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Review

Horizontal gene transfer in cowpea (*Vigna unguiculata L.Walp*) through genetic transformation

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Cowpea (*Vigna ungiuculata* L.) is a crop with wide environmental adaptability grown in areas with extreme heat and drought, as well as in humid and wet tropical areas. Cowpea represents a crucial source of protein, especially in sub-Saharan Africa, where more than 70% of the crop is produced and consumed. Conventional breeding approaches have contributed substantially to the genetic improvement of cowpea. However, non-availability of genes for resistance to pests and viruses and sexual incompatibility with sources of resistance makes the development of insect- and virus-resistant cowpea increasingly difficult. Genetic modification of cowpea with genes of agronomic importance has the potential to overcome these problems. In this review, we summarize the key aspects of cowpea transformation work carried out in research centers around the world. We also discuss the approaches employed and the obstacles militating against efficient regeneration of transgenic cowpea expressing genes of interest.

Keywords: Genetic transformation, cowpea, *Agrobacterium tumefaciens*, ballistics electroporation.

INTRODUCTION

Cowpea (*Vigna ungiuculata* L.) is a crop with wide environmental adaptability grown in areas with extreme heat and drought as well as in humid and wet tropical areas. It is an important protein supplement to carbohydrate-rich staple food consumed in sub-Saharan Africa. About 7.56 million tons of cowpea are produced annually over an area of 12.76 million hectares, with 70% of the production coming from sub-Saharan Africa (www.iita.org/cms/detail/cowpea_project_details).

Production of cowpea is severely limited by biotic constraints, which reduce the overall grain yield to an average of 0.37 ton per hectare (Waddington *et al*. 2010). Among the biotic constraints, the most important are insect pests and viruses, which cause enormous losses in the yield of cowpea across the growing regions. Yield can be substantially increased with constant spraying of insecticides; however, most farmers, particularly in sub-Saharan Africa, are poor and cannot afford the cost of insecticide application. Unlike with insects, the direct control of viral diseases is not possible due to the fact that virucidal chemicals are not yet available. The most viable approach for the control of this menace is by developing plants with inherent resistance to pests and pathogens. Conventional breeding approaches have contributed substantially in the genetic improvement of cowpea (Singh and Awika, 2010). Improvement in insect and virus resistance is becoming increasingly difficult due to the absence of genes for these traits in the genome of cultivated cowpea. Recent molecular analysis of the cowpea genome (Coulibaly *et al.,* 2002; Kouam *et al.,* 2012) revealed an extensive gene flow from wild to cultivated forms, suggesting a single domestication event and narrow genetic base in cultivated cowpea. Attempts to diversify the genetic base of cowpea by interspecific

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hybridization with wild relatives such as *Vigna vexillata* (L) A. Rich have not been successful because of the compatibility barrier (Singh *et al.*, 2000). Therefore, horizontal gene transfer through transgenic technologies holds the key to successful improvement of cowpea. This review summarizes a study of trends in genetic transformation of cowpea and the novel genes introduced so far. It also discusses what improvement in gene delivery systems holds for the commercial production of transgenic cowpea.

Genetic Transformation in Cowpea

Genetic transformation is a process that involves the introduction and expression of foreign genes in a host organism. This expression can result from the extrachromosomal or episomal presence of genes in nuclei that may persist if the introduced DNA has a mechanism for replication (Handler, 2008). Genetic transformation is increasingly used as a method of introgressing genes into cowpea, and it promises to give scientists the opportunity to circumvent the genetic barrier. In addition, it provides the means of overcoming challenges posed by insects and viruses for which little or no natural resistance has been identified. The general strategy employed in genetic transformation of cowpea involves the regeneration of the plant through tissue culture following gene delivery (Citadin *et al.*, 2011). Success in genetic manipulation of cowpea therefore depends on the availability of *in vitro* regeneration systems that will provide totipotent cells capable of regenerating complete plants following gene delivery. Cowpea appears to be recalcitrant for *in vitro* manipulations, especially via *de novo* regeneration (Aragão and Campos, 2007). Due to difficulty in regeneration from callus culture and genotype specificity in response to tissue culture, studies that involve *in vitro* culture of cowpea are difficult to carry out. However, some regeneration systems of cowpea have been reported (Muthukumar *et al*., 1995; Pellegrineschi, 1997; Brar *et al.*, 1997; Anand *et al.*, 2000; Ramahrishnan *et al.,* 2005; Aasim *et al.*, 2009), thus making cowpea transformation feasible. Assessment of research progress has indicated that Agrobacterium*,* particle bombardment and electroporation approaches are employed in genetic transformation of cowpea (Figure 1).

