Chapter 5
Genetic Transformation and Transgenics of *Jatropha curcas*, a Biofuel Plant

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Abstract *Jatropha curcas* is considered as a potential biodiesel feedstock plant. To date, however, it remains a semi-wild species. Transgenic modification is one of the most effective and rapid approaches to accelerate its breeding process. Various methods of genetic transformation, such as *Agrobacterium*- and particle bombardment-mediated transformation, have been attempted and improved over the past 10 years. This chapter presents a comprehensive account of the influence of several important factors on the genetic transformation of *Jatropha*. It also introduces studies on transgenic *Jatropha* involving functional genes for novel agronomic traits, including plant morphology, flowering time, seed development, seed oil content, oil composition and yield, as well as biotic and abiotic stress tolerance. Moreover, improvements in genetic transformation and the completion of genomic sequencing analysis give *Jatropha* the potential to become a new model species for studies on gene function and genetic improvement in woody plants.

Keywords Explants · *Jatropha curcas* · Selective agent · Transformation efficiency · Transgenic plant

5.1 Introduction

Jatropha curcas L. (hereafter referred to as *Jatropha*) is considered a potential oilseed plant for biofuel production because its seeds contain 30–40% oil (Kandpal and Madan 1995; Agarwal and Agarwal 2007; Tapanes et al. 2008), which can be easily converted to biodiesel or bio-jet fuel and used to partially or fully replace fossil fuels (Fairless 2007; Makkar and Becker 2009; Juan et al. 2011). The plant is simple to propagate, exhibits rapid growth and wide adaptability, and is ideally

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suited for growing in marginal or wastelands that are unsuitable for food production (Maghuly and Laimer 2013). However, at present, the potential of *Jatropha* is far from being realized in the biofuel industry because its seed yields are generally low in many areas (Singh et al. 2014). Investments in the large-scale planting of *Jatropha* have progressed ahead of scientific studies that aim to fully explore this plant and understand its limitations (Sanderson 2009).

Jatropha continues to remain semi-wild, and ideal commercial varieties with profitable yields are still lacking. Varieties that produce high and stable yields need to be bred to develop *Jatropha* into a successful biofuel crop (Chikara et al. 2013; Montes and Melchinger 2016). Although conventional breeding methods have been widely used to improve many plant species, this procedure is slow, especially for perennial species such as *Jatropha*. Additionally, the narrow genetic diversity among *Jatropha* germplasm (Sun et al. 2008; Rosado et al. 2010) has limited traditional cross- and selective breeding for the genetic improvement of this plant. Therefore, genetic transformation technology is needed to provide an additional tool for the genetic improvement of this crop. Compared to traditional breeding, genetic transformation techniques have many advantages, such as the directional cultivation of new breeds, reduced costs, a shorter breeding period, and the ability to introduce genes for desirable traits that may not be available within the species or that may be difficult or impossible to manage via traditional breeding methods (Li et al. 1996; Visarada et al. 2009; Herr and Carlson 2013). Moreover, genetic transformation has become an important method for basic research on gene functions and for the generation of new transgenic plants with excellent traits.

The genetic transformation of *Jatropha* has been pursued for the last 10 years, and many transformation methods have been established. However, the transformation efficiency of these methods varies greatly, ranging from only 4% to 95.4% (Li et al. 2008; Pan et al. 2010; Khemkladngoen et al. 2011; Kajikawa et al. 2012; Toppo et al. 2012; Liu et al. 2017). Several transformation techniques have been adopted for transgenesis and for the analysis of gene function (Tsuchimoto et al. 2012; Joshi et al. 2013; Li et al. 2014; Gu et al. 2015; Maravi et al. 2016; Hu et al. 2017). In this chapter, we will provide a brief overview of several common genetic transformation techniques and recent progress on gene function research in *Jatropha*.

