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Genetic engineering in cowpea (*Vigna unguiculata*)

History, status and prospects

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In the last three decades, a number of attempts have been made to develop reproducible protocols for generating transgenic cowpea that permit the expression of genes of agronomic importance. Pioneer works focused on the development of such systems vis-à-vis an in vitro culture system that would guarantee de novo regeneration of transgenic cowpea arising from cells amenable to one form of gene delivery system or another, but any such system has eluded researchers over the years. Despite this apparent failure, significant progress has been made in generating transgenic cowpea, bringing researchers much nearer to their goal than 30 years ago. Now, various researchers have successfully established transgenic procedures for cowpea with evidence of inherent transgenes of interest, effected by progenies in a Mendelian fashion. New opportunities have thus emerged to optimize existing protocols and devise new strategies to ensure the development of transgenic cowpea with desirable agronomic traits. This review chronicles the important milestones in the past 30 years that have marked the evolution of genetic engineering of cowpea. It also highlights the progress made and describes new strategies that have arisen, culminating in the current status of transgenic technologies for cowpea.

Introduction

Cowpea (*Vigna unguiculata* L. Walp.) is one of the most important and widely cultivated legumes in many parts of the world, particularly in Africa, Europe, Latin America and some parts of Asia and the United States (www.faostat.fao.org/faostat; http://old.iita.org/).

Data available from the Food and Agriculture Organization (FAO) show that approximately 4 million tons of dry cowpea grains are produced annually in an area covering about 10 million hectares worldwide (www.faostat.fao.org/faostat). However, these numbers are actually underestimated because they do not include data from Brazil, India and some other countries.[1] This underestimation is even more apparent when we consider the fact that in Central America and East and Southern Africa, where common bean (*Phaseolus vulgaris*) in addition to *Vigna unguiculata* is grown and statistics from both crops are often mixed up.[2] In Brazil, about 482,000 tons of the crop is produced in an area covering about 1.25 million hectares.

The cowpea grain represents a very important source of carbohydrates, lipids, minerals and vitamins, including folate, thiamin and riboflavin.[3] It is well adapted to dry areas because of its tolerance to water scarcity and, when grown under the right conditions, the crop presents high productivity. These advantages make it a crop of choice for many farmers, who often cannot afford the investment required in managing other crops.[4]

Although considerable progress has been made in cowpea breeding leading to the development and introduction of several improved varieties in over 63 countries, a number of agronomic traits are still unavailable (http://old.iita.org/). For example, the development of *Maruca vitrata* resistant cowpea via traditional breeding has faced great challenges, requiring biotechnology based approach. Transgenic technologies therefore represent important avenues for the development of cowpea with desirable and improved traits.

In this review, we discuss the efforts and the success recorded so far in the global attempt to generate transgenic cowpea in order to meet the challenging demand for improving the crop through different transgenic approaches, spanning a period of three decades. We also report on the application of these protocols in generating transgenic cowpea with improved agronomic traits in a number of research works and some ongoing research projects, giving the current status and future prospects for the development of genetically engineered cowpea.

Genetic Transformation

In the last three decades, a number of attempts have been made to generate transgenic cowpea.[5-8] The general approach in most cases has been through the employment of *Agrobacterium tumefaciens* as a gene delivery system via tissue culture (**Fig. 1**). By manipulating different factors such as target tissues, reporter genes, selection regimes, culture conditions and gene delivery system, researchers from different countries have been

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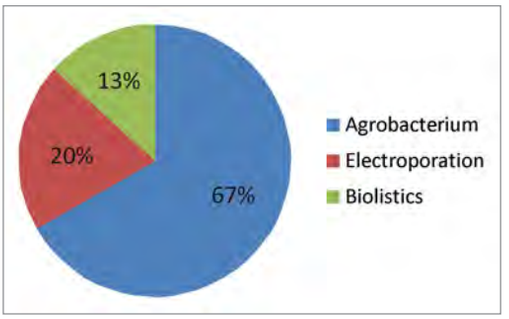

