Biotechnological Means for Genetic Improvement in Castor Bean as a Crop of the Future

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Mulpuri Sujatha and Muddanuru Tarakeswari

Abstract

Profitable cultivation of castor bean is beset with problems of vulnerability of cultivars and hybrids to a multitude of insect pests and diseases. The presence of the toxic proteins ricin and hyperallergenic *Ricinus communis* agglutinin (RCA) in the endosperm restricts the use of deoiled seed cake as cattle feed. Due to this crop's low genetic diversity, genetic engineering can be an efficient approach to introduce resistance to biotic and abiotic stresses as well as seed quality traits. Recently, castor oil gained attention as a sustainable second-generation feedstock for biojet fuel that would reduce carbon dioxide emissions. Because of a growing interest in castor oil as a biofuel and the presence of the powerful toxin ricin in its seed, metabolic pathways and regulatory genes involved in both oil and ricin production have been analyzed and characterized. Genetic engineering of castor bean offers new possibilities to increase oil yield and oxidative stability, confers stress tolerance, and improves other agronomics traits, such as reduced plant height to facilitate mechanical harvesting. However, difficulties in tissue culture-based regeneration and poor reproducibility of results are major bottlenecks for genetic transformation of castor bean. Despite advances in tissue culture research over the past four decades, direct or callus-mediated adventitious shoot regeneration systems that are genotype-independent remain a much sought-after goal in castor bean. Genetic transformation attempts to develop insect-resistant and ricin-free transgenic castor bean lines have been based on shoot proliferation from meristematic tissues. This chapter describes new transformation methods under development and the progress achieved so far in genetic engineering of castor bean for agronomically desirable attributes.

14.1 Introduction

Castor bean (*Ricinus communis*) is a hardy crop easily adaptable for tropical, subtropical, and temperate regions. However, it is reported to be vulnerable to a wide array of pests and diseases that affect vegetative and reproductive tissues during the entire crop cycle. In addition, it is susceptible to drought, requiring at least 600–700 mm of rainfall per year, and it is sensitive to salinity, poorly drained soils, and frost, particularly during early growth stages. Genetic

M. Sujatha (✉) · M. Tarakeswari
ICAR-Indian Institute of Oilseeds Research, Rajendranagar, Hyderabad 500030, Telangana, India
e-mail: mulpuri.sujata@icar.gov.in

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improvement of castor bean has been mostly limited to the exploitation of natural genetic variability and focused on the selection for high yield, desirable branching types, sex morphotypes, nonshattering capsules, disease resistance (e.g., Fusarial wilt), and high seed oil content.

Extensive cultivation under high inputs and without proper scientific management and crop rotation has made castor bean vulnerable to a number of biotic and abiotic stresses. About 100 species of castor bean insect pests have been recorded at different phenological stages of the crop. Among those pests, castor semilooper (*Achaea janata*), capsule borer (*Conogethes punctiferalis*), tobacco caterpillar (*Spodoptera litura*), red hairy caterpillar (*Amsacta albistriga*), jassids (*Empoasca flavescens*), and white fly (*Trialeurodes ricini*) cause considerable damage (Lakshminarayana and Raoof 2005). Seed yield losses are estimated to be in the range of 35–50%, depending on the crop growth stage and the pest type. Severe outbreaks of castor semilooper and red hairy caterpillar during early crop growth that resulted in complete crop loss have been reported. Castor bean can tolerate up to 25% leaf damage without affecting seed yield, while damage caused to spikes and capsules often leads to significant yield losses. Diseases, such as wilt (*Fusarium oxysporum* spp. *ricini*), root rot (*Macrophomina phaseolina*), bacterial blight (*Xanthomonas ricini*), botrytis gray rot (*Botrytis ricini*), and seedling blight (*Phytophthora colocasiae*), are considered important (Kolte 1995; Lakshminarayana and Raoof 2005).

New sources of abiotic and biotic stress tolerance are in constant demand by breeders. *Ricinus* is considered to be a monotypic genus, and *R. communis* is the lone species encompassing the many polymorphic types known around the world (Weiss 1983). Several of these types were designated as species (*R. communis, R. macrocarpus, R. microcarpus*), but they are intercrossable and fertile and therefore not true species. All castor varieties that have been investigated cytologically are diploids with a $2n$ number of 20 chromosomes (Richharia 1937), and the species is a secondary balanced polyploid with a basic number of $x = 5$ (Singh 1976).

Successful breeding for yield stability is thus limited by a lack of exploitable genetic variability of productivity and disease and pest resistance traits among castor bean germplasm. Breeders have to resort to alternative approaches like mutations, wide (intergeneric) hybridization, and biotechnology for the creation of genetic variability and incorporation of desired traits into castor. The effect of irradiation on castor bean seeds and seedlings has been the subject of a number of studies that aimed at identifying mutants with specifically required characters. The importance of induced mutations in castor has been demonstrated in the development of productive semidwarf lines with high yield potential, early maturing mutants, and sex expression variants (Kulkarni and Ankineedu 1966; Ankineedu et al. 1968). However, mutants that are resistant or tolerant to biotic stresses or mutants that have reduced seed toxicity have not been obtained by radiation mutagenesis so far.

In several crops, wild relatives have often been used in breeding programs as they constitute important reservoirs of genetic variability (Harlan 1976). Morphologically, the genus *Jatropha* ($2n = 22$) resembles *Ricinus* and has attracted interest because it possesses useful traits not found in castor. Although both genera, as well as *Manihot* (cassava), belong to the Euphorbiaceae family, successful introgression of some of the desirable genes of *Jatropha* and *Manihot* into castor bean remain virtually untapped, because of the incompatibility of intergeneric crosses between these genera (Reddy et al. 1987a; Sujatha 1996; Gedil et al. 2009).

In addition to castor bean's susceptibility to insect pests and the presence of toxic proteins in the endosperm, its perennial and indeterminate growth habit are further challenges for breeding early maturing varieties that are amenable for mechanical harvesting.

Castor bean produces various industrially useful fatty acids, and renewed interest in this crop is due to its potential to produce biofuels and epoxy oil through genetic engineering. Because epoxy oil and castor oil have very similar chemical structures, only minor genetic

modifications would be necessary to synthesize epoxy oil in castor bean. Genes involved in triacyl glycerol assembly and fatty acid metabolism, such as stearoyl-acyl carrier protein desaturases (*SAD2, SAD3, SAD4*), diacylglycerol acyl transferases (*DGAT, DGAT2*), enolases, lipid transfer proteins, fatty acid desaturases, hexose transporters, 6-acyl-Sn-glycerol-3-phos phate acyltransferases (*LPAAT2, LPAAT4, LPAAT5*), oleate desaturases (*FAD2*), 3-keto-acyl-ACP synthases (*KASI, KASII, KAS III*), oleosins (*Ole1* and *Ole2*), and lipid transfer proteins (Weig and Komor 1992), can increase oil content and modify the plant's fatty acid profile. Genes coding for the highly toxic protein ricin is also important for the development of cultivars with reduced toxicity. Such genes could be used to generate improved cultivars by genetic engineering. Furthermore, castor bean is an excellent model system for seed development due to its large-sized seed with high lipid and protein content (Kermode et al. 1989), as well as to study sucrose and amino acid uptake mechanisms (Komor 1977; Robinson and Beevers 1981) and nutrient transport through phloem (Orlich and Komor 1992) and xylem (Schobert and Komor 1992).

