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Regeneration and genetic transformation in cowpea

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Abstract
Over the last three decades, sporadic efforts have been made to develop regeneration and transformation systems in cowpea (*Vigna unguiculata* L. Walp). This paper reviews the progress made to date, including highlights of culture media and explants used for regeneration and chimeric gene constructs employed in transformations. Progress has been slow, mainly due to limited resources, since very few laboratories have been involved. There is an urgent need for more focused and consistent efforts to develop genotype, and tissue-culture dependent and independent approaches for obtaining stable genetic transformation in cowpea.

Introduction

Cowpea faces several biotic and abiotic stresses for which conventional breeding alone may not provide ultimate solutions. For example, grain yield losses are mainly due to damage caused by insect pests and diseases, as well as abiotic stresses such as heat and drought (Singh et al. 1997). Plant molecular biology and genetic engineering approaches offer alternative ways of overcoming these stresses. In addition to direct transfer of genes of agronomic interest, genetic transformation techniques can be used to answer many basic questions pertaining to cowpea biology such as understanding of gene function and regulation of physiological and developmental processes (Gelvin 1998). These benefits require the development of reliable, efficient, and reproducible methods for cowpea transformation and regeneration.

Although legumes are considered "recalcitrant" to regeneration and transformation, routine protocols for obtaining stable transformants are now available for the major grain legumes such as the common bean (*Phaseolus vulgaris*), soybean (*Glycine max*), pea (*Pisum sativum*), peanut (*Arachis hypogea*), and alfalfa (*Medicago sativa*), as well as the model legume, barrel medic (*Medicago truncata*) (Christou 1992; Puonti-Kaerlas et al. 1990; Russell et al. 1993). In contrast, development of tractable gene transfer systems in cowpea has been impeded by several constraints. Cowpea is not of major economic importance to the most technologically advanced countries in North America and Europe. This crop is mainly grown in tropical Africa, Asia, and Latin America where technical expertise and infrastructure for biotechnology research are either lacking or poor. Therefore, comparatively little work has been done to develop and optimize regeneration and transformation procedures, relative to temperate crops that are of economic importance in the North, including recalcitrant cereals (Komari et al. 1998). This paper reviews

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previous work on cowpea cell and tissue culture and transformation. It also highlights future research directions that could hold promise for the establishment of reliable gene transfer systems for a crop that has tremendous potential as a rich source of dietary protein for millions of people in Africa and Asia.

Cell and tissue culture

The two methods commonly used for regeneration of plants from cell cultures are somatic embryogenesis and organogenesis. Both methods are controlled by plant hormones and other factors added to the culture medium. As the name suggests, somatic embryogenesis involves the generation of embryos from somatic tissues, such as roots, cotyledons, leaves, stems, and reproductive organs. The proliferating somatic embryos are either induced in liquid culture or on solid medium. Since embryogenic tissues are very prolific and usually originate from single cells, the embryos are considered excellent targets for transformation (Hansen and Wright 1999). This is why somatic embryogenesis is the method of choice for most genetic transformation protocols for recalcitrant legumes and monocots such as soybean, maize, and rice, respectively (Komari et al. 1998; Puonti-Kaerlas 1993; Trick et al. 1997). In cowpea, induction of somatic embryos has been reported to occur in suspension cultures of calli derived from seedling leaf explants (Ganapathi and Anand 1998). Embryogenic calli were induced on solid Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 1.5 mg/liter (mg/L) of 2-, 4-dichlorophenoxy-acetic acid (2-, 4-D). The maximum frequency of somatic embryos was obtained when callus was transferred to liquid MS medium supplemented with 0.5 mg/L 2-, 4-D. This work is repeated in other laboratories, including characterization of the stages and processes of somatic embryo development. Additionally, other explant sources other than young leaves should also be investigated for their ability to produce somatic embryos in solid and liquid suspension cultures. The basal medium developed for embryo development by Pellegrineschi et al. (1997) could form a starting point for formulating media for growth of somatic embryos in vitro. Growth medium supplements that enhanced embryo development included addition of sucrose, casein hydrolysate, and any one of three commonly used cytokins, namely zeatin, benzyl amino purine (BAP), and kinetin, for enhancing embryo maturation.