Agrobacterium-mediated Transformation

Agrobacterium-mediated transformation allows for stable integration of the low number of copies of transgenes into the plant genome. It also results in fewer rearrangements and an improved stability of transgene expression over generations than direct DNA delivery methods (Hu *et al*., 2003). *Agrobacterium*-mediated transformation is the most widely used approach in genetic transformation of cowpea (Figure 1). Various starting explants, which include leaf disc, mature cotyledon, cotyledonary node, immature cotyledon and embryonic axes, are employed as target tissues in *Agrobacterium*-mediated transformation studies in cowpea (Figure 2). However, the cotyledonary node is the preferred and most widely used explant. In addition to explants, a number of disarmed *Agrobacterium* strains are used in cowpea transformation. Successful cowpea transformation has been reported by co-cultivation of various explants with common strains such as C58CI, pUCD2614, AGL1 EHA105 LBA4404 and pGV3850.

Proof of concept for exploiting the *Agrobacterium*-mediated transformation technology for the transfer of foreign genes into *Vigna unguiculata* was first demonstrated by Garcia *et al.* (1986 & 1987).

Primary leaf explants were co-cultured with *Agrobacterium* strain C58CI harboring binary vector pGV3850, transferring kanamycin resistance (*nptII*) gene (Garcia *et al.,*1986) and viral RNA fragment (M-RNA) *of Cowpea Mosaic Virus* (CPMV) (Garcia *et al.,*1987), under the control of *Agrobacterium* nopaline synthase (*nos*) and 35S promoter from the *Cauliflower Mosaic Virus (CaMV),* respectively. Transgenic calli expressing Kanamycin resistance and presence of full-length DNA copy of *CPMV* M-RNA (3481 bases long) were reported. This report made two fundamental observations: first, the possibility of using *A. tumefaciens* as a vehicle to deliver exogenous DNA and RNA into the genome of cowpea; secondly, the efficacy of 35S promoter from the *Cauliflower Mosaic Virus (CaMV),* over the *Agrobacterium* nopaline synthase (*nos*) promoter in expressing exogenous DNA or RNA in cowpea. Although Garcia *et al.* (1986 & 87) reported expression of the integrated foreign genes at a detectable level, attempts to regenerate whole plants from the transgenic callus were not successful.

It took almost a decade after the pioneering report from Garcia *et al*. (1986 & 1987) before regeneration of the first transgenic cowpea plant was reported by Muthukumar *et al*. (1996), following 48-hour co-cultivation of cotyledons with disarmed *A. tumefaciens* strain pUCD 2614 carrying pUCD2340 plasmid clone with the hygromycin phosphortransferase (*hpt*) gene, which confers resistance against the antibiotic hygromicin. Transgenic shoots at a frequency of 15-19% were regenerated by subjecting infected cotyledons to organogenesis on B5 medium supplemented with 8×10^{-6} M BAP and 25mg/L Hygromicin-B. Stable integration of the *hpt* gene in the transgenic plants was confirmed by southern blot hybridization analysis. Although four out of the six surviving transgenic plants were fertile and produced seeds, none of the seeds germinated, indicating a possible pleotropic effect of the transgene.

It took another decade before progress was made with the first report of *Agrobacterium*-mediated stable transformation in which progeny transmission of the transgene with Mendelian segregation was achieved