5.2 Establishment of Transformation Methods

The efficiency of genetic transformation in plants is affected by several important factors, including the ability of explants to regenerate, the methods of gene transfer, and the selection conditions (Li et al. 2008; Kajikawa et al. 2012; Mao et al. 2013; Fu et al. 2015; Kumar et al. 2015). Table 5.1 presents a summary of various transformation studies conducted on *Jatropha*, along with various factors that may influence transformation efficiency.

Table 5.1 Genetic transformation methods for *Jatropha*

Explant tissue	Transformation method	*Agrobacterium* strain	Selection agent/concentration (mg/l)	Transformation efficiency (%)	References
Cotyledon	*Agrobacterium-mediated*	LBA4404	Phosphinothricin/1	13	Li et al. (2008)
Cotyledon	*Agrobacterium-mediated*	LBA4404	Kanamycin/20	30.8	Pan et al. (2010)
Cotyledon	*Agrobacterium-mediated*	LBA4404	Kanamycin/20	53	Khemkladngoen et al. (2011)
Cotyledon	*Agrobacterium-mediated*	LBA4404	Bispyribac-sodium/4.5	4.3	Kajikawa et al. (2012)
Cotyledon	*Agrobacterium-mediated*	AGL1	Hygromycin/3–5 or glufosinate ammonium/1	Not available	Mao et al. (2013)
Cotyledon	*Agrobacterium-mediated*	EHA105	Kanamycin/40	56.0	Fu et al. (2015)
Cotyledon	*Agrobacterium-mediated*	LBA4404	Bispyribac-sodium /4.5–11.25	23.3	Nanasato et al. (2015)
Leaf	*Agrobacterium-mediated*	LBA4404	Hygromycin/5	29	Kumar et al. (2010)
Young leaf	*Agrobacterium-mediated*	LBA4404	Kanamycin/40	23.9	Zong et al. (2010)
Leaf/ hypocotyl	*Agrobacterium-mediated*	EHA101	Kanamycin/50	5/4	Misra et al. (2012)
Leaf	*Agrobacterium-mediated*	GV3101	Mannose/20 $\times 10^3$	50	Chen et al. (2015)
Petiole	*Agrobacterium-mediated*	EHA105	Hygromycin/3	95.4	Liu et al. (2017)
Plantlet	*Agrobacterium-mediated*	EHA105	Phosphinothricin/2 $\times 10^3$	62.7	Jaganath et al. (2014)
Germinating seed	*Agrobacterium-mediated*	GV3101	Not available	15	Patade et al. (2014)
Shoot apex	Particle bombardment-mediated	Not applicable	Kanamycin/25	Not available	Purkayastha et al. (2010)
Embryo axes	Particle bombardment-mediated	Not applicable	Hygromycin/5–7	44.7	Joshi et al. (2011)

5.2.1 Explant Selection

The establishment of a highly efficient tissue culture-based regeneration system is a prerequisite for a successful transformation system. An efficient regeneration system includes highly regenerative explants and the corresponding culture medium components. Various explants from *Jatropha*, such as embryos, cotyledons, epicotyls, hypocotyls, leaves, petioles, nodal segments, axillary nodes, and shoot apices, have been successfully used to regenerate shoots (Sujatha and Mukta 1996; Khurana-Kaul et al. 2010; Kumar and Reddy 2010; Mazumdar et al. 2010; Purkayastha et al. 2010; Singh et al. 2010; Sharma et al. 2011; Toppo et al. 2012). Some explants, such as cotyledons, young leaves, and petioles, have been successfully utilized in genetic transformation protocols that employ *Agrobacterium tumefaciens* (Li et al. 2008; Kumar et al. 2010; Pan et al. 2010; Khemkladngoen et al. 2011; Misra et al. 2012; Mao et al. 2013) (Table 5.1). In addition, shoot apices and embryo axes have been utilized in particle bombardment (Purkayastha et al. 2010; Joshi et al. 2011) (Table 5.1). Among these explants, cotyledons are generally preferred for transformation because they exhibit high regeneration frequencies and produce more genetically transformed plants than other explants do. Additionally, the cotyledons of *Jatropha* are more susceptible to *A. tumefaciens* infection than are explants from other tissues, such as petioles, hypocotyls, epicotyls, or leaves (Li et al. 2006). However, cotyledons cannot maintain the genetic character of the mother plant because *Jatropha* is frequently cross-pollinated. Young leaves can maintain the good characters of the mother plant and are also available in large quantities for transformation. The methods of *Jatropha* transformation should be further developed with a focus on enhancing the transformation efficiency of leaf explants.