With the availability of a draft sequence of the castor bean genome, most of the genes underlying oil metabolism, ricin, agglutinin, and disease resistance have been identified (Chan et al. 2010). Malik (2013) suggested the possibility of combining genes for heat tolerance, drought tolerance, and resistance to insect pests and diseases to improve tolerance and high oil yield in castor. Also, new genome editing tools could be applied to eliminate ricin from seed meal and make epoxy oil in castor bean.

One popular strategy to mitigate insect pest susceptibility is the introduction of suitable resistance genes through transformation (Sharma et al. 2000). The 110-fold hectare increase of cultivation of transgenic crops in two decades between 1996 and 2016 makes genetic engineering the fastest adopted crop technology in agriculture (James 2016). Of the 185 million ha of cultivated biotech crops during 2016 globally, 12.5% were occupied by insect-resistant crops, and 41% were crops with stacked double and triple traits.

During the past three decades, intensive studies on plant regeneration and transformation have led to the production of transgenic plants in many crop plants. The introduction of foreign genes by genetic engineering techniques often requires an efficient in vitro regeneration system for the desired plant species. Such a system must be rapid, reliable, and applicable to a broad range of genotypes. However, facile techniques for tissue culture, regeneration, and gene transfer have not been established for castor bean, despite decades of research. The protocols for stable transformation that are in place for castor bean utilize meristematic tissues, which could lead to the recovery of chimaeras, but there is potential for genetic improvement of this species through in vitro techniques and biotechnological tools.

14.2 Castor Bean Tissue Culture

The major hindrance for genetic transformation of several crop plants is the availability of a reproducible system for plant regeneration. In many cases, transformation methods target young apical meristems or other tissues that ultimately give rise to gametes (Birch 1997). In castor bean, the development of an efficient and highly reproducible system for tissue culture and regeneration remains a major challenge for genetic transformation experiments. Castor bean proved to be highly recalcitrant to in vitro manipulations, which is a major impediment for the development of transgenic lines (Reviewed by Sujatha et al. 2008). Early attempts at establishing castor bean tissue culture were carried out during 1960s using endosperm tissue mostly because its large endospermic seeds facilitated in vitro culture. However, these experiments resulted in continuously growing tissue that did not undergo organogenic differentiation. Tissue culture studies of castor bean undertaken during 1980s aimed at obtaining whole plantlet regeneration from seedling tissue. These experiments were conducted on young seedlings, but regeneration of complete plants was rather limited.

Plant regeneration was mainly derived from pre-existing meristematic centers (Athma and Reddy 1983; Sangduen et al. 1987; Reddy and Bahadur 1989; Molina and Schobert 1995; Alam et al. 2010; Li et al. 2015a), and a maximum of 40 and 47 shoots from embryo axes and shoot tip explants, respectively, was reported (Sujatha and Reddy 1998). Callus-mediated shoot regeneration from hypocotyl explants, young stem segments, leaves, and cotyledonary leaves have been reported, but morphogenic differentiation was sporadic, unreproducible, and with very low frequency of shoot regeneration, showing only 1–5 shoots per responding explant (Reddy et al. 1987b; Genyu 1988; Bahadur et al. 1992; Sarvesh et al. 1992). Subsequently, Ahn et al. (2007), Sujatha and Reddy (2007), and Ganesh Kumari et al. (2008) reported relatively higher shoot induction frequencies from seedling explants, obtaining 22–24 shoots per explant. They used growth adjuvants and amino acids to improve caulogenic ability. Studies by Zhang et al. (2016) suggested that a 6-day dark treatment significantly increased the average number of shoots (37) per explant when cultured on medium supplemented with 0.3 mg/L thidiazuron (TDZ). Li et al. (2015b) compared WUSCHEL (*WUS*) gene expression in castor bean tissues under different inducing conditions and optimized a regeneration system based on embryogenic cell induction. These studies showed a positive correlation between *WUS* gene expression and embryogenic cell induction, which had direct influence on the rate of shoot budding. Nevertheless, the reproducibility of these methods across laboratories and different genotypes has to be ascertained as very few genotypes were used in most of these studies.

Meristematic explants were tested in castor bean, and embryo axes showed high proliferative ability as compared to shoot apices and nodal explants (Sujatha and Reddy 1998). Shoot proliferation rates reported in this investigation were very high and were 40 and 81.7 from embryo axes and 46.7 and 22.0 shoots per explant from shoot tips on induction and subculture media, respectively. The study demonstrated a carryover effect of TDZ in embryo axes from mature seeds for 2–3 subculture cycles. TDZ-habituated embryo axes tended to produce shoots continuously, reaching up to 81.7 shoots per explant when cultures were transferred from medium with 5.0 mg/L TDZ to medium supplemented with 0.5 mg/L benzyladenine (BA). The elevated proliferation rate of zygotic embryos on medium supplemented with TDZ paved the way for genetic transformation studies in castor bean (Sujatha and Sailaja 2005; Malathi et al. 2006).

Genotypic differences were not significant in shoot proliferation experiments on medium supplemented with TDZ. Studies of Ahn et al. (2007) were in agreement with those of Sujatha and Reddy (1998), which showed a threefold increase in cytokinin activity of TDZ relative to BA for promoting shoot regeneration from mature zygotic embryos. Pretreatment of embryo axes in the dark for 7 days increased the number of shoots regenerated per explant by 82 and 36% with TDZ and BA, respectively (Ahn et al. 2007). Likewise, preincubation of cotyledon explants from mature seeds cultured on medium with 5 μM TDZ in the dark for 7 days resulted in a maximum of 25 shoots per explant (Ahn and Chen 2008). In both studies, histological analysis indicated adventitious origin of the shoots from the meristematic region, including the shoot apex and the cotyledonary leaf axil. TDZ showed a beneficial effect on adventitious shoot regeneration as well. Ahn et al. (2007) reported a high frequency of shoot regeneration from hypocotyls on medium supplemented with 0.25 mg/L TDZ and 4.5 mg/L BA. Zalavadiya et al. (2014) reported shoot regeneration from hypocotyl-derived callus on medium supplemented with 0.5 mg/L kinetin and 0.25 mg/L BA, which was reproducible across genotypes and in different laboratories. However, this protocol suffers from lack of rooting of the regenerated shoots even after 30 days in culture.