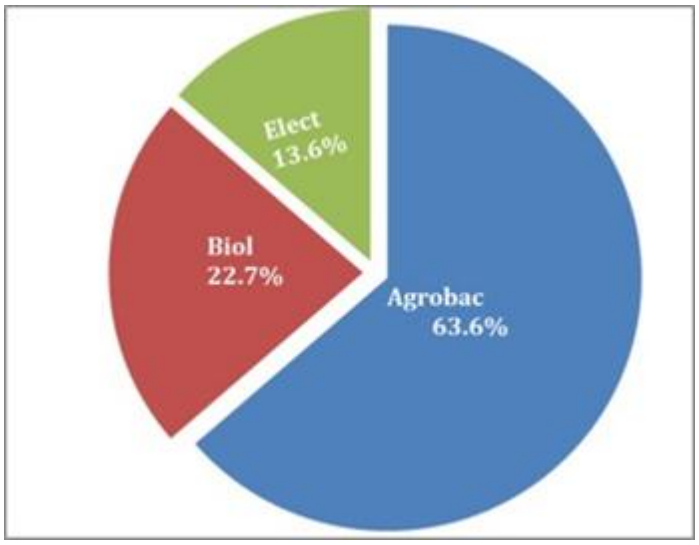

Figure 1. Percentage distribution of gene delivery systems in reported cases of successful transformation of cowpea from 1986 to2014, *Agrobac*-Agrobacterium, *Biol*- Biolistic, *Elect*- Electroporation.

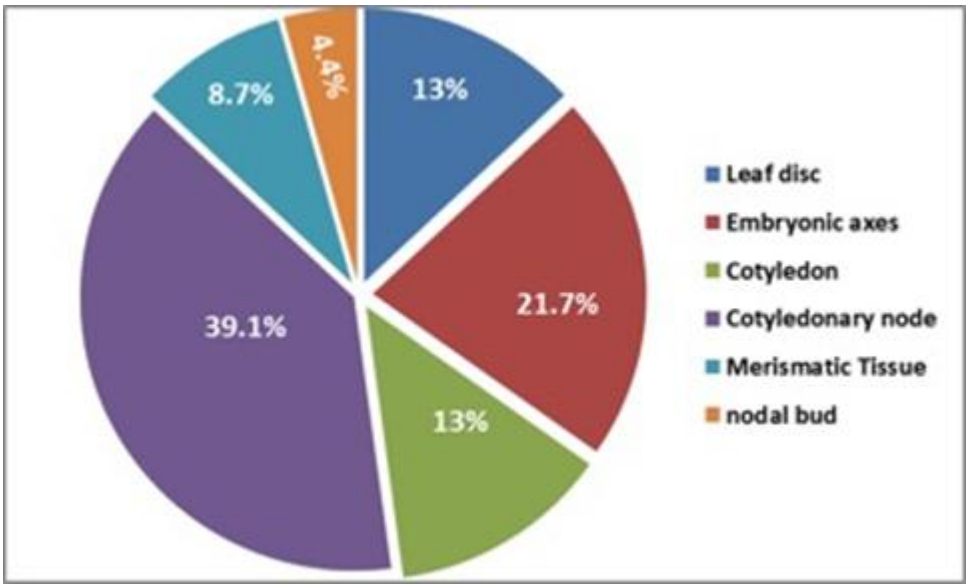

Figure 2. Percentage distribution of explants in reports of successful transformation of cowpea from 1986 to 2014.

(Popelka *et al.*, 2006). In an attempt to achieve stable transformation, Popelka *et al.* (2006) co-cultured longitudinally bisected embryonic axes with cotyledons attached, but without shoot and root apices, with *Agrobacterium* strain *AGL*1 carrying the vector pBSF16 clone with selectable marker gene (*bar*) and reporter gene (*uid*A). They adopted a strategy in which critical parameters that will ensure successful infection and regeneration of transgenic plants were considered. Among the parameters exploited are (i) selection of cotyledonary node from developing or mature seed as the starting explant, (ii) modification of culture medium by withdrawing auxin and supplementation with low-level BAP during shoot induction and elongation stages, (iii) Addition of thiol-compounds during infection and co-culture with *Agrobacterium* to ensure improved T-DNA delivery by inhibiting the activities of plant-pathogen-inducing and wound-response enzyme (Olhoft *et al.*, 2001) and (iv) choice of *bar* gene for selection with phosphinothricin. A transformation frequency of 0.15%

was achieved using this strategy; however, regeneration of fertile transgenic plant that transmits transgenes to progeny was as low as 0.1%. Despite the low regeneration frequency (0.1%) of transgenic plants with stable transformation, this work represents a watershed in the historical development of *Agrobacterium*-mediated transformation of cowpea. This work laid the foundation for generating transgenic cowpea lines expressing genes of Agronomic importance.