Figure 1. Distribution of the gene delivery system in the production of transgenic cowpea from 1987 to 2010.

engaged in generating transgenic cowpea with the ultimate aim of integrating genes of interest and their segregation in progenies arising from one generation to another in a Mendelian fashion (**Table 1**). In all of these approaches, one important aspect has been the establishment of in vitro propagation techniques capable of generating amenable culture that could produce totipotent cells with the potential to regenerate complete plants following gene delivery. Unfortunately, cowpea appeared to be recalcitrant to most in vitro manipulations, particularly via de novo regeneration.[20] While researchers have been rushing to break this totipotency jinx in the bid to establish de novo regeneration and genetic transformation protocols since the late seventies, no such breakthrough has been recorded so far. However, with the development of novel strategies that do not necessarily rely directly on the use de novo regeneration systems, these problems appear to have been circumvented, with reports of some success stories.[8,11,12,15]

The first of such reports appeared in 1986, when Garcia et al.[5] transformed leaf discs from primary leaves of cowpea using *A. tumefaciens* designed with a Ti plasmid harboring two identical chimeric genes for kanamycin resistance. The group obtained transgenic callus expressing nopaline synthase and amino glycoside phosphotraferase confirmed by DNA gel blot analysis. Following this, the same group used the protocol to express a viral RNA fragment (M-RNA) of Cowpea Mosaic Virus (CPMV) in callus of cowpea.[6] The rationale behind this second strategy was to study the virus using two different constructs; one containing 35S promoter of the Cauliflower Mosaic Virus (CaMV) and the other harboring nopaline synthase promoter of *A. tumefaciens*, to ensure elimination of possible escapes. This approach resulted in a 10-fold increase in the number of transcripts of the transgene using 35S CaMV promoter rather than nopaline synthase, leading to increased efficiency and thus applicability of the promoter in the transformation of cowpea.[6] Indeed, the work underscored the possibility of studying cowpea parasites using this approach because of its applicability in different plant tissues. More importantly, it demonstrated for the first time that *A. tumefaciens* could be used as an exogenous gene delivery system on the one hand, and as means of studying virus associated with cowpea on the other. Although the vast majority of transformation experiments in the period between this report and 2010 have relied heavily on the Agrobacterium mediated system of gene delivery, a few other experiments have used direct systems of gene delivery (**Fig. 1**).

Despite the pioneering work of Garcia et al.[5,6] and a number of other attempts to develop transformation and regeneration systems,[7,9,10,21,22] it took close to a decade to produce the first transgenic cowpea plants when Muthukumar et al. co-cultured explants of cotyledons with disarmed *A. tumefaciens* expressing the gene for resistance against hygromicin, which were subjected to organogenesis leading to the generation of transgenic shoots.[11] Analysis of data from the work showed that one third of the total shoots arising from co-cultured explants thrived well after acclimatization in soil following rooting. Although a good number of these plants attained maturity, seeds arising therefrom could not germinate.[11]

It was another decade before any progress was made in cowpea transformation, when Ikea et al. reported on the production of the first transgenic cowpea via particle bombardment with evidence of molecular integration of the gene observed in T1, T2 and T_3 generations. In the experiment, meristematic tissues were pre-cultured 24 to 48 hrs before bombardment with a plasmid containing the reporter gene *gus* along with the selectable marker *bar* under control of CaMV 35S promoter. Although less than one percent of total explants bombarded were recovered and even fewer progenies expressing the transgenes were obtained, this work represented an important milestone in the historical trajectory of cowpea transformation. Indeed, it is a measure of this importance that the work reported for the first time transformation events from one generation to another, although the progenies generated did not fully obey the Mendelian law.

