule possesses a sporophytic self-incompatibility (SSI) system, in which the success or failure of generating viable seeds depends not only on the genotype of the stigma in the maternal genotype, but also on the genotype of the paternal sporophyte (pollen-producing plant). Although the self-incompatibility feature makes diploid guayule an obligate outcrosser and cannot generate homozygous progenies, the resulting high degree of heterozygosity in the populations facilitates long-term evolutionary adaptation in this species (Estilai and Ray 1991).

6.4.1.3 Apomixis

Most currently available guayule germplasm consists of triploid and tetraploid accessions, which are facultative apomicts (Coffelt et al. 2015; Estilai and Ray 1991). The meiotic division in apomictic guayule is blocked in the megaspore mother cells (MMCs), resulting in the unreduced embryos with the same chromosome complement as the maternal parent. However, unlike common obligate apomixis, facultative apomixis requires fertilization of male gametes to realize endosperm development and thus potentially results in four types of seeds: (1) asexual seeds derived from non-reduced female gametes without fertilization, (2) sexual seeds derived from non-reduced female gametes and fertilized by meotically reduced male gametes, (3) polyhaploid seeds from reduced female gametes without fertilization and (4) sexual seeds derived from reduced female gametes and fertilized by reduced male gametes (Cruz et al. 2017; Esau 1946; Estilai and Ray 1991; Gore et al. 2011; Hammond and Polhamus 1965). This complexity in apomictic progenies results in the presence of various ploidy levels and genotypes among progeny, leading to new genetic variation for further use in plant selection and breeding programs. Previous researchers hypothesized that at least three loci are involved in guayule apomixis: one inhibiting meiosis in MMCs, a second controlling fertilization and the third controlling seed development (Hammond and Polhamus 1965; Kupzow 1969; Powers 1945; Powers and Rollins 1945).

6.4.2 Breeding Objectives

Among all guayule breeding objectives, improving rubber yield has been paramount. Other breeding objectives such as rubber quality, resin yield, plant vigor and regrowth ability after clipping, disease resistance, drought tolerance and salinity tolerance are all important traits of interest, and should also be improved (Coffelt et al. 2015; Estilai and Ray 1991; Ray et al. 2010; Thompson and Ray 1988).

In order to breed for traits of interest, breeders need to first determine the heritability of these traits, whether they are controlled by single or multigene families, the environmental effects and the number of generations required to fix a trait. Rubber yield is equal to plant biomass multiplied by rubber concentration (%), meaning that either increasing plant biomass or rubber concentration can improve rubber yield. However, selecting for large biomass involves additional costs in harvesting, transportation and handling, resulting in reduced efficiency in the entire processing chain. Therefore, selecting normal-size plants with high rubber concentration seems to be a solution. This may be difficult because previous researchers have found that there was a negative correlation between rubber concentration and plant biomass in guayule (Coffelt et al. 2015; Dierig et al. 1989; Thompson et al. 1988). However, fresh and dry weights, and plant height and width were found to be highly positively correlated with rubber yield (Dierig et al. 1989; Ray et al. 1993; Thompson et al. 1988), suggesting that plant biomass can be used as a primary selection index for rubber yield (Coffelt et al. 2015). The heritability of eight components of rubber yield was estimated in a study using parent-offspring regression in an open-pollinated population derived from single-plant selections (Ray et al. 1993). None of the parent-offspring regressions were significantly different from zero among all these components, indicating a low heritability for all these traits. In addition, a larger phenotypic variation was found among parents than progenies (Ray et al. 1993), which was probably compounded by the environmental effects since parents were 1 year older than the progenies (Coffelt et al. 2015; Dierig et al. 1989). Based on these results, family selections, either full-sibs or half-sibs, should be considered in future breeding programs rather than single-plant selections as described in this study (Ray et al. 1993).