The establishment and maintenance of embryogenic cultures as well as recovery of plants can be an extremely labor intensive and lengthy process that has the added risk of encountering morphological abnormalities and sterility among regenerants. In contrast, multiple shoot formation via organogenesis is simpler once a suitable explant has been identified. Various laboratories have independently reported successful regeneration of cowpea by direct organogenesis from a variety of explants. These include roots, stem pieces, intact immature cotyledons or protoplasts derived from them, leaves, stem apices, stimulated shoot bud formation following gamma irradiation, or germination of mature seeds in the presence of the herbicide thidiazuron (Kartha et al. 1981; Subramaniam et al. 1968). Shoot regeneration has also been reported using axenic cowpea hypocotyls and cotyledons excised from green immature pods of advanced breeding lines and varieties developed at the International Institute of Tropical Agriculture (IITA) in Ibadan, Nigeria, (Pellegrineschi 1997). The apical parts of the embryos were removed and the hypocotyls were transferred to regeneration

media modified from a formulation that was previously employed for embryo rescue (Pellegrineschi et al. 1997). Fertile cowpea plants have been regenerated successfully using nodal thin cell layer (TCL) explants. The TCL, approximately eight cells thick, was obtained by cutting twice over each cotyledonary node, followed by regeneration on MS media containing either 1.1 mg/L zeatin and 0.05 mg/L indole butyric acid (IBA) or 1.1 mg/L BAP and 0.05 mg/L IBA.

Among these explants, direct organogenesis from cotyledons, cotyledonary nodes, epicotyls, and primary leaves cultured on MS containing optimal levels of either N^6-benzyladenine (BA) or BAP appear to be reproducible and hold promise for use in transformation (Brar et al. 1999; Muthukumar et al. 1995; Obembe et al. 2000a; Pellegrineschi 1997). At IITA, organogenesis has been obtained in several genotypes such as 90K-277, 89D-288, 83D-442, 86D-1010, 93K-624, Vita 3, and Ife Brown (Fig. 1a). Shoot meristem regeneration on MS media supplemeted with either the herbicide thidiazuron or BAP has been successfully demonstrated in various genotypes, including CB5, TARS 36, SUV-2, 283, 1137, 275, TN88-63, B301, Tvu 9062, Vita 3, Vita 4, and 58-57 (Kononowicz et al. 1997; Monti et al. 1997). Brar et al. (1999) have recently reported a regeneration system that was applicable to 17 US commercial cowpea cultivars and breeding lines. Cotyledons were initiated on 1/3 MS medium containing 15–35 mg/L of BA followed by shoot regeneration on MS containing 1.0 mg/L of BA. Depending on the genotype, regeneration percentages ranged from 1 to 11, with 4–12 multiple shoots produced per explant. For rooting of cowpea plantlets, the report of Brar et al. (1999) and our results show that hormone-free MS medium works well. However, addition of 1.0 mg/L of indole-3-acetic acid (IAA) or 0.05 mg/L of nephthalene acetic acid (NAA) significantly enhances rooting and survival of plantlets in soil during the hardening and acclimatization phase following transfer from tissue culture conditions (Obembe et al. 2000a). A procedure for protoplast isolation from leaf mesophyll cells and regeneration leading to production of microcalli has also been described. However, plant regeneration from protoplast-derived calli was not possible, rendering the system inapplicable for heritable gene transfer.

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Figure 1A. In vitro cowpea regeneration from cotyledonary nodes cultured on 0.5 mg/l of benzyl amino purine (BAP).

Transformation systems

Currently used methods for genetic transformation have been classified into natural and non-natural or in vitro methods (Gelvin 1998). The latter include DNA microinjection (Neuhaus and Spangenberg 1990), direct DNA uptake into protoplasts with or without the use of electroporation (Shillito 1999), use of silicon carbide whiskers (Kaepplar et al. 1990) and biolistic bombardment (Hadi et al. 1996; McCabe et al. 1998; Shillito 1999). Natural methods involve the use of viral vectors that will result in transient but not stable transformation (Choi et al. 2000; Masuta et al. 2000) and *Agrobacterium tumefaciens* T-DNA-mediated transformation (Zupan et al. 2000).

