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Opportunities for biotechnology in cowpea*

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Abstract

Several applications of biotechnology have been successfully used recently in cowpea. A molecular map of cowpea has been constructed using RFLP markers, and this has already facilitated the localization of certain quantitative trait loci; gene chromosome localization using in situ hybridization is in progress. Appropriate bioassays have been developed that have facilitated the identification of candidate genes for insect pest resistance in cowpea, including *Bacillus thuringensis* protoxin genes, and genes coding α-amylase inhibitor, protease inhibitor, and lectins. Since cowpeas are "recalcitrant" to regenerate "in vitro", several attempts have been made to develop a reliable protocol for differentiating shoots from calli obtained through in vitro tissue cultures. Thus far, only regeneration from already meristem-rich tissues has been obtained. The best results were obtained using the herbicide, thidiazuron, as a growth regulator to induce multiple bud proliferation. *Agrobacterium*-mediated plant genetic transformation remains an approach that requires considerable further work to be efficient. Direct plasmid DNA transfer into meristematic cells has also been attempted using microprojectile bombardment; rates of genetic transformation are too low to be useful. Recently, two new transformation methods were set up on in vivo plants: the first is based on electroinjection of plasmid DNA directly into meristematic cells, and the second involves the inoculation of buds with *Agrobacterium;* these two methods do not need in vitro regeneration and are giving promising results.

Introduction

The role of cowpea in the nutrition and farming systems of Africa is well known; also well known are the reasons for the poor yields of this crop, prominent among which is its susceptibility to several insect pests and diseases. Though improved varieties have been obtained with modifications of the plant habit and the introduction of genetic resistance to some diseases, the crop still suffers from several insect pests: the cowpea pod borer, flower bud thrips, and the pod-sucking bug complex cause very large losses. No good source of resistance against these pests has been found in cowpea, which is why an international network of institutes was promoted by the International Institute of Tropical Agriculture (IITA), with the purpose of exploring the use of innovative technologies to solve such

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intractable problems. This international effort was considered necessary, because cowpea, compared with cash crops where biotechnologies have been successfully applied in industrialized countries, had received no attention from biotechnologists, being considered a "minor" crop.

This paper reports the main results obtained by the use of molecular techniques to improve our knowledge of the cowpea genome, and to transfer genes from other species into it. As most of the efforts are directed toward obtaining cowpea transformed for insect resistance, studies reported here focus on genes that are, at present, the best candidates for this purpose.

Genome analysis

As with many other seed legume species, there is little knowledge of the cowpea genome. Only recently has each of the 11 chromosomes of *V. unguiculata* been characterized (Saccardo et al. 1992), and cytological differences among some *Vigna* species described.

Improved chromosome characterization of cowpea has been obtained using C-banding techniques. The combined use of C-banding and of fluorochromes (CMA and DAPI) led to the identification of two classes of heterochromatin (Galasso et al. 1993). Further improvement came from the application of molecular cytogenetic techniques. Two rDNA probes were co-localized on metaphase chromosomes of cowpea and wild allies, demonstrating a constant association of one of these probes to the CMA-bright heterochromatin type. Additionally, a repetitive sequence of ~ 500 bp was cloned from cowpea and hybridized in situ on metaphase chromosomes and on membrane to genomic DNA digests from several species of the *Phaseoleae*. The results demonstrate that this sequence, named pVuKB1, is species-specific and localized in the centromeric heterochromatin blocks of cowpea (Galasso et al. 1995). Finally, the extension of the analyses of other accessions of cowpea allowed the recognition of a polymorphism for the number of rDNA sites.

Because of limited conventional genetic studies, only a few morphological and physiological marker genes are known in cowpea. The development of restriction fragment length polymorphism (RFLP) and random amplified polymorphic NDA (RAPD) marker technologies has contributed in the past two years to the construction of a cowpea linkage map. Using clones from common bean, mung bean, soybean, and cowpea (Fatokun et al. 1993a), ~ 100 loci were identified as being distributed into 10 linkage groups. The current map has already facilitated the localization in two genomic regions of some major quantitative trait loci controlling seed weight (Fatokun et al. 1992). The average distance between adjacent markers is ~ 7 cM and an increase in marker density is expected, since other segregating populations are being investigated (Menancio-Hautea et al. 1993). Once a more complete DNA marker-based map is obtained, marker-assisted selection for agronomically important traits will be facilitated. An RFLP map of moderate density in the *Vigna* genus will also improve our knowledge of the origin of cowpea, its evolution, and its phylogenetic relationships with closely related species (Fatokun et al. 1993b).