Other than heritability, an accurate estimation of genotypic, environment effects and genotype × environment interactions is another aspect in improving rubber yield. A 2-year field trial in Maricopa, AZ showed that the main effects of location, line and plant age were significant, but the interactions were not significant for plant growth, biomass and latex content (Majeau et al. 2003). Moreover, the impact of environment on guayule was very significant, which accounted for over 50% of the phenotypic variation (Majeau et al. 2003). However, since only four lines were used in this field trial, the results might be overestimated. Some of the nonsignificant interactions may become significant with a larger or wider germplasm base (Majeau et al. 2003). Dierig et al. (1989) also observed significant environment effects within a single field trial. Small or slight genotype × environment interactions are desired in breeding programs since this suggests that variety selection can be done in one location without considering the impact of different environments on the lines and that selected superior lines could maintain their superiority in different locations (Coffelt et al. 2015). This has happened with several AZ lines, which gave similar results when evaluated under different locations such as in the USA, Spain, Australia, South Africa and China (Coffelt et al. 2005, 2015; Ray et al. 2010).

Other traits related to resin and latex have also attracted research interest. Thompson et al. (1988) found that rubber concentration was significantly correlated with resin concentration, meaning that simultaneously breeding for new lines with both high rubber and resin concentration is possible. This feasibility was confirmed by Ray et al. (1999). Improving both traits is important in determining the value of guayule end products for successful commercialization. Studies to explore the relationship between latex and rubber concentration, however, have not always been consistent (Coffelt et al. 2009a, b). A recent field trial conducted in six environments in Arizona and Texas showed moderate to high heritability and positive correlations among dry biomass, and rubber and resin content (Abdel-Haleem et al. 2018). The phenotypic variation, genetic variation and genotype by environment interactions were found to be significant in both

2-year- and 3-year-old plants, meaning that multiple-location field trials are required (Abdel-Haleem et al. 2018).

Cold tolerance identified in accessions 11,591 and N6–5 in a 3-year field trial on the Texas High Plains suggested that sufficient variation does exist in response to cold stress among contemporary germplasm and can be used to expand guayule planting areas (Foster et al. 2011). Other desirable characteristics to facilitate guayule commercialization include: improvements of drought tolerance, salt tolerance, seed quality, reduced postharvest degradation and the elimination of wild characteristics such as intermediate flowering, seed shattering, seed dormancy and complicated reproduction systems (Coffelt et al. 2015; Estilai and Ray 1991; Ray et al. 2010).

To conclude, although several field trials (Dierig et al. 1989; Estilai and Ray 1991; Majeau et al. 2003; Thompson et al. 1988) listed above were attempted in the recent decades, few studies have been consistently continued due to the lack of funding resources. Therefore, continuous multiple-year yield trials under various environmental conditions need to be implemented in order to accurately evaluate the phenotypic effects, genotypic effects, environmental effects and genotype × environment interactions for traits of interest in future breeding programs. Accurate phenotyping is of great significance but not easy, especially for rubber/resin/latex traits, because the quantification analyses are often time consuming, labor intensive and cost-inefficient, which largely limits the sample size that can be processed. In addition, moisture content, deterioration of latex and rubber during the processing procedures can obscure results in rubber and latex quantification (Teetor et al. 2009). Therefore, rapid and accurate phenotyping strategies such as near infrared (NIR) spectroscopy techniques could be used to speed up the quantification processes (Kopicky 2014). However, establishing a reliable NIR model with high R^2 is another important issue to be addressed.

6.4.3 Traditional Breeding Methodologies

6.4.3.1 Single-Plant Selection

Single-plant selection among apomictic polyploids has been the most widely used approach in guayule breeding. This could be the simplest and most efficient way when the heritability of desired traits is high because genetic gains can be increased rapidly in a relatively short time, but in the long run, only modest genetic gains can be achieved due to the lack of new genetic combinations. Therefore, whether this method succeeds or not depends on several factors, including (1) the heterogeneity of the population, (2) the degree of genetic effects and (3) population size that can be effectively screened (Coffelt et al. 2015; Thompson et al. 1988). Previous studies successfully utilized this method to increase annual rubber yields from 300 to 1000 kg/ha by simultaneously selecting individuals with both high rubber concentration and high biomass production (Coffelt et al. 2015; Estilai and Ray 1991; Ray et al. 1999). However, when heritability is low, as mentioned above, family selection, either by full-sibs or half-sibs, might be more efficient to evaluate parent plants. In this case, parents are selected based on the performance of their progenies instead of on their own generation (Coffelt et al. 2015). This method, although possibly lengthening generational intervals, could be successfully conducted in guayule, a perennial shrub with continuous flowering and seed production from a single parent plant.