In another approach aimed at improving transformation efficiency, Chaudhury *et al*. (2007) co-cultured cotyledonary node explants with Agrobacterium strain EHA105, harboring binary vector pCAMBIA2301, carrying *uid*A and *npt*II as reporter and selectable marker genes respectively, on Murashige and Skoog's basal salts and Gamborg's B_5 vitamins (MBM) fortified with 10µM BAP. This was then followed by regeneration/selection in the same medium supplemented with 5 µM BAP, 85 mg l^{-1} kanamycin and 500 mg l^{-1}cefotaxime. Transgenic T_0 plants were regenerated with transformation efficiency of 0.76% and evidence of *npt*II transmission to T_1 confirmed by southern analysis.

Spurred by the reports of Popelka *et al.* (2006) and Chaudury*et al.* (2007), in which stable transformation of cowpea was achieved with evidence of progeny transmission in Mendelian fashion, a number of scientists have explored the potential of *Agrobacterium* as a tool for producing transgenic cowpea between the period of 2007 and 2014 (Table 1).

Introduction of genes of Agronomic importance

While most of the reports from 1986 to 2014 (Table 1) dealt with the regeneration of transgenic cowpea plants expressing reporter or selectable marker genes, the ultimate goal of the cowpea transformation project is the stable integration of genes of agronomic importance. It took over two decades after the pioneering work of Garcia *et al*. (1986 & 87) before the first transgenic cowpea plant carrying a gene of agronomic importance was produced. Solleti *et al.* (2008) co-cultured cotyledonary nodes with hyper-virulent *Agrobacterium* strain LBA4404, harboring vector pSB1 cloned with α-amylase inhibiting protein (*aAI-1*) from *P. vulgaris,* as a means of conferring resistance against cowpea storage insects. The *aAI-1* is a lectin-like α-amylase inhibitor that binds to the active site of α-amylase, blocking substrate binding by the formation of an inhibitor-enzyme complex, and it has been extensively used in generating transgenic plants due to its insecticidal properties. To ensure efficient transformation, the authors used additional copies of *vir* G, *vir* C and *vir* B genes in the presence of dithiothreitol and L-Cysteine and subsequent selection using geneticin. This strategy resulted in optimum T-DNA delivery with enhanced recovery of transgenic plants to an average of 1.67% (Solleti *et al.*, 2008). The workers also reported a decrease in susceptibility of up to 82.3% and 72.2% when transgenic plants were subjected to *Callosobruchus chinensis* and *Callosobruchus maculatus,* respectively.

In a similar approach, Higgins *et al*. (2010) reported, in separate experiments, the stable integration of *Cry1Ab*-a, popular gene for protein toxin from *Bacillus thuringiensis,* and *aAI-1* in cowpea, following the co-culture of longitudinally dissected embryonic axes or intact embryonic axes with *Agrobacterium* strain *AGL*1 and selection in the presence of geneticin. Transgenic plants with the stable integration of the transgenes were regenerated at a frequency of 0.3%. An insect bioassay with T_3 progenies carrying *aAI-1* showed complete protection against bruchid larvae, while those carrying *Cry1Ab* gave excellent protection against *Helicoverpa armigera* and *Maruca vitrata.*

Recently, in an attempt to improve transformation efficiency using *Cry1*Ac, Bakshi *et al.* (2011 and 2013), evaluated two different approaches in *Agrobacterium* mediated transformation of cowpea. In one approach, Bakshi *et al.* (2011) employed sonication and vacuum infiltration of *Agrobacterium* with subsequent selection in the presence of kanamycin. They reported a 88.5% increase in transformation efficiency over the normal *Agrobacterium* transformation with transgene transmission in Mendelian fashion. In another approach, Bakshi *et al.* (2013), preconditioned the explants by treating them with Thidiazuron, with subsequent regeneration in the presence of Benzylaminopurine and kinetin, following co-cultivation with *A. tumefaciens* strain EHA105, harboring the binary vector pSouv:*cry1*Ac. The authors reported 48.7% increase in transformation frequency over previous reports in cowpea. They also reported *Cry1*Ac transmission in a Mendelian fashion in the transgenic plants. However, no evidence of protection against target insects was reported.