Tissue culture research conducted so far in castor bean has revealed a strong recalcitrance to in vitro culture and identified the development of an adventitious shoot regeneration system as a major challenge. The observed sporadic shoot regeneration indicates the presence of a few morphogenic cells interspersed within

nonmorphogenic tissues. Although morphogenically competent cells are more likely to be found in juvenile than in mature tissues of flowering plants, suppression of competent cells by overgrowth of noncompetent cells may have been the reason for the failure to isolate a competent line in castor bean. The occasional appearance of shoots could also be due to the activation of recalcitrant calli to undergo caulogenesis caused by rare exogenous and endogenous inductive stimuli. Hence, a large number of genotypes and growth regulator combinations need to be assessed for determining caulogenic ability and to understand recalcitrance in castor bean tissues in vitro. Until an efficient and reproducible system for plant regeneration is developed, genetic transformation of castor will be a meristem-based shoot proliferation system.

14.3 Genetic Transformation Methods for Castor Bean

Castor bean has been reported to be susceptible to crown gall disease caused by *Agrobacterium tumefaciens* (Lippincott and Haberlein 1965), which is broadly used for plant transformation due to its capacity to introduce DNA into plant cells. Genetic transformation methods tried for castor include *Agrobacterium*-mediated transformation and particle gun bombardment methods with each of these methods having its own advantages and disadvantages.

14.3.1 *Agrobacterium*-Mediated Method

Owing to the poor caulogenic ability of in vitro cultured tissues in castor, transformation was carried out with tissues with pre-existing meristems like decotyledonated embryo axes or using alternate methods bypassing tissue culture-based regeneration such as floral bud transformation or *in planta* transformation.

Agrobacterium-mediated transformation is one of the most efficient methods for gene transfer and takes advantage of the naturally evolved crown gall-inducing mechanisms of DNA present in *A. tumefaciens*—a ubiquitous gram-negative soil pathogen. The uniqueness of this soil microorganism lies in its capability of transfer of DNA to eukaryotic cells. Although it is genotype-dependent, it is the most preferred method of gene transfer owing to its simplicity, cost-effectiveness, and generation of stable transformants with a single or few copies of the inserted DNA fragment, which can be relatively large and with defined ends (Gelvin 2003).

The *Agrobacterium*-mediated transformation process involves a number of steps which include: (a) isolation of the genes of interest from the source organism; (b) development of a functional transgenic construct harboring the gene of interest along with promoters to drive expression and marker genes (selectable such as antibiotic or herbicide markers or reporter genes like *GUS* or *GFP*) to facilitate tracking of the introduced genes in the host plant; (c) insertion of the transgene into the Ti plasmid; (d) introduction of the T-DNA-containing plasmid into *Agrobacterium*; (e) cocultivation of plant cells with transformed *Agrobacterium* to allow transfer of T-DNA into plant chromosome; (f) regeneration of the transformed cells into putative transformants; (g) confirmation of integration and expression of the introduced transgene through molecular analysis; and (h) assessing the trait performance through appropriate assays at lab, greenhouse, and field level.

In planta transformation methods like floral dip method that have been developed for *Arabidopsis* are incredibly simple and extended to transformation of several other crops. In floral bud transformation method of castor, the flower buds of an intact plant are wounded and an *Agrobacterium* strain carrying the transgene is infiltrated into the wound (US Patent No 6.620.986). The flower buds are allowed to set seed, and those seeds that harbor the transgene (which typically includes a selectable marker) are grown under the selective medium. However, in this method, transformation of the germline (so as to enable the progeny carry the transgene) is extremely difficult because the floral initials are

usually at different stages of differentiation along the racemes. Another method is to prick the shoot apical meristems of 10–15-day-old seedlings with fine needles followed by immersion in *Agrobacterium* suspension and transfer to soilrite in bottles for a week (Kumar et al. 2011). Subsequently, the infected seedlings were transferred to pots and grown to maturity in the greenhouse. Transformed lines will be selected in the next generation through antibiotic/herbicide screening. The advantage of *in planta* method lies not only in the simplicity and reliability but also in obtaining genetically uniform (nonchimeric) transformed progeny as somaclonal variation associated with tissue culture and regeneration is minimized. Although the method is simple, it requires extensive analysis of the T_1 plants for identification of plants carrying the transgene.

14.3.2 Particle Gun Bombardment

The development of particle gene gun has revolutionized DNA transfer technology, bypassing limitations imposed by *Agrobacterium* host specificity and cell culture constraints, and has allowed the engineering of almost all the major crops including formerly recalcitrant cereals, legumes, woody species, and other organisms. It is a physical method in which rapidly propelled tungsten or gold particles coated with DNA are blasted to deliver biologically active DNA into plant cells. Direct gene transfer through particle gun bombardment is generally genotype-independent, facilitating multiple gene cotransformation, but the success depends on several physical parameters and is often reported to result in transgenics with multiple copies of the transgene.

Regardless of the method employed, most transformation studies in castor bean have targeted meristematic tissues, which are prone to result in chimeras. Because of its highly proliferative ability on medium supplemented with a strong cytokinin like TDZ or a combination of TDZ and BA, castor bean embryo axes have been the most used tissue for transformation, regardless of the method. Success depends on the precise targeting of the introduced DNA to the wounded meristematic tissues and the conditions (*Agrobacterium* culture density, period of cocultivation, use of *vir* gene inducers like acetosyringone and other phenolic compounds) for enhancing the virulence (the transfer of T-DNA from *Agrobacterium* to its host).

McKeon and Chen (2003) obtained 12 genetically engineered castor bean plants through vacuum infiltration of wounded flower buds in *Agrobacterium* suspension (US Patent No 6.620.986). Sujatha and Sailaja (2005) described the first successful attempt at development of a stable transformation system for castor bean using decotyledonated embryo axes. In this method, embryo axes following cocultivation with *Agrobacterium* were subjected to expansion and proliferation on Murashige and Skoog (1962) (MS) medium supplemented with 0.5 mg/L TDZ followed by three cycles of selection on medium with 0.5 mg/L BA and increasing concentrations of hygromycin (20–40–60 mg/L) or kanamycin (50–100–200 mg/L) depending on the selectable marker (*hpt, npt* II). Selected shoot clusters were transferred to medium with 0.5 mg/L BA for proliferation and 0.2 mg/L BA for shoot elongation. Elongated shoots were rooted on half-strength MS medium supplemented with 2.0 mg/L NAA. By employing this protocol, a primary transformant was obtained within 5 months from cultured embryo axes with an overall transformation efficiency of 0.08%. As the protocol does not involve an intervening callus phase, no abnormal phenotypes were reported through this procedure. A similar shoot proliferation method with minor modifications was followed for direct gene transfer using the particle gun bombardment method achieving a transformation efficiency of 1.4% (Sailaja et al. 2008). Transformation frequencies of castor bean reported to date are generally low (Table 14.1).