5.2.2 Transformation Methods

Agrobacterium- and particle bombardment-mediated genetic transformations are two popular methods used to produce stably transformed plants. Only three studies have used particle bombardment to genetically transform *Jatropha* (Purkayastha et al. 2010; Joshi et al. 2011, 2013). Purkayastha et al. (2010) established the particle bombardment-mediated transformation method by comparing the size of the gold particles, the bombardment pressure, the target distance, the travel distance of the macrocarrier, and the type and duration of osmotic pretreatment. Joshi et al. (2011) also genetically transformed *Jatropha* via particle bombardment and achieved 44.7% transformation efficiency. Using this method, salt-tolerant transgenic *Jatropha* shoots harboring *35S:SbNHX1* were created (Joshi et al. 2013). The advantages of *Agrobacterium*-mediated genetic transformation, including minimal equipment cost, are the potentially single- or low-copy transgene insertions, and preferential integration into transcriptionally active regions of chromosomes (Newell 2000), which make

this method the most widely used to generate transgenic *Jatropha* (Li et al. 2008; Pan et al. 2010; Zong et al. 2010; Kajikawa et al. 2012; Mao et al. 2013).

In the *Agrobacterium*-mediated transformation method, the *A. tumefaciens* strain is one of the most important factors in the efficiency of genetic transformation. Several *A. tumefaciens* strains have been used for the genetic transformation of *Jatropha*, including LBA4404 (Li et al. 2008; Kumar et al. 2010; Pan et al. 2010; Kajikawa et al. 2012), EHA105 (Jaganath et al. 2014; Fu et al. 2015; Liu et al. 2017), GV3101 (Patade et al. 2014; Chen et al. 2015), AGL1 (Mao et al. 2013), and EHA101 (Misra et al. 2012). However, the transformation efficiency in *Jatropha* varies greatly among these *Agrobacterium*-mediated transformation methods (Table 5.1). Different *Jatropha* explants may have different susceptibilities to different *Agrobacterium* strains (Li et al. 2008; Khemkladngoen et al. 2011; Kajikawa et al. 2012; Nanasato et al. 2015). In addition, several other conditions and factors also affect the transformation efficiency, such as the pre-culture of the explants, the density of *A. tumefaciens*, supplementation and concentration of acetosyringone, and the time course of the co-culture period (Kumar et al. 2013; Sujatha et al. 2013).

Recently, *in planta* transformation has also been successfully established in germinating *Jatropha* seeds (Patade et al. 2014) and plantlets (Jaganath et al. 2014). Both methods result in high transformation efficiency, reaching 15% and 62.66%, respectively (Table 5.1). These transformation methods are simple and easy to implement without tissue culture and can generate a relatively large number of transgenic plants in a relatively short time. We look forward to further research on gene function and transgenic breeding in *Jatropha* using this method.

5.2.3 Selection Conditions

In addition to the development of genetic transformation methods, the types of selection agents and the selection pressure are integral to developing an efficient transformation system. Kanamycin, hygromycin, and herbicides have been used widely as selection agents in genetic transformation systems. The ideal selection agent and pressure can suppress or kill untransformed cells and simultaneously allow the preferential proliferation of the transformed cells (Que et al. 2014), which may then efficiently regenerate plantlets. Li et al. (2008) first reported *Agrobacterium*-mediated transformation methods using the herbicide phosphinothricin as a selection agent. Subsequently, several groups used hygromycin (Kumar et al. 2010; Joshi et al. 2011; Mao et al. 2013), kanamycin (Pan et al. 2010; Zong et al. 2010; Khemkladngoen et al. 2011; Misra et al. 2012), bispyribac-sodium salt (Kajikawa et al. 2012), or mannose (Chen et al. 2015) as selection agents.