There are two major causes for the delay in the development of methods for the genetic transformation of legumes, in comparison to other dicotyledonous species. First, is the problem of recalcitrancy to regeneration by somatic embryogenesis and organogenesis, as already discussed. Secondly, transformation mediated by the soil bacterium *A. tumefaciens* was not, initially, readily applicable to legumes. Therefore, attempts at gene transfer initially focused on direct DNA delivery, especially by microprojectile (particle) bombardment which is still a popular technique since it is species- and genotype-independent (Christou 1992; McCabe et al. 1998). It has now been demonstrated that *A. tumefaciens* can efficiently transform legumes such as soybean (Trick et al. 1997). In the following section, we will review the methods and results of previous work that has been done on genetic transformation in cowpea.

Agrobacterium-mediated transformation

The earliest report on *Agrobacterium*-mediated cowpea transformation was based on the tobacco leaf disc transformation method (Horsch et al. 1985). Cowpea leaf discs were punched from primary leaves obtained from 6-day old seedlings and co-cultivated with *A. tumefaciens* strains harboring tumor inducing (*Ti*)-derived vectors containing two copies of a chimeric kanamycin resistance gene (Garcia et al. 1986a, 1986b). *A. tumefaciens* strain C58CI harboring the non-oncogenic *Ti* plasmid pGV3850::1103*neo,* or its derivatives, strain LBA 1010 containing the octopine type *Ti* plasmid pTIB6 and strain LBA 958 containing a nopaline type Ti plasmid were all infective on cowpea leaves and stems. For selection of transformed tissues, G418 (50 mg/L) was initially incorporated into the culture media, but tissues were transferred and selected on kanamycin (100 mg/L) during later subcultures. This procedure resulted in stable transformation of callus, but no transgenic plants were regenerated. The full length cDNA of cowpea mosaic virus (*CPMV*) gene under the control of either the cauliflower mosaic virus (*CaMV* 35S) or nopaline synthase (nos) promoter was stably transferred and expressed in cowpea calli (Garcia et al. 1986b). The *CaMV* 35S was also more than ten times stronger than the nos promoter. Moreover, this work showed that 7-day old cowpea plants (stems) are susceptible to *Agrobacterium* infection, since both oncogenic *Agrobacterium* strains LBA 1010 and LBA 958 induced crown galls at wounded stem sites. An earlier study by Saedi et al. (1979) showed that cowpea seedlings fail to develop tumors after being inoculated with *A. tumefaciens* if, at times earlier than one day later, they were inoculated on the primary leaves with a cowpea mosaic virus that systemically infects them. Inoculation with buffer or with a virus that is restricted to a localized infection, or to which the cowpea is immune, did not interfere with the subsequent development of tumors. These observations indicated that systemic virus infection may induce in cowpeas a translocated substance that prevents tumor induction

by *A. tumefaciens*. Therefore, the pathology of cowpea tissues may be an important factor to consider during *Agrobacterium*-mediated transformation. We have found LBA 4404 (carrying octopine type plasmid pTiA6) to be least virulent on cowpea tissues cultured in vitro, compared to AGL1, a disarmed, hypervirulent strain harboring mannopine-type *Ti* plasmid pTiBo542. PGV3850, another disarmed, wide host range hypervirulent strain harboring a nopaline-type *Ti* plasmid pTiC58, is also very virulent on cowpea (Obembe et al. 2000b).

Only a few other reports have appeared in scientific literature concerning *Agrobacterium*-mediated transformation of cowpea since the excellent early work of Garcia et al. (1986a, 1986b). Perkins et al. (1987) and Filippone (1990) were able to show stable transformation of callus by co-cultivation of mature embryos, cotyledonary node buds, epicotyls, and apical meristems with *A. tumefaciens*. Cowpea accessions used in Filippone's work were IT81D-994, Tvu 9062, and cv VITA4. Transformations utilized the hypervirulent *A. tumefaciens* strain 6044 containing plasmid pGA472 carrying the neomycin phosphotransferase (*NPTII*) gene. Selection of transformed calli was carried out on 100 mg/L kanamycin or 50 mg/L geneticin. When cowpea embryos were used, the parts most amenable to transformation were the collar and epicotyls (Filippone 1990). Penza et al. (1991) reported the production of chimeric beta-glucuronidase (*gus*) (Jefferson 1989) in transgenic cowpea plants from mature embryos co-cultivated with *A. tumefaciens*. Using excised, ungerminated embryos was seen as a way of bypassing problems associated with regeneration from callus and differentiated tissues. Co-cultivation of embryos with the disarmed *A. tumefaciens* strain C58 (pGV2260/p35SGUSINT) carrying a *gus* intron resulted in chimeric, transformed shoots derived from axillary buds. Transformed cells were mostly located in subepidermal regions of the plant stems where the L2 meristematic layer is positioned (Fletcher and Meyerowitz 2000). Since the L2 layer potentially can contribute to flower buds, it still remains unclear why the transgenes were not transmitted through the germline, despite extensive plant propagation through nodal culture (Penza et al. 1991). The ability to regenerate cowpea *in planta* (Machuka 2000) as well as the use of positive selection systems (Joersbo et al. 1998) may provide avenues for recovery of stable transformed plants. If successful, the mature embryo co-cultivation method would be simple and easy to use for large-seeded legumes such as cowpea. Using excised leaf, epicotyl, and hypocotyl explants, stable callus transformation was obtained after co-cultivation of the explants with LBA 4404 carrying the *gus*-intron plasmid p35SGUSINT. Through co-cultivation of these explants with *A. rhizogenes,* the same workers demonstrated production of transgenic hairy roots following in vitro selection on kanamycin. Hairy root transformation was also reported earlier (Suzuki et al. 1993). These workers used a soybean cell wall protein gene (*SbPRP1*) promoter-GUS construct to show localization of *SbPRP1* in actively growing roots (apical and elongating regions) during cowpea seedling growth.