Table 14.1 Genetic transformation studies in castor bean

Transformation method	Gene inserted	Explant	Trait	Transformation frequency (%)	References
Agrobacterium-mediated	*Gus*	Wounded flower buds	Transformation protocol	–	McKeon and Chen (2003)
Agrobacterium-mediated	*Gus, hpt, npt*	Embryo axis	Transformation protocol	0.08	Sujatha and Sailaja (2005)
Agrobacterium-mediated	*cry1Ab*	Embryo axis	Insect resistance (semilooper)	0.42	Malathi et al. (2006)
Particle gun method	*Gus, hpt, npt*	Embryo axis	Transformation protocol	1.4	Sailaja et al. (2008)
Agrobacterium-mediated and particle gun methods	*cry1EC*	Embryo axis	Insect resistance (semilooper and Spodoptera)	0.82 and 0.69, respectively	Sujatha et al. (2009)
Agrobacterium-mediated	*Chitinase – Chi 1*	Cotyledonary node	Fusarium wilt	–	Ganesh Kumari (2010)
Agrobacterium-mediated *in planta* methods	*cry1ACF*	Two-days old seedlings	Insect resistance (Spodoptera)	1.4	Kumar et al. (2011)
Agrobacterium-mediated	*Ricin toxin A chain*	Cotyledonary node	Reduced seed toxicity	–	Chen et al. (2013)
Agrobacterium-mediated	*Ricin toxin A chain*	Epicotyl	Reduced seed toxicity	–	Li et al. (2014)
Agrobacterium-mediated	*SbNHX I*	Embryo axis	Salt stress	2.8–5.9	Patel et al. (2015)
Agrobacterium-mediated	*TFL1*	–	Determinate and early maturity	–	Peles et al. (2017)
Agrobacterium-mediated	*Ricin toxin A chain*	Embryonic axis	Reduced seed toxicity	0.85	Sousa et al. (2017)

14.4 Genetic Transformation of Castor Bean with Insect Resistance Genes

Before embarking on a genetic engineering program to address a pest problem, it is imperative to identify suitable insect resistance genes, which could be introduced into castor bean against major pests. Several candidate genes, such as crystal protein (*Cry*) genes of *Bacillus thuringiensis* (*Bt*) produced during the sporulation stage, vegetative insecticidal *Bt* proteins (VIPs) induced during the vegetative stage, proteinase inhibitors, lectins, α-amylase inhibitors, insect chitinases, and novel genes of plant origin, can be deployed into crop plants for imparting protection against insect pests. However, the most commonly used and commercially exploited insect resistance genes are the *Bt Cry* genes. Insecticidal δ-endotoxins of *B. thuringiensis* have acquired great significance because of their specificity to target pests, nontoxicity to humans and beneficial insects, toxicity at low concentration, and environment-friendly nature. *B. thuringiensis* var. *kurstaki* strains produce several lepidopteran toxic proteins such as Cry1Aa, Cry1Ab, Cry1Ac, Cry1IA, and Cry1B. Information on the reaction of the major lepidopteran pests attacking castor bean to Cry

proteins in the toxin specificity database (http://www.glfc.forestry.ca/bacillus/web98.adb) is limited. Experiments were undertaken at the Indian Institute of Oilseeds Research to assess the efficacy of various purified crystal *Bt* proteins which are lepidopteran-specific against major defoliators of castor bean (Lakshminarayana and Sujatha 2005; Sujatha and Lakshminarayana 2005).

Bioassays against neonate larvae of *Achoea janata* (castor semilooper), *S. litura*, *Spilosoma obliqua*, and *Euproctis fraterna* using Cry toxins (Cry1Aa, 3A, 2B, 1C, 2A, 1E, 1Ac, 1F, 9A, 1Ab) at concentrations ranging from 4 to 1500 ng/cm^2 were done using leaf paint assays. With regard to semilooper, the Cry proteins 1Aa, 1Ab, 1E, and 2A were found to be the most effective, resulting in 100% mortality within 48 h, while the other proteins gave nil or delayed mortality at the highest concentrations tested. Among the effective proteins, Cry1Aa was found to be superior to other proteins in giving early mortality even at lower concentrations (125 ng/cm^2) (Sujatha and Lakshminarayana 2005). In the case of Spodoptera, none of the proteins gave 100% mortality even after 96 h of treatment at the highest concentration tested (1500 ng/cm^2), except for Cry1Aa, which gave 50% mortality at 1500 ng/cm^2. Increasing the concentration of the proteins up to 3000 ng/cm^2 also failed to cause larval mortality. However, feeding cessation in terms of low larval weight was recorded in treatments with Cry1Aa and Cry 1Ab (Lakshminarayana and Sujatha 2005). The Cry proteins 1Aa, 1E, 1Ab were found to be effective against *S. obliqua*, while 1Ac, 1Aa were effective against *E. fraterna.*

Due to Cry1Aa protein's efficacy against all tested pests, genetic transformation of castor bean has been initiated using the *cry1Aa* gene modified for plant codon usage. *S. litura*, which is a polyphagous lepidopteran insect that affects castor bean, is tolerant to most of the known δ-endotoxin proteins. Hence, a hybrid Cry1Ea/Cry1Ca δ-endotoxin protein called Cry1EC was developed by replacing amino acid residues 530–587 in the low-activity Cry1Ea protein with a highly homologous 70-amino acid region of Cry1Ca in domain III (Singh et al. 2004). The soluble Cry1EC protein produced with an expression vector was fourfold more toxic to the larvae than Cry1Ca, the most effective δ-endotoxin against *Spodoptera* sp. This Cry1EC hybrid endotoxin conferred complete protection against *S. litura* when deployed in tobacco and cotton. Based on these studies, the genes *cry1Aa* and *cry1Ab* have been selected to confer protection against major foliage feeders of castor bean, and transgenic castor bean lines harboring the chimeric genes *cry1EC* (Sujatha et al. 2009) and *cry1AbcF* (Kumar et al. 2011), as well as the synthetic δ-endotoxin gene *cry1Ab* (Malathi et al. 2006), have already been generated.

The vector-mediated and direct gene transfer methods (Sujatha and Sailaja 2005; Sailaja et al. 2008) were employed for transformation of castor bean cv. DCS-9 using appropriate vectors containing the *cry1Aa* (Sujatha M, unpublished) and the *Bt* fusion gene *cry1EC*, driven by an enhanced 35S promoter (Sujatha et al. 2009). With the *cry1EC* gene, 81 and 12 putative transformants were obtained following selection on hygromycin and kanamycin, respectively. The integration and inheritance of the introduced genes were demonstrated up to T_4 generation by PCR and Southern blot analysis. Field bioassays against *S. litura* and castor semilooper, conducted for eight transformation events in T_1–T_4 generations under net confinement, lead to identification of promising events conferring resistance to the two major defoliators (Fig. 14.1).