6.4.3.2 Recurrent Selection and Mass Selection

Although apomictic polyploids ensure the uniformity of genetic background in selected varieties, new desirable genes from diverse resources need to be introduced into long-term breeding programs. Sexual diploids are potentially useful to introduce new genetic combinations because cross-pollination is the predominate mode of reproduction in guayule. Mass selection is a simple, common and established breeding method, in which large numbers of varieties are selected by collecting seeds from selected plants to propagate the next generation (Romero and Frey 1966). Recurrent selection, as an extension of mass selection, is the intermating of selected plants/varieties for generation after generation (Hayes and Garber 1919). In diploid guayule, problems such as reduced biomass, lower rubber content and increased susceptibility to root diseases have occurred; however, previous researchers have successfully used modified recurrent selection breeding schemes to increase yield (Estilai and Ray 1991) and mass selection to develop *Verticillium*-resistant lines (Ray et al. 1995). Newly-released varieties were exploited as follows (Estilai and Ray 1991): (1) if biomass production and rubber concentration are both economically acceptable in the population, the open-pollinated seeds can be released or they can be continuously crossed to other diploids to generate more genetic variation for later selections; (2) if rubber concentration is high but biomass is low, they are crossed to selected apomictic polyploids to improve biomass; (3) after the improvement is done, the improved diploids may have their chromosomes doubled chemically to produce autotetraploid cultivars. Following this scheme, Cal-3, a diploid germplasm, was released (Hashemi et al. 1989). These breeding efforts have resulted in only limited advancements because most financial support grants are for a period of 3 years, but plant breeding programs need continuous funding. This is compounded in guayule because it is a perennial plant, requiring several years to mature before phenotyping, and a large number of samples must be collected requiring corresponding labor, time and costs. Finally, since guayule is grown several years before harvest, genetic effects can become masked by the compounded environmental effects; therefore, the ability to make early selections is very important.

6.4.3.3 Hybridization

Intraspecific Hybridization In the past, intraspecific hybridization was sparingly used in guayule due to the lack of genetic markers to separate progenies from asexual apomixis and from sexually reproduced hybrids. Keys et al. (2002) identified a method to distinguish plants expressing high levels of sexuality from asexual apomicts. By this method, seeds with predominately apomictic features can be released as a new variety, and seeds with predominately sexual features used for self-pollination, cross-pollination and backcrossing (Coffelt et al. 2015).

Interspecific Hybridization Only limited efforts have been made to generate interspecific hybrids in guayule due to the dramatic decrease in rubber content, which needs many backcrossing generations to restore as well as to keep the new desirable traits such as increased vigor, resin content, biomass, disease and insect resistance, regrowth capability after clipping and cold tolerance (Coffelt et al. 2015; Estilai and Ray 1991; Ray et al. 2010). Despite these difficulties, three germplasm lines (Cal-1, Cal-2, Cal-5) derived from crossing guayule and three different *Parthenium* species were released by the University of California Riverside (Estilai 1985, 1986). These three germplasm lines have improved vigor, biomass production and *Verticillium* resistance. AZ-101 is another interspecific hybrid derived from natural crossing in a guayule field, between guayule and *Parthenium tomentosum*, and maintains both high vigor and biomass production (Estilai and Ray 1991).