Direct DNA Transfer

Techniques that involve direct DNA delivery into plant cells have been developed for species not susceptible to *Agrobacterium* and those known to be recalcitrant. The most commonly used naked DNA delivery techniques are electroporation, biolistic and polyethylene-glycol. However, biolistic and electroporation techniques are preferred because transformation using polyethylene-glycol requires the tedious process of regenerating plants from protoplast. The two techniques are being used in the development of genotype-independent transformation systems in those species that are difficult to transform, including cowpea (Rech *et al*., 2006).

Biolistic method

Since it was first reported (Sanford *et al.*, 1987), and since its subsequent modification using helium-power acceleration system (Sanford *et al.*, 1991), the biolistic

Table 1. Historical development of transgenic cowpea (*Vigna unguiculata* L.)

Explant	Gene(s) of Interest	Delivery system	Rational	Gene Integration Frequency	Authors
Leaf disc	*nptII*	A	Establishment of selection condition for transformation using Agrobacterium	Transgenic calli	Garcia *et al.*,1986
Leaf disc	CMPV M-RNA: *nptII*	A	Study the efficacy of 35S promoter from *Cauliflower Mosaic Virus* and infectivity of the clone viral DNA	Transgenic Calli	Garcia *et al.*, 1987
Leaf discs	*uid*A	E	Protocol development; test delivery and expression of *uid*A	Transgenic zygotic embryos	Penza *et al.*, 1992
Mature embryos	*uid*A, *hpt*	E	Protocol development; test exogenous gene delivery and expression using *uid*A	Transgenic zygotic embryos	Akella and Lurquin, 1993
Cotyledons	*Hpt*	A	Protocol development; establish selection condition for Agrobacterium-mediated transformation	Regenerated transgenic plant	Muthukumar, *et al.*, 1996
Cotyledonary node	*αA1-1*	B	Protocol development;insertion and expression of *αA1-1*	Transgenic plants / no progeny transmission	Kononwicz *et al.,* 1997
Meristematic tissue	*uid*A, *bar*	B	Protocol development; using selectable marker *bar* and reporter gene *uid*A	Progeny transmission/no Mendelian segregation	Ikea *et al.*, 2003
Cotyledonarynode	*uid*A, *bar*	A	Protocol development; using selectable marker *bar* and reporter gene *uid*A	Transgenic plants (0.15%) with Mendelian progeny transmission	Popelka *et al.*, 2006
cotyledonary node	*uid*A, *nptII*	A	Protocol development; study the integration and transmission of *uid*A, and *nptII*	Transgenic plants (0.76%) with Mendelianprogenytransmission	Chaudhury *et al.*, 2007
Meristematic tissue	*uid*A,*ahas*	B	Protocol development; study the integration and transmission of *uid*A, and *ahas genes*	Transgenic plants (0.9%) with Mendelian progeny transmission	Ivo *et al.*, 2008
Cotyledonary node	*αA1-1, nptII, uid*A	A	Insertion and expression of *αA1-1* and test if it can confer resistance against bruchids	Transgenic plants (1.64%) with	Solletie*t al.,* 2008
Nodal buds	*Cry1*Ab, *nptII*	E	Insertion and expression of *cry1*Ab and test its ability to confer resistance against *Marucavitrata*	Progenytransmission/not Mendelian Mendelian progeny transmission segregation	Adesoye *et al.*, 2008
Embryo	*uid*A	A	Protocol development; transform embryos by vacuum infiltration of *Agrobacteruim* cells	Transgenic plants (3.9%) with Mendelian segregation not reported	Adesoye *et al.*, 2010
Embryonic axes, cotyledon	*αA1-1, bar, nptII*	A	Insertion of *αA1-1* to provide protection against *Callosobruchusmaculatus*	Progeny transmission with Mendelian segregation	Higgins *et al.*, 2010
Cotyledonary node	*uid*A, *nptII*	A	Protocol development; study the integration and transmission of *uid*A, and *nptII*	Transgenic plant regenerated (1.61%)	Raveendar and Ignacimuthu, 2010
Cotyledonary node	*cry1*Ac, *nptII, uid*A	A	Protocol development; study the effect of sonication and vacuum infiltration on the integration and transmission of *cry1*Ac, and *nptII*	Transgenic plant with Mendelian segregation	Bakshi *et al.*, 2011
Cotyledonary node	*pmi*	A	Protocol development using mannose as selectable agent	Transgenic plant (3.6%) with Mendelian segregation	Bakshi *et al.*, 2012