Publications on stable *Agrobacterium*-mediated transformation incorporating southern analysis of primary transformants are available (Muthukumar et al. 1996; Kononowicz et al. 1997; Monti et al. 1997). Muthukumar and co-workers used mature de-embryonated cotyledons excised from 2–3-day old seedlings. The cotyledons were co-cultivated with *A. tumefaciens* and transformed tissues selected on 25 mg/L hygromycin. Our preliminary work on the effect of hygromycin on in vitro regeneration and rooting of untransformed cowpea has established significant inhibition levels at ≥20 mg/L (Obembe et al. 2000b).

Although Muthukumar et al. (1996) reported that 15–19% of explants produced shoots on hygromycin selection medium, 13 out of 17 putative transformants died. Unfortunately, seeds from the four remaining plants failed to germinate, thus leaving us without reproducible evidence of stable transformation. Research teams at Purdue University (USA) and the University of Naples (Italy) obtained transformed T0 plants using the *gus* reporter gene as well as two useful genes. However, results from further analysis to establish proof of stable transformation and reliability of the protocols have not been forthcoming. Despite this, the work was useful in many respects. For example, tests pertaining to the virulence of *Agrobacterium* strains revealed that A281, a hypervirulent oncogenic strain, was most infective, followed by EHA 101, whereas LBA 4404 had the lowest virulence (Kononowicz et al. 1997; Monti et al. 1997). For many plant species, *Agrobacterium*-mediated transformation is relatively efficient, and a low copy number of intact, nonrearranged transgenes are frequently integrated into the plant genome (Zupan 2000). These observations and the foregoing discussion indicate that *Agrobacterium*-mediated transformation in cowpea is feasible and may yet be the preferred choice for laboratories that work or plan to begin work on genetic transformation in cowpea.

Transformation with naked DNA

Microprojectile bombardment can be performed with any tissue of most species; however, the process is relatively inefficient because few cells are stably transformed. When DNA is delivered by this method, the conversion rate from transient expression to stable integration is estimated to be <1 to 9% (Hansen and Wright 1999; Finer et al. 2000). This method of transformation has been used on cowpea cotyledon segments, immature embryos, and shoot meristems (Ikea 1998; Kononowicz et al. 1997; Monti et al. 1997). However, convincing molecular evidence of transformation in T1 and subsequent progeny was not provided. In the work of Kononowicz et al. (1997) and Monti et al. (1997), some chimeric gene constructs used in transformations contained the phosphoinothricin (*bar*) resistance, *gus* and *NPTII* genes, driven by *CaMV 35S* or nos promoters. Other constructs contained sequences encoding the common bean α-amylase inhibitor or Bex (2S albumin) protein from Brazil nut, under control of phaseolin (seed-specific) or CaMV 35S (constitutive) promoters. Putative transformed tissues were selected on 50 mg/L kanamycin, which is probably not stringent enough to prevent escapes.