6.5 Functional Genomics, Tissue Culture, Genetic Engineering and Molecular Characterization

Limited studies of guayule have been conducted utilizing advanced biotechnology techniques. These are limited to a few transgenic, functional genomic, physiological and biochemical studies, and polyploidy analyses using flow cytometry. Most of these investigations were designed to understand the rubber biosynthesis pathway. No comprehensive molecular breeding studies have been carried out so far in guayule. Despite that, a 1.6 Gb guayule genome of a diploid guayule accession was sequenced, assembled and around 40,000 transcribed, protein encoding genes were annotated (Valdes Franco et al. 2018). This study describes the first partial draft genome of guayule and is expected to enhance research topics important to guayule genetic improvement and the future application of modern biotechnology, molecular breeding, functional genetics and comparative genomics.

6.5.1 Tissue Culture

Only a few tissue culture studies have been carried out on guayule. Guayule micropropagation has been achieved from callus and shoots. Although there have been several reports that somatic embryogenesis (callus culture) could realize regeneration of shoots, or even whole plants (Dhar et al. 1989; Finnie et al. 1989; Zavala et al. 1982), induction of callus requires high concentrations of growth regulators and leads to somaclonal variation. Therefore, since the 1990s, researchers have used direct organogenesis through shoot culture, followed by rooting and subsequent acclimation of plantlets to simplify and improve the efficiency of guayule tissue culture (Castillon and Cornish 2000; Dong et al. 2006; Pan et al. 1996; Staba and Nygaard 1983; Trautmann and Visser 1990). These studies laid the foundation for subsequent creation of genetically-engineered guayule plants by mediating their metabolic pathways to produce rubber.

6.5.2 Genetic Engineering

Two studies have been conducted to evaluate genetically-engineered guayule lines. Veatch et al. (2005) overexpressed three allylic pyrophosphate initiators including farnesyl pyrophosphate (FPP), geranylgeranyl pyrophosphate synthase (GGPP) and hexa-heptaprenyl pyrophosphate synthase (H-HPP) in three transgenic guayule lines (AZ101, AZ-2, N6-5). Even though the rubber concentration was not significantly altered in transgenic lines, resin production was enhanced.

Dong et al. (2013) evaluated transgenic guayule lines with the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) overexpressed. HMGR is a key regulatory enzyme of mevolanate (MEV) carbon flux in mammals, microbial systems and possibly in plants since it converts 3-hydroxy-3-methylglutaryl-CoA to mevalonate, a precursor of isopentenyl pyrophosphate (IPP) (Dong et al. 2013; Kirby and Keasling 2009). In this study, overexpression of the HMGR gene produced a 65% rubber increase in one out of five genetically-modified guayule lines (HMGR6), but there was no significant differentiation in rubber production in the other lines in this lab-based experiment. Although differences were observed in size, biomass and plant morphology of HMGR6 in the field, rubber and resin content were not significantly different from the control lines. However, the survival rate during regrowth was significantly improved for these transgenic lines, indicating that the overexpression of HMGR enhanced carbon flux to produce important secondary isoprenoid metabolites (e.g. growth phytohormones).

6.5.3 Functional Genomics

Rubber biosynthesis in guayule increases as night temperatures fall and accumulate to the highest degree in the cold winter months (Benedict et al. 2008; Cornish and Backhaus 2003). It is important to examine the effect of cold temperatures on this biosynthetic pathway, to understand the regulation of rubber biosynthesis in guayule, if the goal of improving guayule as a domestic rubber crop is to be realized, by means of breeding or genetic engineering. Ponciano et al. (2012) conducted a transcriptome analysis using 11,748 expressed sequence tags (ESTs) to evaluate expression of genes potentially related to rubber biosynthesis. Contrary to a previous study by Cornish and Backhaus (2003), Ponciano et al. (2012) found that none of the genes encoding 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS) and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), farnesyl pyrophosphate synthase (FPP), squalene synthase, small rubber particle protein (SRPP), *cis*-prenyltransferase (CPT) and allene oxide synthase (AOS) were positively correlated with air temperatures, except for CPT, which reached a peak in gene expression right after a sudden increase in night temperature 10 days before harvest. They concluded that either the critical members of the rubber transferase complex are yet to be identified, or the enzymatic activity of the rubber transferase complex is more likely controlled by posttranslational modifications rather than gene expression. Moreover, it is possible that the low abundance of proteins (~1%) present in rubber particles or participating in rubber biosynthesis accounted for only a very small part of the entire guayule transcriptome, which might be masked by the high threshold established by other differentially expressed genes. Similar results were also found in transcriptome studies of other rubber-producing plants (Ko et al. 2003; Luo et al. 2017; Priya et al. 2007).