Many plant species, including *Jatropha*, are hypersensitive to these selection agents, and this hypersensitivity most likely causes the low transformation efficiencies observed in previous studies (Pan et al. 2010; Kajikawa et al. 2012). Alternative selection strategies, including adjusting the concentrations of the selection agents

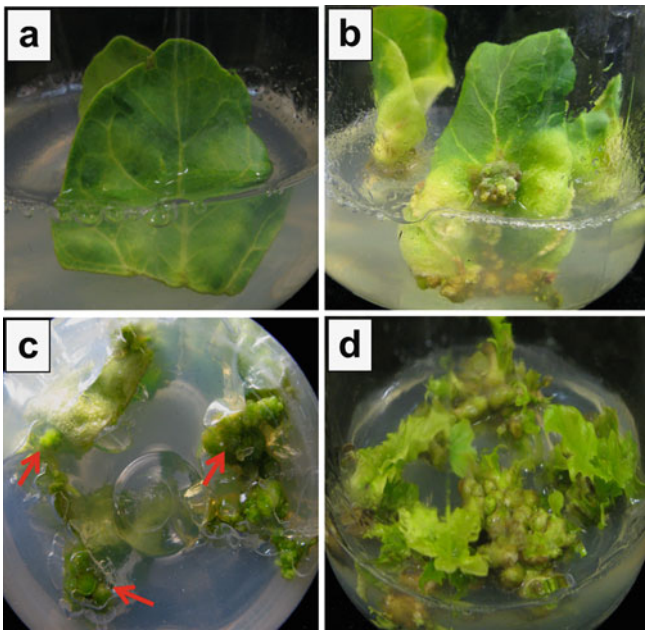

Fig. 5.1 Different stages of the *Agrobacterium*-mediated genetic transformation of *Jatropha*. (**a**) Inserting cotyledon explants into shoot-inducing medium (SIM) after cultivation on callus-inducing medium (CIM) for 7 days to induce resistant calli. (**b**) Three-week-old resistant calli in SIM after a 1-week delay in selection with 40 mg/l kanamycin. (**c**) Resistant calli (indicated by red arrows) in SIM. (**d**) Resistant shoot buds on SIM in the third cycle of selection. (Source: Fu et al. 2015)

and delaying selection, have successfully yielded transgenic apple (Yao et al. 1995) and almond plants (Miguel and Oliveira 1999; Ramesh et al. 2006). In our study, the percentage of β-glucuronidase (GUS)-positive shoots reached an average of 56.0% using 40 mg/l kanamycin with a 1-week delay in selection (Fu et al. 2015). This screening strategy also yielded transgenic *Jatropha* plants when the selection agent hygromycin (0.8–10 mg/l) or the herbicide glyphosate (1 mg/l) was used (unpublished data). In addition, the success of selection also depends on increasing the contact between explants and the selection medium through inoculation (Bhatia et al. 2005). In our method, cotyledon explants were transferred from callus-inducing medium (CIM) to shoot-inducing medium (SIM) with approximately 1 cm of their cut ends embedded in the medium (Fig. 5.1a). After 2–3 weeks, green calli and adventitious buds developed at the cut ends of the cotyledon explants in the medium (Fig. 5.1b, c), and some adventitious buds developed into transgenic shoots (Fig. 5.1d). This improved inoculation method could also lead to a reduction in escapes from kanamycin selection. The overall scheme of this transformation method is presented in Fig. 5.2.