(Particle bombardment) transformation system has been utilized in the transformation of various plant species. It is now an efficient transformation system in which biologically active DNA is driven at high velocity across the cell membrane into the cell cytoplasm and nucleus. Particle bombardment offers a number of advantages, including: (i) bypassing the biological limitation of *Agrobacterium* specificity; (ii) development of genotype-independent transformation system;(iii) gene stacking (introduction of multiple genes); and (iv) transformation of chloroplast and

Table 1. Continued.

Cotyledonary node	*GmIFS*	A	Insert and express *Glycine max isoflavone* synthase (*IFS*) gene in cowpea	Transgenic callus and roots	Kaur and Murphy, 2012
Immature Cotyledone	*bar, uid*A	A	Protocol development; insertion and express of *bar*	Progeny transmission/Mendelian segregation not reported	AASIM *et al.*, 2013
Cotyledonary node	*Cry1*Ac, *nptII*	A	Protocol development, insertion and expression of *Cry1*Ac in cowpea	Transgenic plant with Mendelian segregation (2.44%)	Bakshi and Sahoo, 2013
Embryonic axes	*Atahas*	B	Development of herbicide resistant cowpea	Progeny transmission with Mendelian segregation reported	*Citadin et al.*, 2013
Embryonic axes	ΔCSMVCABMV containing cp gene of CABMV and 32K protein of CPSMV	B	Development of virus-resistant cowpea using RNA interference construct to silence proteinase cofactor gene of CPSMVand coat protein gene of CABMV	Transgenic plant (1.04%) with Progeny transmission/Mendelian segregation	Cruz &Aragao, 2014

mitochondrial genome.

Kononowizs et al.(1997) were the first to report successful transformation of cowpea cotyledonary nodes via the biolistic method of gene transfer. Transgenic plants carrying *aAI-1* were reported, but no bioactivity or progeny transmission of the transgene were reported. Six years later, Ikea *et al.* (2003) reported regeneration of the first transgenic cowpea, in which progeny transmission of the transgenes to T_1, T_2 and T_3 generations was achieved. The authors bombarded meristematic tissues with plasmid-carrying reporter gene (*uid*A) and selectable marker gene (*bar*) under the control of *CaMV*35S promoter following pre-conditioning for 1 to 2 days on CP3 medium (Kononowicz*et al.*, 1997). Even though the transformation frequency was low (<1%), this work represents the first report of progeny transmission of transgenes in cowpea.

Following the success reported by Ikea *et al.* (2003), the first stable transformation of cowpea with Mendelian segregation of the transgenes was reported by Ivo *et al.* (2008). In this report, a genotype-independent protocol for stable transformation of cowpea was developed by bombarding the apical dome of embryonic axes following careful removal of primordial leaves. The plasmid constructs used contains the *gus* reporter gene and mutated *ahas* from *Arabidopsis thaliana* for selection using the herbicide imazapyr under the control of the *act2* promoter (*act2p*). The work no doubt demonstrated, for the first time, efficient and stable integration of transgenes and their subsequent segregations according to Mendelian law. With relatively high transformation efficiency (0.9%), this report opened up the possibility of using the system to obtain transgenic cowpea expressing genes of Agronomic importance.

Introduction of genes of Agronomic importance

It took over one and a half decades after the initial attempt by Kononowicz*et al.* (1997) to introduce the α-amylase inhibiting protein (*aAI-1*), before the first transgenic cowpea plant carrying a gene of agronomic importance was produced using particle bombardment. Citadin et *al.* (2013) obtained herbicide-resistant transgenic cowpea following bombardment of embryonic axes with vector pAC321 (Aragao*et al.*, 2000) cloned with acetohydroxyacid synthase coding gene (*Atahas*) from *A. thaliana,* which confers tolerance to imidazolinone. Transgenic plants were obtained at a frequency of 0.9% with T_1, T_2 and T_3 plants, demonstrating high tolerance of up to fourfold (400g/ha) concentration of imazapyr recommended for commercial weed control. In addition to high tolerance to imazapyr expressed by the progenies, one interesting peculiarity of this work is that it represents the first report in the history of cowpea transformation in which regeneration of non-chimeric transgenic plants with stable integration and transmission of the transgene with Mendelian segregation was achieved.