A similar procedure was used for the production of semilooper-resistant transgenic castor bean that expresses a synthetic δ-endotoxin *cry1Ab* gene driven by CaMV (cauliflower mosaic virus) 35S promoter (Malathi et al. 2006). The construct harboring the insect resistance gene carried the herbicide resistance gene (*bar*) for selection of putative transformants. The presence of the introduced gene, its stable integration, expression, and inheritance was confirmed through PCR, Southern blot analysis, ELISA, and progeny tests. The transformed plants showed a Cry1Ab protein concentration between 0.23 and

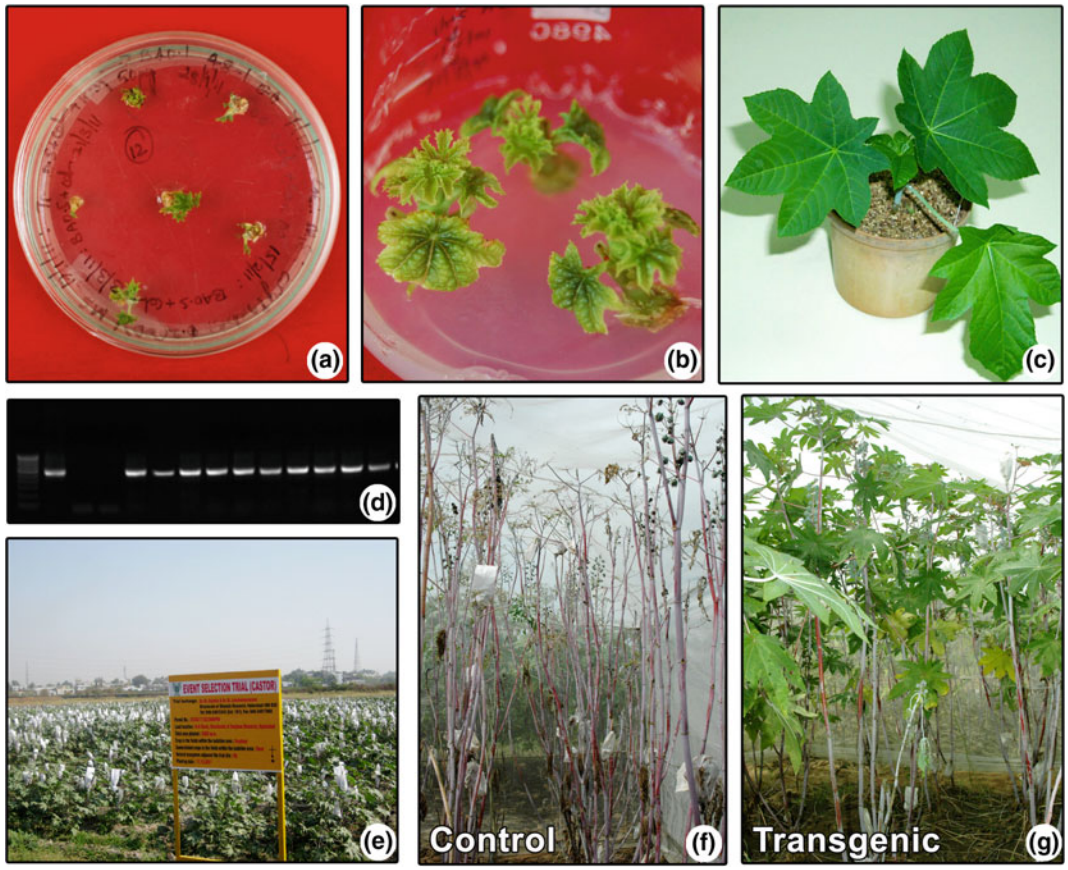

Fig. 14.1 Genetic transformation in castor for insect resistance. **a** Selection of putative transformants on kanamycin; **b** elongation of putative transformed shoots; **c** acclimatization of transformed shoots; **d** identification of homozygous line through PCR analysis; **e** event selection trial for generation advancement and insect bioassays, **f** reaction of control (untransformed) plants showing complete defoliation; **g** reaction of the transgenic lines showing less foliar damage compared to control plants

0.47 ng/mg in the leaf tissue. Biological assays in the laboratory with neonate larvae of semilooper showed a marked feeding inhibition associated with reduced larval growth and substantial mortality (88.9–97.3%) on different primary transformants as compared to the untransformed control plants (13.9%). This and the above study (Sujatha et al. 2009) used the castor bean genotype DCS-9 (Jyoti), which is a cultivated commercial variety and a parental line for the hybrid DCH-177, and therefore, it will not be necessary to transfer the transgene between experimental and commercial varieties.

Kumar et al. (2011) used the *in planta* transformation procedure through *Agrobacterium*-mediated transfer for introduction of the *cry1AbcF* gene against Spodoptera. In this method, two-day-old seedlings were infected with *Agrobacterium* strain EHA105/pBin*Bt*8 harboring the *cry1AcF* gene and transformed rooted shoots were established in the greenhouse. Subsequently, T_1 generation seedlings were screened on 300 mg/L kanamycin to select putative transformants and five highly expressing transgenic lines were identified through molecular and expression analyses. These transgenic lines showed high mortality of semilooper and the stability of the transgene up to T_2 generation was successfully demonstrated.

The studies dealing with evaluation of transgenics for conferring protection against the two major castor bean foliage feeders were mostly confined to laboratory bioassays except for the study dealing with *cry1EC* transgenics (Sujatha et al. 2009) where whole plant assays were conducted. In the case of *cry1Ab* transformants, T_0 plants were bioassayed against semilooper (Malathi et al. 2006), while T_1 and T_2 transformants harboring the *cry1AbcF* gene were assessed for their reaction to Spodoptera in detached leaf assays in the laboratory (Kumar et al. 2011). The transformants harboring the *cry1EC* gene were tested for their reaction to castor bean semilooper and Spodoptera under laboratory conditions using detached leaves and at whole plant level under net-contained field conditions (Sujatha et al. 2009). Insect bioassays coupled with ELISA studies revealed variation in the plants' pest response and in protein expression levels, depending on transformant generation, crop stage, and growing conditions.

The identification of transformation events that result in plants bestowed with resistance to foliage feeders is just a beginning of generating pest-resistant castor bean lines. It is then necessary to determine toxin expression levels in different transformants and developmental stages. It is also essential to assess the toxicity of the candidate genes to beneficial insects. Eri silkworm (*Samia cynthia ricini*) larvae are reared on castor leaves for silk production. Kumar et al. (2016) observed high toxicity of purified Cry1Aa and Cry1Ab crystal proteins of *B. thuringiensis* against *eri* silkworm.