Research by Valdes Franco et al. (2018) revealed 15 families of SaTar (satellite targeted) elements with unique chromosomal distribution profiles. These transposable elements (TEs) are frequently observed in multimeric linear arrays of unrelated individual elements.

6.5.4 Flow Cytometry Analyses

Flow cytometry has been used fairly often in guayule genetic studies, compared to other modern genotyping techniques, to characterize ploidy levels of guayule accessions (Gore et al. 2011; Ilut et al. 2015; Sanchez et al. 2014). These studies provide a foundation for interspecific hybridization and genome sequencing studies in guayule breeding programs. Cruz et al. (2017) utilized flow cytometry to estimate the rate of apomixis and validate the reproduction mode in guayule polyhaploids, they found that seed embryo and endosperm DNA content varied during seed development and maturity stages, and that the nuclear DNA content is more consistent when seeds are collected during middle to late development stages.

6.6 Conclusions and Prospects

Among more than 2000 plant species known to produce rubber, based on its origin in Southern Texas and Northern Mexico, guayule is a good candidate for arid and semiarid sustainable agricultural systems as an alternative rubber crop to *Hevea*. Commercialization of guayule as a new/alternative crop is possible and advisable due to the disadvantage of being totally dependent upon *Hevea*. To establish guayule as a new crop, we must continue genetic improvement and establish best agronomic practices. Farming strategies have been developed for guayule, but there are still agronomic challenges to be met. For example, in order to replace transplanting with the more cost-efficient direct-seeding strategy, problems of seed quality, seedling vigor and salt tolerance remain to be addressed. Even though guayule is a semiarid, drought-tolerant shrub requiring only low nutrient inputs, suitable irrigation and fertilization applications still need to be adjusted and optimized according to different growing conditions and maximum sustained production targets. The control of water quality is also very important because salt tolerance of guayule at emergence and seedling stages is reportedly very low, even lower than carrots, one of the most salt-sensitive crops grown in the southwestern USA. Clipping can improve input/output efficiency, but lines vary in their capability to regrow after clipping. Therefore, selecting lines with high regrowth potential should be considered by breeders and agronomists in future studies prior to releasing new germplasm. As a perennial crop, one of the major challenges in guayule is to shorten the plant growth cycle and maximize rubber production, which can be complicated by the strong influence of environmental effects and changing climate.

While previous attempts to improve rubber yield achieved limited success, a better understanding of the genetics of rubber production will help by providing an estimate of the number of genes controlling rubber production. Traditional breeding methods including the recurrent or mass selection in sexual diploids, the selection and hybridization of apomictic polyploids and backcrossing of interspecific hybrids have produced new and improved germplasm. As result, since 1970, improved germplasm has been released with higher rubber and resin concentrations, faster growth with high biomass and disease resistance. Fortunately, with the development of advanced biotechnology and molecular markers, these activities can be accelerated. For example, GBS technology has provided fast and precise discovery of SNP markers in guayule; this technology could be used to identify interspecific hybrids or sexual intraspecific hybrids at an early stage of selection and recurrent or mass selections could be accelerated by marker-assisted recurrent selection (MARS). Molecular breeding integrated with marker-assisted selection (MAS) may be more effective for the quantitative traits controlled by a small number of genes. Both MAS and MARS could be used to select for simple and complex traits in early generations and stages of a breeding program. One of the major steps toward using