Genetic engineering

Several attempts are in progress to overcome interspecific barriers to gene flow, in order to transfer pest resistance traits that are present in wild species into cowpea. Histological

studies have shown that after crossing *V. unguiculata* with *V. vexillata*, F_1 embryos start to develop but collapse while still in the globular stage (Barone and Ng 1990; Ng 1992). Successful crossing of *V. luteola* and *V. oblongifolia* resulted in hybrid plants that can be used as bridges for crosses to cowpea (Schnapp et al. 1990).

Molecular technologies have opened up new opportunities for crop breeders by enabling them to use isolated single genes derived from other organisms, and these techniques can now be applied also to cowpea, where recently successful regeneration and genetic transformation experiments were carried out.

In transferring selected genes from one species to another using recombinant DNA techniques, priority has been given to insect resistance genes. This kind of research requires (1) the setting up of effective bioassays for discovering resistance genes for specific pests; (2) the use of those bioassays to search through the plant, fungal, animal, and microbial kingdoms for suitable genes; and (3) the understanding of insects' physiological and biochemical systems that are vulnerable to resistance genes.

Cowpea has many insect pests, but we are still at a rudimentary stage in the process of using biotechnology for practical improvement of cowpea for insect resistance. At present, effective bioassays are available only for a storage pest, the cowpea weevil, and a field pest, the cowpea pod borer. For flower thrips, bioassays are yet to be developed that would allow the identification of candidate resistance genes. For some of the other pests, such as the pod-sucking bugs, preliminary collaborative studies between IITA and Purdue University indicate that the artificial seed system developed for the cowpea weevil may be useful for evaluating candidate genes for control of these pests. Further, it is important to remember that cowpea is grown over a wide geographic area, not only in Africa and the Americas, but in Asia as well. Some of the pests are cosmopolitan, and it is possible that they will exhibit a wide range of adaptations and variabilities, such that one population may be invulnerable to a gene that controls another population. For these reasons, and for the reason that virulent biotypes may emerge against single, highly active genes, it is apt to continue the search for additional genes that can be pyramided or deployed over time to ensure that biotechnological management tools are both effective and durable.

Candidate genes for pest resistance

Following the bioassay methods above mentioned, the active substances coded by known genes were tested on cowpea weevil and on *Maruca vitrata* (formerly *M. testulalis).*

"B.t". Despite some concerns about the practical implementation and sustainability of genes from *Bacillus thuringiensis* "B.t." used in transgenic crop cultivars, research has made it clear that B.t. genes have potential for controlling a number of the insect pests of cowpea. Bioassays carried out at Purdue University in collaboration with Auburn University have demonstrated that the cowpea pod borer, *M. vitrata,* is susceptible to several different forms of the B.t. crystal toxin when these are fed in its diet. Concentrations that caused 50% mortality (LC_{50}'s) ranged from 0.03 µg/g of diet for CryIA(b) crystal toxin to 1.0 µg/g for Cry IA(a) crystal toxin; whereas CryIA(c), CryIC, and CryIIA have an activity intermediate between the two. Genes encoding several of these proteins are available for cowpea transformation and could be used to impart resistance to *M. vitrata.*

Joint research efforts involving Purdue and Auburn universities have also provided evidence that B.t. crystal toxins that are effective against the cowpea weevil may be found through systematic screening. Several well known forms of B.t. crystal toxin (e.g., CryIA(b), a lepidopteran-active form) proved totally inactive in feeding bioassays against *Callosobruchus maculatus*, as did a beetle-active B.t., *Bacillus thuringiensis tenebrionis*. The most active B.t. found to date is CryIA/CryIB, which caused a significant mortality of *C. maculatus* when incorporated into the diet at a level of 16 µg/g, and > 90% mortality at a level of 128 µg/g. (W. Moar and R.E. Shade, Purdue and Auburn Universities, USA, personal communication). These are levels of proteins that could easily be attained in the protein-rich seeds of cowpea.