Traditional capsule borer, *Conogethes (Dichocrosis) punctiferalis* which used to be at low key, has increased in India causing up to 50% capsule damage in castor (Duraimurugan et al. 2015). The management of defoliators like semilooper and *Spodoptera* is relatively easy as the plant has the ability to tolerate a certain degree of defoliation, and these pests have potential natural enemies and are susceptible to a wide range of insecticides. Contrary to this, the management of capsule borer is rather difficult as the pest attacks the inflorescence and growing capsules, which directly translates into yield reduction. Capsule borer is less susceptible to chemical insecticides and hence is difficult to manage. Suitable management practices other than chemical control are not available. For capsule borer, the major challenge lies in optimizing a rearing technique prior to testing the effective agents and identification of candidate gene(s) for introduction through genetic engineering tools.

14.5 Transgenic Castor Bean for Abiotic Stress Tolerance

The first generation of genetically modified crops that have been cultivated since the 1990s in a number of countries harbor single genes that confer tolerance to herbicides or insect attacks (www.isaaa.org). Second-generation transgenic crops with stacked genes or traits that allow crops to tolerate environmental stresses such as drought, cold, salt, heat, or flood are now being tested in contained field trials around the world. In the case of castor bean, transgenic development is still in its infancy and there are no commercially released lines as of today. With regard to abiotic stresses, an Israeli biotechnology company—Rahan Meristem (http://www.rahan.co.il)—is developing castor bean transformation protocols to generate salinity and drought resistance using genes from unicellular algae, which are expected to improve the performance of castor bean in low rainfall climates.

In order to enhance salt stress in castor, Patel et al. (2015) introduced the vacuolar Na^+/H^+ antiporter gene *SbNHX1* into castor bean with an improved *Agrobacterium*-mediated transformation method that uses spermidine (1 mM) and acetosyringone (200 μM). Stable integration and expression of the transgenes were confirmed by PCR and Southern blot hybridization. Physio-biochemical analyses such as quantification of chlorophyll, water, proline, malondialdehyde (MDA), Na^+ and K^+, as well as

measurements of K^+/Na^+ ratios, and electrolytic leakage under varying NaCl concentrations showed that ectopic expression of *SbNHX1* enhances salt tolerance in transgenic plants by modulating physiological process.

14.6 Introduction of Other Agronomically Desirable Traits into Castor Bean

14.6.1 Altering Plant Architecture

Flowering plants exhibit either determinate or indeterminate type of inflorescence architecture. Determinate inflorescences are ideal for uniform and high-density planting, and it is amenable for mechanization and combined harvesting. The inflorescence of cultivated castor bean, including ruling hybrids and varieties, is predominantly indeterminate making the crop less amenable for mechanical harvesting.

Castor bean genotypes with low tendencies to branch have been selected using the pedigree method, but successive rounds of self-pollination of these cultivars reduced plant vigor (Baldanzi and Pugliesi 1998). According to Severino et al. (2012), the selection for short and nonbranching castor bean plants is usually difficult due to their high genotype versus environment interaction. According to Brigham (1980), the transition from the vegetative to the reproductive stage of the main stem corresponding to the formation of the main raceme leads to production of axillary shoots. From these shoots, the second and subsequent order racemes originate and the sequence of development continues as long as the plants remain alive and healthy, thus producing an indeterminate growth habit.

Because terminal flowers are produced in determinate types, two important regulators of the flowering pathways, viz. FLOWERING LOCUS T (FT) and TERMINAL FLOWER 1 (TFL1) which function in diverse signaling pathways have been studied. FT promotes the transition to reproductive development and flowering, while TFL1 represses it. A loss of function of the TFL-1 gene has been associated with a determinate phenotype and early flowering. An early flowering and determinate castor bean inbred line have been developed through transgenic downregulation of TFL1 activity. The resulting plants exhibited stable vigor for at least five generations (Peles et al. 2017).

14.6.2 Seed Traits

The demand for castor oil for expanded industrial applications in manufacturing of biodegradable products and as a source of biofuel is increasing. Consequently, there is a need for developing nontoxic, low-ricinine, and nonallergenic castor bean for safe utilization of the seed meal for animal feeding besides increasing the oil content and modification of oil quality for varied purposes.

14.6.3 Toxin-Free Castor

The major toxic constituents of castor bean seed meal are the two-chained polypeptide ricin, the alkaloid ricinine, the four-chained polypeptide *R. communis* agglutinin (RCA), and the allergenic protein polysaccharide CB-1A (castor bean allergen). Due to the presence of these deadly natural poisons and allergens, castor bean's high-protein meal is only used as fertilizer or it is incinerated. Enhanced expression of ricin, 2S albumin, and ricinoleate production start at 26 days after pollination (Chen et al. 2007). Ricin is a type II ribosome inactivating protein (RIP) and is deadly when inhaled, ingested, or injected (Lord et al. 1984; Hartley and Lord 1993). Chemical detoxification procedures are available, but the energy input needed to treat the deoiled seed meal to destroy the toxic proteins and allergens limits the economic competitiveness of such procedures. A major objective of castor bean genetic transformation is to develop ricin-free varieties that are safe for growers and seed processors while increasing the value of the high-protein meal remaining after castor oil extraction. This problem is being tackled through a multipronged approach. Conventional and

biotechnological approaches are being attempted to develop cultivars with reduced levels of toxin. Traditional breeding methods involving crosses of the high-yielding dwarf line 'hale' with two lines that show low levels of seed toxins resulted in the development of a dwarf plant line with a 70–75% reduction in ricin and RCA content (Auld et al. 2003). Gene silencing approaches to reduce the levels of toxins/allergens through crop genetic engineering have been successful. The ricin gene has been isolated and sequenced, and analyses of the transcription of ricin, allergen, and numerous lipid biosynthetic enzymes during seed development led to the identification of promoters that can be used to express genes that suppress toxin and allergen production. A combination of breeding and transgenic approaches is expected to produce castor plants that have potential for 99.9% reduction in ricin content in segregating populations (Auld et al. 2001). However, transgenic strategies through antisense silencing resulted in limited toxin reduction as ricin production is controlled by multiple genes. Studies by Pinkerton et al. (1999) indicated that ricin concentration is controlled by multiple genes and is also influenced by the environment.