To avoid environmental safety issues and public concerns regarding transgenic plants, an antibiotic marker-free system has been used in *Jatropha* (Qu et al. 2012;

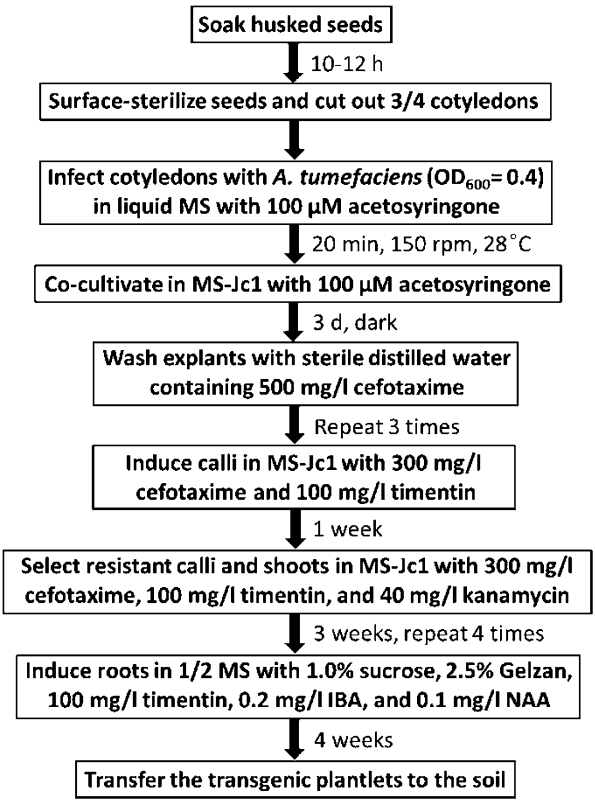

Fig. 5.2 Schematic representation of the protocol for the *Agrobacterium*-mediated transformation and regeneration of *Jatropha* using cotyledon explants. (Source: Fu et al. 2015)

Gu et al. 2014, 2015). In this technique, transgenic plants are transformed and regenerated using the conventional transformation process with a selectable marker; subsequently, the marker is removed from the host plant genome using a chemically inducible Cre-lox-mediated site-specific recombination system (Zuo et al. 2001; Guo et al. 2003). This improved technique lays the foundation for the broad planting of transgenic *Jatropha* in the field.

5.3 Progress in Transgenic *Jatropha*

In recent years, many studies on transgenic *Jatropha* have been reported by several laboratories. Transgenic *Jatropha* plants have been modified with respect to aspects such as plant architecture, flowering time, seed size, oil yield, fatty acid (FA) composition, toxic components, and biotic and abiotic stress tolerance (Table 5.2).

Table 5.2 Research on transgenic *Jatropha*

Promoter/trait genes	Promoter/ selectable marker gene	Phenotypes	References
SUC2:JcFT	*35S:NPTII*	Early flowering	Li et al. (2014)
G10-90:JcFT	*NOS:HPT*	Early flowering	Ye et al. (2014a)
35S:JcLFY	*35S:NPTII*	Moderately early flowering	Tang et al. (2016a)
35S:JcAP1	*35S:NPTII*	No effect on flowering time	Tang et al. (2016b)
35S:JcTFL1a, 35S: JcTFL1b 35S: JcTFL1c	*35S:NPTII*	Extremely late flowering	Li et al. (2017)
35S:JcTFL1b-RNAi	*35S:NPTII*	Moderately early flowering	Li et al. (2017)
35S:JcARF19	*35S:HPT*	Increase in seed size and seed yield	Sun et al. (2017)
JcUEP:JcGA2ox6	*35S:NPTII*	Dwarf phenotype with dark-green leaves and smaller reproductive organs	Hu et al. (2017)
35S:JcCYP735A-CRISPR/Cas9	*35S:NPTII*	Retardated shoot growth	Cai et al. (2018)
P_{Gm7S}*: JcFAD2-1-RNAi*	*NOS:HPT*	Increased oleic acid levels of seed oil	Qu et al. (2012)
P_{JcSDP1}*:JcSDP1-RNAi*	*NOS:HPT*	Enhanced accumulation of seed total lipid	Kim et al. (2014)
35S:AtDGAT1	*NOS:NPTII*	Dramatic increase in lipid content in leaves and seeds	Maravi et al. (2016)
$P_{JcCurcin1}$*:Curcin1*	*NOS:HPT*	Curcin-deficient in seeds	Gu et al. (2015)
P_{JcLEA1}*:dsR366*	*35S:HPT*	Reduction of phorbol ester content in seeds	Li et al. (2016)
P_{JcLEA1}*:dsSET12*			
35S:GSMT+35S: DMT	*NOS:NPTII*	Enhanced glycine betaine synthesis	Tsuchimoto et al. (2012)
35S:SbNHX1	*35S:HPT*	Enhanced salt tolerance	Joshi et al. (2013)
2X35S:ICMV-RNAi	*NOS:HPT*	Gemini viruses resistant	Ye et al. (2014b)
P_{ZmPepc}*:Cry1Ab/1Ac*	*NOS:HPT*	Strong insecticidal activity to *Archips micaceanus*	Gu et al. (2014)