Three years later, the first report appeared in which stable transformation was successfully achieved and progenies were generated with Mendelian segregation.[8] In a brilliant adoption of different strategies of transformation systems of legumes using *A. tumefaciens*, Popelka et al.[8] were able to obtain transgenic cowpea plants with a transformation frequency of 0.15%. Critical parameters exploited by the authors included use of cotyledon nodes of mature or developing seeds as explants and removal of auxins in the initial proliferative stage of the explants, with supplementation of low level of BAP during shoot formation and elongation as well as addition of thiol compounds during transfection and co-culture with Agrobacterium.

An improvement on this was recorded using an *A. tumefaciens* system in which a number of modifications were made, like the use of different cultivars, explant type, Agro strain and binary vector, co-culture medium and duration, type and application of selective agent and marker, and shoot and root induction media, ensuring higher frequency of transformation.[13]

Following this, our group developed an efficient cowpea transformation protocol which did not appear to be variety specific and presented a high frequency of transformants following Mendelian segregation.[15] The system comprised of biolistic transformation of meristems using *gus* as a reporter and a novel selection regime based on the use of the herbicide imazapyr. This feat was achieved by introducing a mutant *ahas* gene in the vector used for transformation. The practicability of this approach, as well as the use of imazapyr, brought to the fore the enormous

Table 1. The historical trajectory of the development of transgenic cowpea (*Vigna unguiculata*)

Gene of intrest	Selectable marker	Gene delivery systems	Target tissue	Transformation effeciency	Degree of gene integration	Reference
nptII	kanamycin	*A*	Leaf discs	NR	Transgenic callus	5
CPMV M-RNA	kanamycin	*A*	Leaf discs	NR	Transgenic Callus	6
gus	-	*A*	Mature embryos	NR	Transgenic zygotic embryos	7
gus	-	*E*	Mature embryos	NR	Transgenic zygotic embryos	9
gus	-	*E*	Mature embryos	NR	Transgenic zygotic embryos	10
hpt	hygromycin	*A*	Cotiledonary explants	NR	Regenerated transgenic plants	11
gus	phosphinothricin	*B*	Meristematic explants	NR	Progenie transmission/not Mendelian segregation	12
gus	Phosphinothricin	*A*	Cotyledonary nodal cuttings	0.15%	Transgenic plants with Mendelian progenies transmission	8
gus	kanamycin	*A*	Cotyledonary nodal cuttings	0.76%	Transgenic plants with Mendelian progenies transmission	13
gus	geneticin	*A*	Cotiledonary nodal cuttings	1.64%	Transgenic plants with Mendelian progenies transmission	14
gus	imazapyr	*B*	Shoor apical meristem	0.9%	Transgenic plants with Mendelian progenies transmission	15
αAI-1	geneticin	*A*	Cotiledonary nodal cutting	1.67%	Transgenic plants with Mendelian progenies transmission	16
Cry1Ab	geneticin	*E*	Nodal buds	NR	Progenie transmission/not Mendelian segregation	17
gus	Phosphinotricin	*A*	Embryos	3.9%	Progenie transmission/Mendelian segregation was not reported	18
gus	hygromycin	*A*	Cotiledonary nodal cuttings	1.61%	Transgenic plant regenerated	19

A, *Agrobacterium tumefaciens; B*, Biolistics; *E*, Electroporation; NR, Not reported; CPMV, cowpea mosaic virus; *αAI-1,* Alpha-amylase inhibitor-1.

potential of this system in producing transgenic cowpea that expresses traits of agronomic importance. Besides the relatively higher transformation frequency (0.9%), the work demonstrated efficient gene transfer which was inherited by T1 and T2 generations with segregation according to Mendelian law.