The biotech company Arcadia Biosciences has focused on 'knocking out' toxic genes using classical mutagenesis and tilling (targeting induced local lesions in genomes), which identifies single nucleotide polymorphisms (SNPs) in known genes responsible for ricin production (www.arcadiabio.com). Ricin was initially considered to belong to a lectin multigene family composed of eight members (four A and four B chains). Based on this information, research was undertaken at Mississippi State University, USA, USDA-ARS, Albany, USA, and the Indian Institute of Oilseeds Research, Hyderabad, India, to genetically engineer castor for inhibiting ricin expression and/or production. Later on, the annotation of the draft castor bean genome sequence identified 28 putative genes in the ricin family, including potential pseudogenes or gene fragments (Chan et al. 2010).

RNA interference (RNAi) technology has been exploited to silence the ricin genes in castor bean seed endosperm to develop cultivars with reduced toxicity (Chen et al. 2013; Li et al. 2014; Sousa et al. 2017). Sousa et al. (2017) cloned two copies of a 460 bp fragment from the ricin toxin A-chain gene (RTA) in concatenated sense and antisense orientations under the control of the 35S CaMV promoter in order to generate an intron-hairpin RNAi cassette (Δ*ricin*). The cloning vector also contained the reporter *gus* gene and a mutated *ahas* gene from Arabidopsis, which confers tolerance to the imidazolinone class of herbicides. Results indicated effective silencing of the ricin genes in the transgenic plants and ricin proteins could be not detected by ELISA. Protein extracts from transgenic seeds lacked hemagglutination activity and were not toxic to rat intestine epithelial cells or Swiss Webster mice. Chen et al. (2013) also succeeded in obtaining transgenic castor bean plants containing a 762 bp fragment of the RTA by *A. tumefaciens*-mediated transformation of cotyledonary nodes. Li et al. (2014) selected a 351-bp fragment of the RTA to produce an RNAi construct that was introduced into castor bean through *Agrobacterium*-mediated transformation of epicotyls. Semiquantitative analysis showed an apparent decrease in the RTA expression level, further suggesting that RNAi is an effective approach for reducing ricin mRNA expression. The biodetoxified castor bean cake which is very rich in valuable proteins can be used for animal rations.

The introduction of ricin into heterologous systems is also being pursued for uses related to biodefense, treatment of AIDS, cancer immunotherapy, and disease model systems involving apoptosis. Production of pharmaceutically important compounds in plants is an area of interest that has expanded during the past two decades. However, the development of genetically engineered therapeutics utilizing type II RIPs such as ricin has been limited owing to the difficulty of their expression in heterologous systems due to improper polypeptide folding, incomplete processing of preproricin, and an overall susceptibility of host ribosomes to depurination (Frankel 1992). Successful processing of preproricin using either prokaryote or eukaryote expression systems has not been

reported, while Sehnke et al. (1994) demonstrated the processing of RIP preprotein precursor into a fully active mature toxin when expressed in tobacco. Sehnke and Ferl (1999) developed stable cell cultures from transgenic tobacco expressing ricin that provides a safe and simple means to produce properly processed recombinant ricin. Regardless of its advantages, the development and release of genetically modified ricin-producing species should be approached with caution, following appropriate safety regulations.

14.6.4 Modification of Oil Quality

The presence of the mid-chain hydroxyl group and double bonds imparts unique chemical and physical properties to castor oil that stabilizes the oil against oxidation, making castor oil a vital industrial feedstock. Castor oil contains ricinoleic acid (the 18-hydroxy oleic acid D-12-hydroxy octadec-cis-9-enoic acid), which is industrially important as it is used in the manufacture of polymers, lubricants, polyurethane coatings, cosmetics, and plastics. The ricinoleic acid molecule has three elements of functionality that make castor oil suitable for many chemical reactions and modifications (Ogunniyi 2006): (a) a carboxyl group that allows a wide range of esterifications, (b) a double bond that can be altered by hydrogenation, epoxidation, or vulcanization, and (c) a mid-chain hydroxyl group that can be acetylated, alkylated, or dehydrated to produce semidrying oils.

The uniqueness of castor bean not only lies in the presence of oleic acid hydroxylase to synthesize ricinoleic acid, but also in its capacity to efficiently accumulate high amounts of ricinoleic acid (88–91%) in its seed oil. Most of the genes encoding the key enzymes involved in the biosynthesis of fatty acids and triacylglycerols are single copy genes (Chan et al. 2010). Key areas of research to achieve desired oil quality include increasing oleic acid content in castor bean or producing ricinoleic acid in heterologous systems. Decreasing the ricinoleic acid content with concomitant increase in oleic acid content would enhance the performance of castor oil as a feedstock for biodiesel. Conventional breeding led to the identification of a castor bean mutant line (OLE-1), which has a 20-fold increase in oleic acid (C18:1) and a sixfold decrease in ricinoleic acid content (C18:1-OH) (Rojas-Barros et al. 2005). These studies concluded that the desaturation of oleic to linoleic acid was controlled by a major gene whose action was modified by a second gene. The recessive gene *ol* present in OLE-1 could affect the action of the oleoyl-12-hydroxylase enzyme preventing the hydroxylation of oleic acid to synthesize ricinoleic acid. The recessive alleles at the modifier (*M1*) locus would suppress the effect of the *ol* allele on the oleic/ricinoleic trait.

Genetic engineered production of ricinoleate in heterologous systems in order to replace castor bean has been a scientific and technical challenge. It would benefit ricinoleate-related industries by producing a valuable renewable resource in greater quantity without the complications of castor bean toxins. Incorporation, modification, or suppression of key metabolic pathways leading to over-accumulation of desired fatty acids are viable metabolic engineering approaches. Although it is relatively easy to genetically engineer biosynthetic pathways, achieving high levels of the industrial fatty acids in heterologous systems is not always successful. For example, transgenic expression of the fatty acid hydroxylase (*Fah12*) gene from castor bean under the control of strong seed-specific promoter (*napin*) from *Brassica napus* resulted in very low levels of accumulation of ricinoleic acid in the heterologous host (<1% in tobacco, 17% in Arabidopsis), making the approach commercially inviable (Brown and Somerville 1997). The major limitation for the accumulation of unusual fatty acids (UFAs) such as ricinoleate in heterologous systems is the inefficient transfer of the UFAs from the site of synthesis on phospholipids to the triacylglycerols (TAG) where the oil is assembled and stored. Lin et al. (2002) demonstrated that labeled ricinoleate is preferentially inserted into TAG in castor bean endosperm microsomes, while the same substrate failed to be efficiently incorporated in other