Protease inhibitors. Limited studies have been carried out to evaluate the impact of proteinase inhibitors on insect pests of cowpea. Lima bean, Bowman-Birk, and Kunitz trypsin inhibitors had no effect on developmental rates or mortality of *M. vitrata* larvae when present in the diet at levels of 1% (w/w). Protease inhibitors I and II (PTI-I, -II) from potato, by contrast, exhibited measurable activity, causing slight developmental delays and increased mortalities at dietary levels of 1.0%. When the diet contained 1% (w/w) of each potato inhibitor, all insects died. In view of the high dose necessary to have a substantial effect on *M. vitrata* it is doubtful if the transfer of the PTI-I or -II genes into cowpea would be worthwhile. However, in view of the fact that low levels of trypsin inhibitors may markedly enhance the activity of B.t. crystal toxins (MacIntosh et al. 1990), knowledge of effective trypsin inhibitors against *M. vitrata* may prove useful.

Resistance of cowpea variety TVu 2027 was, for many years, widely held to result from an elevated level of a trypsin inhibitor (Gatehouse et al. 1979). Seeds of TVu 2027 were reported to contain levels of trypsin inhibitor almost twice as high as those in susceptible seeds. This interesting hypothesis has not been upheld, for numerous reasons. First, several laboratories (e.g., Xavier-Filho et al. 1989) have been unable to verify that this variety has higher levels of trypsin inhibitor than do other, susceptible, varieties. Second, when an appropriate bioassay is utilized (Zhu et al. 1994), the cowpea weevil is not affected by dietary levels of cowpea trypsin inhibitor twice as high as those originally reported by Gatehouse et al. (1979). Third, cowpea weevil larvae do not use a serine protease to digest their dietary protein (Gatehouse et al. 1985; Kitch and Murdock 1986), making it unlikely that a serine proteinase inhibitor could disrupt protein digestion.

Cowpea weevil larvae are susceptible to dietary cysteine proteinase inhibitors. When artificial seeds are made up containing E-64, a specific cysteine proteinase inhibitor, doses as low as 0.02% (w/w) significantly reduce growth rates, mortality, and fecundity (Murdock et al. 1988). The effects of E-64 were reversed by adding free amino acids to the diet (R.E. Shade and L.L. Murdock, unpublished data), indicating that the negative effect of E-64 was to restrict the supply of free amino acids. E-64, from *Aspergillus japonicus*, is an unusual tripeptide, which contains agmatine and trans-epoxysuccinic acid. Multiple genes are involved in its biosynthesis; thus it is not practical to think of using E-64 to protect cowpeas through gene transfer. There are, however, a few proteinaceous inhibitors of cysteine proteinases whose genes might be used to confer protection against cowpea weevil. Such an inhibitor from soybean seed is effective in vitro against the digestive protease of the cowpea weevil (Hines et al. 1991). Further studies are needed with this and

other proteinaceous cysteine protease inhibitors before their potential can be fully assessed.

Lectins. The most thorough studies on lectins, thus far, have focused on the cowpea weevil. Purified *Phaseolus vulgaris* lectin, which occurs in leukocyte agglutinating form (PHA-L), in erythrocyte agglutinating form (PHA-E), and as a mixture (PHA-P), has no effect on cowpea weevil when fed in the diet at concentrations of 1% (w/w) and above (Murdock et al. 1990). This result contradicted that of Gatehouse et al. (1986) who had observed that a preparation of PHA was toxic when present at relatively low levels in the diet of the cowpea weevil. Curiously, however, the authors noted that a purified preparation of PHA was less toxic than the impure preparation.

Furthermore, tests of PHA-E or PHA-L separately revealed that they were "largely ineffective" against *C. maculatus* (Boulter 1986). The disagreement of results using the individual purified isolectins with the earlier published results using the impure lectin preparation was explained away with the assumption that there is a synergistic effect of E and L lectin subunits. Unfortunately, this assumption was not tested, but tests with PHA-P at Purdue revealed no biological activity (Huesing et al. 1991). Since an impure preparation of PHA from Sigma Chemical Co. was active when fed to cowpea weevil and since this preparation was found to contain a substantial part (15–20%) of α-amylase inhibitor as impurity—enough to account for its biological activity against the cowpea weevil—there seems no reason to expect that purified PHA, in any of its forms, has any substantial activity against the cowpea weevil.