In a similar drive, Cruz and Aragao (2014) used the transformation system developed by Ivo *et al.* (2008) to generate transgenic cowpea plants expressing a chimeric gene comprising a fragment from the proteinase cofactor gene of *Cowpea Severe Mosaic Virus* (CPSMV) and a fragment of the coat protein gene of *Cowpea Aphid Borne Mosaic Virus*(CABMV). Transgenic cowpea lines were generated with transformation efficiency of 1.04%, and PCR analysis confirmed the presence of DCSMVCABMV in all the primary transformants (T_0). A bioassay using mechanical inoculation of the viruses in T_2 generations and subsequent analysis of the plants indicated that

resistance against the viruses was homozygosis-dependent. Homozygous plants expressed total resistance to CPSMV and CABMV, while hemizygous plants expressed milder resistance to the viruses.

Electroporation

In electroporation, a given gene of interest is made to pass through the cell membrane of the host cell via pores created when electric current is applied across a living surface. The system capitalizes on the relatively weak nature of hydrophobic/hydrophilic interactions in the phospholipid bilayer of the cell membrane and its ability to spontaneously reassemble after disturbance (Purves*et al.,* 2001). Electric pulse results in the formation of temporary aqueous pores and an increase in the electric potentials across the membrane, so that charged molecules like DNA are easily driven across the membrane through the pores.

Since the first report of its application in maize (D'halliun*et al.*, 1992), electroporation has been used to transform a number of plant species. Penza *et al.* (1992) were the first to report the transient expression of a chimeric reporter gene (*uid*A) following electroporation of seed-derived embryos of *V. ungiuculata*. They demonstrated that embryos could take up and transiently express the chimeric gene (*uid*A) following electroporation-mediated DNA transfer. Akella and Lurquin (1993) further demonstrated that electroporation of embryos in the presence of DNA and protectants such as spermine and cationic liposome increased not only the proportion of embryo-derived seedlings expressing the chimeric gene but also the level of gene expression. However, in both attempts, generation of transgenic plants from the transformed embryos was not reported.

Despite the pioneering work of Penza*etal.* (1992) and Akella and Lurquin, (1993), it took close to two decades to produce the first electroporation-mediated transgenic cowpea plant when Adesoye*et al.* (2008) electroporated nodal buds in the presence of plasmid-carrying insect resistance gene (*Cry1*Ab),a selectable marker (*nptII*) driven by *CaMV* 35S promoter. The work reported stable integration of *Cry1*Ab with T3 progenies showing complete protection against Maruca vitrata larvae (Adesoye*et al.*, 2008).

Future Prospects

The demand for genetically improved cowpea with agronomic traits is increasing owing to its immeasurable value for food, feed and soil improvement, especially in semi-arid regions of sub-Saharan Africa. In the past two decades, a number of transformation protocols and their applications in generating cowpea with genes of agronomic importance have been reported (Table 1). Studies so far have been predominantly centered on the development of transgenic protocols for various genotypes. In the majority of the reports, transgenic cowpea lines contain reporter genes and/or selectable marker genes to demonstrate proof of genetic modification, and few reports have actually expressed genes of agronomic importance.

Success has been recorded in the stable integration of *aAI-1 Cry1*Ab,*Cry1*Ac,*Atahas*and development of *si*RNA-mediated resistance against *Cowpea Severe Mosaic Virus* (CPSMV) and *Cowpea Aphid Borne Mosaic Virus* (CABMV) in cowpea (Table 1). These achievements no doubt demonstrated the enormous benefits that the crop stands to gain from transgenic technologies (Citadin*et al*., 2011). Significant resistance to *aAI-1* and *Cry1*Ab in transgenic cowpea lines under field conditions has been reported in Australia and Nigeria (Luthi*et al.*, 2013; Mohammed *et al*., 2014).