Agronomic Problems as a Target for Genetic Engineering

Because of its great economic importance, cowpea has been the target of breeding programs for many years in different centers of research the world over (http://old.iita.org/). The ultimate goal of these programs is to address agronomic problems such as attack by virus, bacteria, pests and diseases.[23,24] Unfortunately, the apparent unavailability of resistant genes in the plant has rendered the conventional breeding approach less attractive in strategies for producing pest-resistant genotypes of cowpea. This task is even more herculean given the cross-incompatibility between the cultivated and wild Vigna species that may possess some inherent resistance.[25-27]

At almost every stage of its development, cowpea is confronted by one form of biological stress or another (http://old.iita.org/). This includes attack by aphids like *Aphis craccivora* which invades leaves and stems of seedlings, extracting juice and infecting plants with cowpea mosaic virus at the same time. During flower formation, the crop is attacked by thrips like *Megalurothrips sjostedti* which prevents the formation of seeds. Bruchid weevils like *Callosobruchus maculatus,* on the other hand, destroy the seeds following harvest. Besides susceptibility to attack by fungi, bacteria, virus and some classes of nematodes, parasitic weeds also retard and sometimes completely prevent its growth (http://old.iita.org/). These factors often result in severe economic losses for farmers and lead to low yields which, in Africa, stand at 350 Kg per hectare (http://old.iita.org/). For example, *M. vitrata* has been reported to cause a 17–53% yield loss in cowpea[28] while *C. maculatus* causes 20–60% grain loss.[29,30] In Brazil and several other Latin American countries, the most common causes of these losses are viruses, which are often transmitted by insects. Foremost among them are Cowpea Severe Mosaic Virus (CPSMV), Cowpea Aphid-Borne Mosaic Virus (CABMV) and

Cowpea Golden Mosaic Virus (CPGMV). Collectively, these viruses cause losses of about 81%.[16,31]

The most obvious means of controlling these insects would appear to be the use of insecticides and other pesticides, but besides being expensive and therefore beyond the reach of the often poor farmers that grow the crop, these may pose serious hazards to users and the ecosystem. Unfortunately, even such imperfect solutions do not exist for combating viruses.

With the limitations encountered in breeding programs, biotechnology-based approaches seem to hold the key to effectively tackling these biological stresses. Genetic engineering offers important strategies with promising results in addressing not only problems caused by pests and diseases, but also by viruses.[20]

Introduction of Useful Agronomic Traits

The first report on the regeneration and stable transformation of cowpea expressing a gene of agronomic importance appeared in 2008 when workers generated transgenic cowpea that expressed some degree of insect resistence.[14] The establishment of an *A. tumefaciens*-mediated transformation protocol using geneticin and supplementation of post-selection media with benzyl aminopurine for over 3 weeks ensured a regeneration frequency of 1.64%.[32] The strategy employed was based on the use of the gene for α-amylase inhibiting protein (*aAI-1*) from *P. vulgaris* as a means of conferring resistance against different insects. The efficiency of transformation in this case was enhanced by using multiple copies of the gene *vir*, co-culture of explants in the presence of thiol compounds and by sequential selection using geneticin.[14] The work reported up to 82.3% decrease in insect susceptibility in transgenic plants when subjected to *Callosobruchus chinensis*.[14] Without doubt, this work represented an important breakthrough in the development of transgenic cowpea and paved the way for several attempts to optimize existing protocols and, in some cases, the development of novel strategies for pest and disease resistance in cowpea.[19]

This successful demonstration of cowpea resistance using *aAI-1* gene was followed by the report of another considerable resistance against *M. vitrata* by T_3 progenies after transformation of nodal cuttings with a plasmid harboring *Cry1Ab*, the now popular gene for protein toxin from *Bacillus thuringiensis*, using *nptII* as a selectable marker under the control of 35S of CaMV.[17] In an attempt to improve on this, Adesoye et al.[18] employed vacuum infiltration of embryos in the transformation of cowpea and reported a fairly high degree of transformation frequency in T1 seeds ranging between 2.5% and 3.9%.

Recently, researchers have reported the use of *Cry1Ab* gene from *B. thuringiensis* to produce several transgenic lines of cowpea with resistence against *Helicoverpa armigera* and *M. vitrata*. These Bt-cowpeas are being field tested in countries like Nigeria with good preliminary results.[33] In addition, a number of field trials have been going on in the last couple of years using cowpea with high degree of resistance against *C. maculatus* in field trials in Puerto Rico and Nigeria with promising results (www.csiro.au).