5.3.1 Plant Architecture, Flowering Time, and Seed Development

Jatropha, a small tree, can reach a height of up to 5 m, which makes seed collection inconvenient. Hu et al. (2017) obtained dwarf transgenic *Jatropha* by transforming the plant with the *JcUEP:JcGA2ox6* vector, and the endogenous GA_4 content decreased. Transgenic *Jatropha* transformed with *JcUEP:JcGA2ox6* produced fewer inflorescences and flowers than those of the wild type. Using the CRISPR-Cas9 system, Cai et al. (2018) obtained transgenic *Jatropha* with the *JcCYP735A* gene knocked out; shoot growth in the *Jccyp735a* mutants was slower than in the wild type. Although *Jatropha* grows rapidly and generates abundant biomass in the growing season, excessive vegetative growth may suppress reproductive growth, resulting in an unstable flowering time and a reduction in inflorescences. FLOWERING LOCUS T (FT) plays a crucial role in the transition from vegetative to reproductive growth (Liu et al. 2013). Two laboratories have reported the function of the *Jatropha FT* ortholog (*JcFT*). The overexpression of *JcFT*, driven by the *SUC2* and *G10-90* promoters, respectively, leads to early flowering and modifies the architecture to a semi-dwarf stature of *Jatropha* (Li et al. 2014; Ye et al. 2014a). The early flowering character can therefore be used to accelerate the genetic modification of key agronomic traits. Li et al. (2017) also reported the function of the *Jatropha TFL1* (*JcTFL1*) orthologs, and all transgenic *Jatropha* that overexpressed *JcTFL1a*, *JcTFL1b*, or *JcTFL1c* showed late flowering. However, transgenic *JcTFL1b-RNAi Jatropha* consistently exhibited a moderately early flowering phenotype. JcFT and JcTFL1 may be key systemic signals regulating growth and flowering time in *Jatropha.* In addition, the overexpression of the *Jatropha LEAFY* ortholog (*JcLFY*) can cause moderately early flowering (Tang et al. 2016a). Overexpressing the *Jatropha* APETALA1 ortholog (*JcAP1*) cannot affect flowering time in *Jatropha*; however, the ectopic expression of *JcAP1* can cause early flowering in *Arabidopsis* (Tang et al. 2016b). The molecular mechanisms that control flowering may differ between herbaceous and woody plants. Sun et al. (2017) found that the overexpression of *Jatropha Auxin Response Factor 19* (*JcARF19*) significantly increased seed size and yield in transgenic *Arabidopsis* and *Jatropha*, indicating the importance of the auxin pathway in controlling seed yield in dicotyledonous plants.