The need to improve transformation technologies for efficiency, especially in generating lines with stably inherited traits, is a priority. This is even more important, especially for the commercial production of transgenic cowpea cultivars expressing genes of agronomic importance. In a commercial transgenic cultivar development program, a large number of transformants (dozens to hundreds) are produced and screened phenotypically to identify the few that have the most desirable expression of the transgenic trait (Bradford *et al*., 2005). This is mainly because the transformants produced generally have various levels of transgene expression (either over expression or down-regulation) and often contain inserts that are either re-arranged or in multiple copies (Wang and Ge, 2006).

On the other hand, low transformation efficiency may be attributable to the current organogenesis-based regeneration protocol employed in the transformation of cowpea. Overcoming the recalcitrant nature of cowpea to *in vitro* manipulations via *de novo* regeneration is an important challenge researchers are yet to overcome. As in many other plant species, the development of a regeneration system that provides totipotent cells will ensure accelerated production of transgenic cowpea expressing genes of interest.

With success recorded in Bt toxin and α-amylase inhibiting protein in cowpea, it is clear that commercially available transgenic cowpea cultivars with an acceptable level of insect resistance will be available in the next few years. Currently, a transgenic common bean (*Phaseolus vulgaris* L.) resistant to *Bean golden mosaic virus* (BGMV) has been generated and made available for commercialization.

However, new techniques are needed in pest management systems, due to continuous development of resistance against the existing control techniques. Development of some resistance to α-amylase inhibiting protein and Bt toxin has already been reported (Jongsma and Bolter, 1997;Tabashnik*et al.*, 2013). Therefore, other candidate genes for insect protection need to be exploited. Potential candidate genes such as fungal *chiti-*

nases, *Streptomyces cholesterol oxidase, isopentenyl-transferase gene* (*ipt*) from *Agrobacterium tumefaciens*, genes encoding insect viral RNAs and other plant-derived genes have already been suggested (Machuka, 2000).

The success achieved in the development of RNA interference-mediated resistance against *Cowpea Severe Mosaic Virus* and *Cowpea Aphid Borne Mosaic Virus* (Cruz and Aragao,2014) has indicated that the approach is emerging as a valuable alternative means of controlling insect pests and pathogens in cowpea. RNAi showed great potential owning to its high specificity, as has been demonstrated, and is a new specific method for the control of western corn rootworm (*Diabrotica virgifera virgifera*), and cotton bollworm (*Helicoverpa armigera*) (Baum *et al.*, 2007; Mao *et al.*, 2007). The vast number of essential genes that can be silenced in insects and pathogens demonstrate the plentiful chances available in the application of dsRNA and the potential of RNA interference in the control of problems associated with cowpea production. With the emergence of promising new tools like the CRISPR (clustered regularly interspaced palindromic repeats)/CRISPR-associated 9 (CRISPR/Cas9) system, it is expected that the speed with which new and desirable traits are effectively inserted into cowpea genome has improved (Zaidi *et al*., 2016).

CONCLUSION

Significant progress has been achieved in the last three decades in the development and refinement of protocols for the regeneration of transgenic cowpea. Cowpea regeneration via organogenesis is now well established and has been utilized in the development of lines expressing genes of agronomic importance. Cowpea lines expressing *αAI-1cry1*Ab, *cry1*Ac At *ahas* and RNAi-mediated resistance against *Cowpea Severe Mosaic Virus* and *Cowpea Aphid BorneMosaicVirus*are now available, and the transmissibility and efficacy of these genes in progenies are being evaluated under field trials in different countries. Although cowpea varieties expressing a gene of agronomic importance are not yet commercially available, the success achieved so far has indicated that goals not reachable by conventional breeding could be achieved in the coming years. While challenges posed by the difficulty of regenerating cowpea, especially via the *de novo* process, seem to have been circumvented, regeneration via somatic embryogenesis is still the best means of achieving a stable transformation system. The recalcitrant nature of cowpea for in *vitro* regeneration, especially via somatic embryogenesis, has so far delayed the development of commercially available cowpea carrying a gene of agronomic impotance. Therefore, much effort needs to be devoted to overcoming cowpea's recalcitrance for in vitro regeneration via the de novo process.

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