MAS and/or MARS in guayule breeding is to identify markers associated with the trait(s) of interest. The key steps in identification of molecular markers linked to trait are: identifying parents differing in specific traits, developing mapping populations segregating for these traits, constructing a linkage map of the population to locate QTLs associated with the traits of interest, identifying molecular markers that cosegregate with the trait of interest and validating the associated markers in different backgrounds. Due to the complexity of apomictic reproduction resulting in a mixture of various ploidy levels among guayule progeny, there are significant hurdles for a population to reach homogeneity. Using sexual diploids is the way to create populations and linkage maps to identify associated markers. The current USDA guayule collection has very few, and narrowly diverse, diploid accessions. Therefore, more collecting trips are needed to discover more wild diploids. Genotyping such large mapping populations will be facilitated by advancements in high-throughput sequencing technologies, making it feasible to create linkage maps for a large population using GBS technology. With the increased ability to discover and map thousands of molecular markers, phenotyping is emerging as the bottleneck for genomics-based approaches, not only in guayule but in many other crops. Complementary, precise and robust technologies of high-throughput phenotyping (HTP) are needed to increase the statistical power and identify marker/trait associations in large populations. HTP technology is a rapidly expanding field, allowing the characterization of complex traits using proximal sensors and imaging systems. We are starting to develop HTP protocols, collect and analyze traits related to guayule growth performance, responses to environments including normalized difference vegetation index (NDVI), canopy height and plant canopy temperature.

Taking into account that guayule is a perennial, and a not a food or feed plant, genetic engineering techniques may be effective for potential simple traits such as herbicide tolerance and insect resistance. Herbicide and insect tolerance are traits that are important during early establishment or regrowth stages in guayule, since growth is usually slow and weed competition is high during this period. Improvements of these two traits will be even more important if researchers want to replace transplanting cultivation systems with a direct-seeding system. In addition, with the development of gene editing technologies such as CRISPR-Cas9, genetic engineering strategies can be more targeted and effective in future applications. Gene editing technology such as CRISPR-Cas9, coupled with functional genomics, could help reveal the genetic architecture behind apomixis in guayule.

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Appendices

Appendix I: Research Institutes Relevant to Guayule

Institution	Specialization and research activities	Contact information and website
USDA-ARS	Agronomy and breeding	Hussein Abdel-Haleem US Arid-Land Agricultural Research Center 21881 North Cardon Lane, Maricopa, Arizona, 85138, USA Tel: 520-316-6355; Fax: 520-316-6330 Hussein.Abdel-Haleem@ars.usda.gov
University of Arizona	Agronomy and breeding	Dennis Ray School of Plant Sciences, Arid Lands Studies 1140 E. South Campus Drive, P.O. Box 210036 303 Forbes Building, Tucson, Arizona 85,721, USA Tel: 520-621-7612 dtray@email.arizona.edu
USDA-ARS	Natural rubber biosynthesis and production	Colleen McMahan 800 Buchanan st, Albany, California 94,710, USA Tel: 510-559-5816; Fax: 510-559-5818 Colleen.McMahan@ars.usda.gov
USDA-ARS	Genetic resources conservation	Claire Heinitz Nat'l Clonal Germplasm Repository (NCGR) 9611 S. Riverbend Ave, Parlier, California 93,648, USA Tel: 559-596-2980 claire.heinitz@ars.usda.gov
Bridgestone Americas, Inc.	Agronomy and breeding	David Dierig Guayule Research Farm, Eloy, Arizona, USA DierigDavid@bfusa.com

(continued)

Institution	Specialization and research activities	Contact information and website
The Ohio State University	Natural rubber biosynthesis and production	Katrina Cornish 108A Williams Hall, 1680 Madison Avenue, Wooster, Ohio 44691, USA Tel: 330-263-3982 cornish.19@osu.edu
Centre de coopération internationale en recherche agronomique pour le développement (CIRAD)	Natural rubber biosynthesis and production	Serge Palu 34398 Montpellier Cedex 5, France Tel: 33-04 67 61 58 99; Fax: 33 04 67 61 65 47 Serge.palu@cirad.fr

Appendix II: Guayule Genetic Resources Worldwide

The guayule genetic resources can be accessed through the Germplasm Resources Information Network: https://npgsweb.ars-grin.gov/gringlobal/taxonomydetail.aspx?id=26802

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