oilseeds. This is mainly because the castor bean acyl-CoA:diacylglycerol acyltransferase (DGAT1) selectively incorporates ricinoleate into TAG. The identification of the castor bean-specific ricinoleate gene phospholipid:diacylglycerol acyltransferase *PDAT1-2* and the manipulation of the phospholipid editing system in transgenic Arabidopsis plants enhanced the accumulation of total hydroxyl fatty acids (HFA) up to 25% (Kim et al. 2011). Subsequent studies involving incorporation of gene combinations, such as DGAT type 2 (*RcDGAT2*) and oleoyl hydroxylase (Burgal et al. 2008), or *PDAT* and oleoyl hydroxylase (van Erp et al. 2011), increased HFA content by 30% in transgenic plants. The conservation of seed oil metabolic pathways across plant species and knowledge of the mechanisms of seed oil accumulation has facilitated the development of designer oil crops by metabolic engineering of fatty acid profiles, and it can be applied to improve castor bean oil content and quality. The expression profiles of genes involved in fatty acid biosynthesis, transport during cellular endosperm development, and accumulation of key enzymes of the triacylglycerol biosynthetic pathway such as diacylglycerol acyltransferase, glycerol-3-phosphate dehydrogenase, and lysophosphatidyl-acyltransferases have been investigated in castor bean (He et al. 2004; Chen et al. 2007; Chan et al. 2010; Xian-Jie et al. 2011; Arroyo-Caro et al. 2013). Reverse engineering strategies are being developed wherein the TAG assembly routes are characterized as a prelude for transgenic expression of the specialized TAG assembly enzymes in oilseeds (Green et al. 2008). Under the Crop Biofactories Initiatives at CSIRO, Australia, several genes involved in the synthesis and storage of UFAs are being cloned and technologies are being developed to engineer high-level synthesis and accumulation of UFAs in transgenic oilseeds including safflower (Green et al. 2008). Expression of splice variants of the specific castor bean *wrinkled1* (*WRI1*) gene belonging to apetala 2 (*AP2*)/ethylene responsive element binding protein (*EREBP*) class of transcription factors in tobacco increased oil content by 4.3- to 4.9-fold as compared to controls and could be potentially used to engineer crops for high oil production (Ji et al. 2018).

Genetic engineering of ricinoleate synthesis has been attempted in *Lesquerella fendleri* for safe castor oil production. *L. fendleri* (L) is valued for its unusual hydroxy fatty acid called lesquerolic acid (C20:1OH), which is produced by a two-carbon elongation of ricinoleate. Therefore, suppression of the elongation step in *L. fendleri* through genetic engineering could generate ricinoleate in *L. fendleri* (Chen 2009).

Ectopic overexpression of castor bean leafy cotyledon (*LEC2*) in Arabidopsis triggered the expression of genes that encode regulators of seed maturation and oil body proteins in vegetative tissues. Consequently, expression of fatty acid elongase 1 (*FAE1*) was increased, inducing the accumulation of triacylglycerols, especially those containing the seed-specific fatty acid eicosenoic acid ($20:1^{\Delta 11}$) in vegetative tissues (Kim et al. 2014).

Fluctuating oil prices, depleting fossil fuel reserves, increasing concerns about climate change, and potential economic opportunity demand renewable sources of fuel. The feedstocks used for biodiesel include edible oil, but the debate on 'food versus fuel' indicates the need for introduction of alternative feedstocks which meet the criterion of (1) sustainability—not compete with food crops for land or water resources, not promote deforestation, the total life cycle of greenhouse gases emissions should be low compared to that of fossil fuels, (2) renewable and continuous availability—reliable supply with potential for large-scale production, and (3) economically feasible—competitive price compared to edible oils and fossil fuels.

Castor bean has attracted the attention of researchers in USA and Brazil because of its high oil content (50%) and relatively high crop yield, which can produce up to 140 gallons of castor oil per acre and provides a safe opportunity for biodiesel production without the risk of displacing food crops. Castor is a summer-grown crop that fits well in Mississippi and other parts of the Southern USA. Estimated CO_2 absorption level

of castor bean plants is 34.6 ton per ha with two growing cycles per year. Lifecycle analysis of biodiesel produced from castor bean showed that greenhouse gas emissions were reduced by 90% when compared to petroleum diesel. Castor oil is the only oil soluble in alcohol and therefore does not have the consequent energy requirement for transesterification as other vegetable oils. Castor oil has a good shelf life when compared to other oils (e.g., four times more stable than olive oil), and it does not turn rancid when subjected to excessive heat. However, its high viscosity, water content, and compressibility, as well as the observed ~10% reduction of its hydroxyl and acid values after 90 days of storage and its premium price, are the major issues limiting the use of straight castor oil as fuel for internal combustion engines (Scholz and da Silva 2008), although the limit values of viscosity, density, and cetane number can be met through transesterification followed by dilution or blending with conventional diesel fuel and other vegetable oils (www.castoroil.in).

Evogene (www.evogene.com) has demonstrated the potential of castor oil as a viable and sustainable second-generation feedstock for the production of biojet fuel as it meets the American Society of Testing and Materials (ASTM) D7566 fuel specification requirements for alternative aviation fuels containing synthetic hydrocarbons. Conversion of ricinoleic-rich castor oil to oleic-rich castor oil can be accomplished through silencing of the fatty acid hydroxylase (*fah1*) gene. Almeria-based researchers have genetically modified castor bean for obtaining oil with a higher proportion of monounsaturated fatty acids (e.g., oleic or palmitic) for use of castor oil as a biolubricant (https://cordis.europa.eu/news/rcn/119449_fr.html).

14.7 Conclusions and Future Perspectives

There is an immediate need for the development of a highly efficient, reliable, and reproducible direct and callus-mediated tissue culture system as a prelude for genetic engineering of castor bean for desirable traits. Transgenic approaches through RNAi technology has been proposed for reduction of the toxic protein ricin and conversion of ricinoleic acid-rich castor oil to oleic-rich oil. Bioengineering of fatty acid metabolic pathways in castor bean has a huge potential for downstream processing and value chain expansion. The availability of draft genome sequences of castor bean (Chan et al. 2010) and other Euphorbiaceae members (e.g., cassava and physic nut) allows genome-wide comparative analyses of stress-responsive genes, fatty acid metabolism genes, the ricin gene family. Their expression profiles help in the identification of key genes, promoters, and transcription factors as suitable targets for genetic engineering. The toxicity of castor bean seed cake due to ricin and RCA in animal feeding experiments poses biosafety and ethics concerns, and hence, suitable strategies should also be simultaneously developed for the assessment of the biosafety of castor transgenics.

With regard to biotic stresses, resistance to capsule borer and *Botrytis* gray rot could be incorporated through transgenic approaches. Considerable research gaps exist in our understanding of these pests' biology in different hosts, their behavior, population dynamics, seasonal abundance, off-season survival, host preference, pest–parasitoid relationships, economic thresholds, and management with suitable insecticides for effective control. The efficacy of crystal protein genes from *Bt* and other proteins against capsule borer has to be evaluated, and the rearing technique is an essential prerequisite for this purpose. Suitable candidate genes with appropriate deployment strategies need to be developed for enhancing resistance to *Botrytis* gray rot.

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