Plant transformation using protoplasts is laborious and requires a lot of finesse. Once isolated mechanically or using enzymes, the protoplasts can be transformed either by *Agrobacterium* or by direct DNA uptake methods, facilitated by polyethylene glycol (PEG) treatment, electroporation, or liposomes (Shillito 1999). The method has the advantage that single cells can be targeted for transformation, provided the protoplasts can regenerate into whole plants. Using cowpea leaf mesophyll protoplasts, stable, PEG-mediated protoplast co-transformation of two plasmids (pGL2 and pMON*GUS*) carrying the hygromycin resistance and *gus* genes were obtained. Stable transgenic microcalli were obtained that could not be regenerated into plants.

Electroporation of cells or tissues in the presence of DNA is used for the introduction of transgenes either stably or transiently into bacterial, fungal, animal, and plant cells (Lurquin 1997; Joersbo and Brunstedt 1991). The method is not often used in plant transformation because of its low reproducibility. However, owing to difficulties encountered in regenerating transformed cowpea cells and tissues in vitro, electroporation of intact

tissues and organs has been resorted to with promising results. Early work using cowpea seed-derived embryos showed that chimeric transgenes could be expressed in cowpea protoplasts and seedlings after passive or electroporation-mediated naked DNA transfer (Akella and Lurquin 1993; Penza et al. 1992). Electropration-mediated DNA delivery into seedling tissues was also demonstrated by Dillen et al. (1995), not only in cowpea but also in other grain legumes such as the common bean, pea, and soybean. Linearization of plasmid DNA markedly increased transient DNA expression levels in intact hypocotyls and epicotyls. It is not clear what is the conversion rate from transient expression to stable integration in the plant genome using electro-transformation, but it is likely to be low (Lurquin 1997; Joersbo and Brunstedt 1990).

Chowrira et al. (1995) at Washington State University, Pullman, provided evidence of both transient and stable expression of the *gus* gene after electroporation of auxillary nodal meristems *in planta*. The branches that grew out of the nodal meristems were chimeric and expressed the introduced gene up to 20 days after electroporation (Chowrira et al. 1996). Transgenic T1 pea, lentil, and cowpea plants were recovered from seeds originating on these chimeric branches as shown by Southern blot hybridization and gus expression. Although transgenic T2 soybean and lentil plants were also obtained, no transgenic T2 cowpeas were reported. Segregation ratios in these populations showed a strong bias against transgene presence or expression. This in vivo transformation approach has at least two advantages. First, electroporation equipment is cheap and the protocols are easy to optimize (Lurquin 1997). Secondly, seeds can be obtained without need for in vitro steps, thereby speeding up the process of generating transgenic plants. The occurrence of chimeras may be reduced if selection systems can be developed for cowpea, such as phosphinothricin (Fig. 1b) and kanamycin painting and chlorophyll fluorescence for phosphinothricin and kanamycin resistance, respectively (Eu et al. 1998; Rasco-Gaunt et al. 1999).

Other promising transformation methodologies

The recent development of simple and routine de novo floral and seedling dipping and/or infiltration procedures for *Agrobacterium*-transformation in *Arabidopsis* and *M. truncata* (Clough and Bent 1998; Trieu et al. 2000) has sparked new optimism to develop similar techniques for other crops. In comparison with these model plants, cowpea has few flowers that would be the key target for transformation. Furthermore, comparatively few seeds are set. Since electroporation of cowpea nodal tissue has already been reported (Chowrira et al. 1996), work is in progress at IITA to maximize the number of vegetative and floral buds produced at every node or at the shoot apex through hormonal applications (Machuka 2000). This procedure has potential for coupling to *in planta* transformation techniques, notably electroporation and dipping of hormone-induced organs in *Agrobacterium* suspensions (Fig. 1c). Transient *gus* expression assays indicate that use of Silwet-L77 in conjunction with acetosyringone enhances expression following vacuum infiltration of excised mature cowpea embyos (Fig. 2). Experiments utilizing these additives in *Agrobacterium* seedlings and floral dipping and infiltration solutions are in progress at IITA. Selection of transformed tissue is likely to be the key obstacle for reliable adoption and exploitation of a de novo cowpea regeneration-based transformation system. Natural plant transformation technologies that include the use of viral vectors for transient transformation should also be explored for cowpea (Choi et al. 2000; Masuta et al. 2000). It is already known that full-length cDNA copies of cowpea mosaic virus RNA cloned downstream of the *CaMV*