5.3.2 Modifying Oil Yield, FA Composition, and Toxin Biosynthesis

The oil content of *Jatropha* seeds is an important economic trait. The oil content can be increased by manipulating the expression levels of key enzymes in the

triacylglycerol (TAG) and FA biosynthetic pathways. The silencing of *sugar-dependent 1* (*JcSDP1*), which encodes a patatin domain TAG lipase, enhances seed oil accumulation in transgenic *Jatropha* (Kim et al. 2014). Recently, the ectopic expression of *AtDGAT1*, which encodes diacylglycerol O-acyltransferase, an enzyme exclusively committed to TAG biosynthesis, was shown to enhance oil accumulation in the seeds and leaves of *Jatropha* (Maravi et al. 2016). In addition, the FA composition and content affect oil quality. A high content of monounsaturated FAs (oleate) and a low content of linoleic acid increase the quality for biodiesel production (Maghuly and Laimer 2017). Using marker-free transgenic technology together with seed-specific RNA interference (RNAi) technology to suppress the expression of 1-acyl-2-oleoyl-sn-glycero-3-phosphocholine delta 12-desaturase (*JcFAD2-1*), Qu et al. (2012) developed transgenic *Jatropha* whose oil had a variation in FA composition; it was modified from 37% oleic acid and 41% polyunsaturated FAs to more than 78% oleic acid and less than 3% polyunsaturated FAs.

Curcin and phorbol esters are the major toxic compounds present in *Jatropha.* The reduction or loss of toxicity in the seeds can allow the use of *Jatropha* in pressed cakes for animal feed. To develop non-toxic *Jatropha* plants, RNAi technology was used to silence the curcin precursor gene in transgenic *Jatropha* (Patade et al. 2014). Further, transgenic *Jatropha* plants have been developed to produce curcin-deficient seeds through endosperm-specific RNAi-mediated gene silencing (Gu et al. 2015). *Jatropha* seeds containing a low amount of phorbol esters have also been generated by disrupting casbene biosynthesis (Li et al. 2016). The improvement of these important traits sets the stage for the development of a commercial variety of *Jatropha*.

5.3.3 Biotic and Abiotic Stress Tolerance

As cultivated areas expand, diseases and pests in *Jatropha* have become prominent problems. To improve tolerance to viruses, Ye et al. (2014b) used RNAi with a hairpin dsRNA that targeted five genes of the DNA-A genome of a geminivirus to produce virus-resistant transgenic *Jatropha*. With 94% nucleotide identity, the transgenic plant showed broad resistance to geminiviruses (Ye et al. 2014b). Gu et al. (2014) developed transgenic *Jatropha* with high insecticidal properties via the expression of the Bt-endotoxin protein Cry1Ab/1Ac.

Although *Jatropha* can be cultivated in marginal lands, thus avoiding competition with food crops for agricultural land, its tolerance to abiotic stress is limited (Cartagena 2017), and its yield is very low under these conditions. Thus, Tsuchimoto et al. (2012) overexpressed genes such as *PAT*, *NF-YB*, *GSMT*, and *DMT* to develop three types of transgenic *Jatropha* plants with improved drought tolerance characters. The glycine content is significantly increased in transgenic *Jatropha* that overexpress *GSMT* and *DMT*. In addition, the *sbNHX1* gene, which encodes an active vacuolar Na^+/H^+ antiporter isolated from the extreme halophyte *Salicornia brachiate*, has been overexpressed in *Jatropha*, resulting in transgenic plants with an

enhanced tolerance to 200 mM NaCl (Joshi et al. 2013). These desirable traits can be used to cultivate crop varieties of *Jatropha*.

5.4 Conclusions

Jatropha has a small genome, a short reproductive cycle, complete genomic information, and effective transformation systems, which are favorable features for its becoming a model woody plant. Although substantial research has been conducted on *Jatropha*, much work is still required for *Jatropha* to become a real biodiesel feedstock. The current priority is to cultivate varieties with improved seed and oil yields through the improvement of agronomic traits such as branching pattern, total flower number, the ratio of female to male flowers, and stress tolerance. We should focus on selecting candidate genes involved in regulating these agronomic traits and then modifying the expression of these genes in *Jatropha* via genetic transformation to produce varieties with the desired traits. Additionally, given the genomic information and efficient genetic transformation systems of *Jatropha*, CRISPR/Cas9 and a large-scale T-DNA insertional method will be prospective developmental tools that can be used to develop a population of *Jatropha* mutants.

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