Based on the foregoing, it appears that transformation and regeneration protocols for the development of transgenic cowpea are well established. The focus now is employment of these techniques in concert with traditional breeding approaches for large-scale production of elite varieties expressing genes of agronomic importance.

What Needs to be Done

Although we are far from resolving the problems associated with the cultivation and utilization of cowpea, the success recorded so far in the development and application of different transgenic technologies to address these problems are clear indicators that these goals are attainable within the next few years.

The success of Bt toxin recorded in several crops including cowpea, demonstrates the enormous benefits the crop stands to gain from transgenic technologies. However, with the possible emergence of insect resistance to Bt toxin, new control strategies involving a different mode of action like the use of RNA interference are emerging and promise to be valuable in managing the problems associated with the production of cowpea.[34-37]

Developed using the natural mechanism of gene regulation in eukaryotes, RNA-mediated gene silencing is of potentially great value thanks to its practicability and effectiveness. The system has been applied in silencing the expression of many target genes mediated by siRNA molecules.[38,39]

Early experiments using RNA gene silencing underscore the high potential of this approach in controlling insects as well as other threats to cowpea. It has been demonstrated that silencing of essential genes in insects mediated by siRNA may cause the cessation of feeding and even death of the insects.[35-37] An endeavor that would develop transgenic cowpea which expresses some siRNA molecules whose sequences could trigger signals for silencing essential genes in insects, bacteria and virus, without compromising the cowpea or the consumer's health seems to be attractive. Indeed, this type of strategy has been applied in a number of crops and among the candidate genes used are insect-vacuolar ATPase and tubulin.[35-37]

The development of genetically engineered common bean with RNAi-mediated resistance to Bean Golden Mosaic Virus[40] opened the possibility of generating cowpea with resistance against the CPSMV using the RNAi strategy in our research group. Plants resulting from this experiment presented a high level of resistance against the virus in greenhouse tests (unpublished). The next phase is to carry out field tests.

Another important candidate gene of great potential in improving cowpea is cystatin, a cysteine proteinase inhibitor with potential as a pest resistance conferring agent.[41-42] We are currently trying to develop transgenic cowpea expressing chicken cystatin with a view to expressing insecticidal activity against bruchids.

Although cowpea is an important source of nutrients, including several amino acids, it is deficient in sulfur-containing amino acids, a trait common in most legumes. Several strategies have been devised to address this using transgenic technology in a number of legumes.[43,44] Our group is using a transgenic approach

to introduce methionine-rich protein in cowpea using the gene for delta-zein from maize.[45]

In spite of its ability to thrive under harsh environmental conditions, the growing demand for cowpea necessitates the development of this legume with some improved resistance against herbicides. In line with this, we are using the protocol established in our laboratory[15] to generate cowpea with resistance against the herbicide imazapyr (unpublished).

Conclusion

In the last few years, significant progress has been made to establish different protocols and their application in the development of transgenic cowpea with one type of characteristic or another. Through the participation of research centers from all over the world, there have been important findings that started with obtaining transgenic callus; from that came transgenic plants that exhibited Mendelian segregation, culminating in recent findings that have led to the production of transgenic cowpea with resistance against *C. maculatus* and *M. vitrata* as a result of the insertion of a gene of agronomic importance in the plant. Currently, various research groups in countries including Australia, Brazil, India and Nigeria possess well-established transformation systems which can be harnessed to improving cowpea, a hitherto unreachable goal by means of traditional breeding. Challenges posed by pests and disease can be tackled through systematic application and optimization of the protocols arising from the efforts of the last three decades.

There are plans for more trials of some of the insect resistant varieties developed, and with the possible emergence of new strategies to use RNA interference, as seen in the development of a genetically engineered common bean with RNAi-mediated resistance to Bean golden mosaic virus,[40] there is likely to be even greater success and more breakthroughs in the development of elite transgenic cowpea.

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