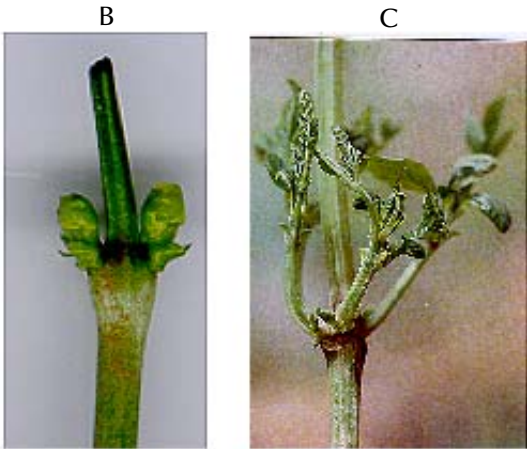

Figure 1 B, C. *In planta* cowpea regeneration of decapitated seedlings (B) and three-week old plants (C) treated with 10 mg/L BAP.

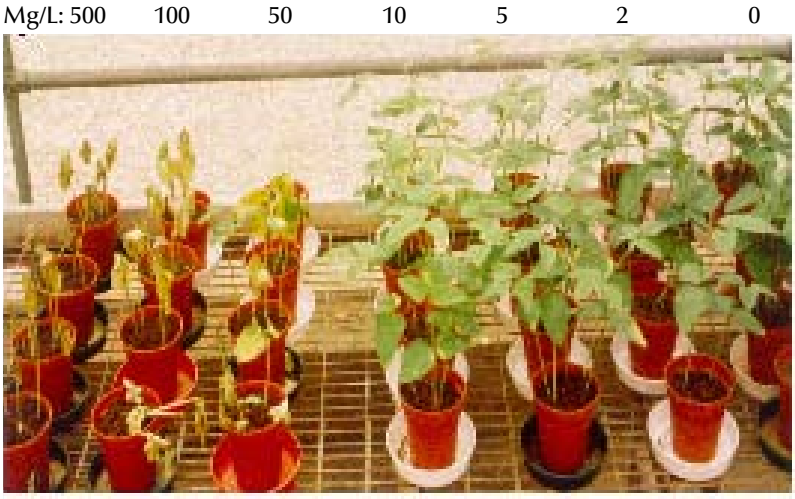

Figure 2. Phosphinothricin (PPT, Duchefa Bichemie, Haarism, Holland) painting of cowpea plants. Numbers represent PPT concentrations. As seen in this photo, survival of seedlings was nil 7 days after spraying with PPT concentrations exceeding 50 mg/L.

35S promoter give rise to cowpea mosaic virus-like symptoms when inoculated onto cowpea plants (Dessens and Lomonossoff 1993). More recently, the clover yellow vein virus has been developed as an efficient vector system for stable foreign gene expression in legumes *in planta* (Masuta et al. 2000).

Techniques for DNA delivery using silicone carbide whiskers (potential carcinogens), microinjection, and laser microbeams (Hansen and Wright 1999) require much finesse and may not be easily adapted for use in African and Asian countries which are likely to

benefit most from genetic modification in cowpea breeding. However, groups working on cowpea transformation need to experiment with techniques that combine the best attributes of *Agrobacterium*-mediated transformation (high efficiency, low copy number, and intact transgenes) with particle technologies (Gelvin 1998). For example, a novel strategy termed "Agrolistic" transformation could be used on cowpea tissues that are susceptible to transformation by particle bombardment (Ikea 1998; Kononowicz et al. 1997; Monti et al. 1997). This technique has the potential to integrate a low copy number of transgenes without integration of plasmid vector sequences (Hansen and Chilton 1996).

Conclusions

The powerful combination of conventional and genetic modification breeding has the potential of greatly enhancing the productivity of cowpeas by increasing resistance to pests, diseases, *Striga*, and abiotic stress, as well as seed quality and other traits that impact on cowpea utilization for fodder and grain. To be of value, genetically modified plants must faithfully transmit their transgenes. From the works surveyed in this review, it is apparent that this has not been achieved in cowpea. Recalcitrance to plant regeneration of transformed tissues, epidermal transformation, and transgene instability are likely causes of failure to achieve stable transformation and transgene transmission. Improvements in existing cell and tissue culture systems to allow regeneration of stable transformed cowpea plants is urgently needed. With so many available advances and new breakthroughs in plant transformation technologies, it is hoped that cowpea's stubborn resistance to genetic engineering will soon be overcome.

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