Several other lectins have been shown to affect the cowpea weevil when present in its diet. The most interesting of these are specific for N-acetyl-glucosamine (GlcNAc) residues (Murdock et al. 1990). The best GlcNAc-specific lectin was wheat germ agglutinin (WGA), which had significant effects on the insect when fed at levels as low as 0.2% (w/w). The vulnerability of cowpea weevil to GlcNAc-specific lectins may be related to the presence of chitin—a polymer of GlcNAc—in the insect gut.

α-amylase inhibitors. Seeds of common bean, *Phaseolus vulgaris*, do not support growth and development of the cowpea bruchid, *Callosobruchus maculatus*, although the females readily oviposit on the beans and the hatchling larvae bore into them. Much of the resistance is due to the presence of a proteinaceous inhibitor of the digestive α-amylase of the bruchid (Ishimoto and Kitamura 1989; Huesing et al. 1991). The kidney bean α-amylase inhibitor, which occurs in common bean seed at levels of ~ 1% (w/w) (Shade et al. 1994), is active against the cowpea weevil digestive amylase and prevents the insect from digesting the complex carbohydrate of the seeds. Microscopic examination of the midgut contents of insects that have fed on diets containing bean α-amylase inhibitor reveals a massive accumulation of undigested starch granules. It is the deprivation of this major nutrient source that presumably accounts for the effectiveness of α-amylase inhibitor in preventing cowpea weevil growth, development, and survival.

While experiments with artificial seeds clearly show the promise of the bean α-amylase inhibitor gene for controlling this important storage pest, transfer of the gene into cowpea to prove its effectiveness awaits the development of an efficient cowpea transformation procedure. In the interim, powerful new evidence has accumulated that such a transfer will,

indeed, generate a new source of cowpea weevil resistance. A multidisciplinary effort by scientists at Purdue University, the University of California at San Diego, and the CSIRO, Canberra, Australia, successfully transferred the bean gene into garden pea, *Pisum sativum*, and expressed it in the pea seeds at levels comparable to those naturally occurring in common bean (Shade et al. 1994). Pea seeds expressing the bean α-amylase inhibitor gene were immune or highly resistant to the adzuki bean weevil. Seeds from the same plants were either immune, highly resistant, or moderately resistant to cowpea weevil, depending on the level of α-amylase inhibitor expression. Differing responses of the two bruchid species to the transgenic seeds reflect the markedly higher sensitivity of the adzuki bean weevil to α-amylase inhibitor compared to the cowpea weevil.

In vitro regeneration

In general, gene transfer methodologies are based on the regeneration in vitro of a complete plant, or at least of new buds, from a transformed single cell or tissue. In grain legumes, except for soybean, regeneration protocols are not so reliable as for other crops. Despite the existence of a few published reports, these protocols seem to be laboratory-dependent, rather than genotype-dependent. To fulfil all the requirements for a good transformation system, a reliable protocol must be: (a) widely applicable, i.e., genotype/laboratory independent; (b) efficient, i.e., generating as many regenerants as possible per cultured explant; (c) reproducible, i.e., without any constraints such as particular chemicals or manipulations; and (d) fast. In this respect, grain legumes gained the negative label of "recalcitrant crops" to in vitro manipulation.

In order to develop a method suitable for cowpea genetic transformation, considerable efforts are under way to differentiate new buds and hence new shoots from differentiated cowpea tissues, or to induce multiple bud proliferation from already present highly morphogenic tissues, by testing different explant sources and several combinations of natural or synthetic plant growth factors.

Shoot differentiation. Scientists at IITA have tested the differentiation ability of young cowpea tissues cultured in vitro on a medium containing coconut water from fresh local coconuts and a high cytokinin concentration. The rationale is that coconut water extracted from fresh coconuts already contains a high level of natural cytokinins (De Wald et al. 1989). After the explants passed through 3 different media and 3 months of in vitro culture, ~ 33% of explants (primary leaves and hypocotyls isolated from germinating seeds) differentiated some shoots (S.Y.C. Ng and G.Thottappilly 1993, IITA, unpublished data). The histology of these explants carried out in Italy showed that a strong cellular proliferation occurred on the explant surface, at the epidermis level, where callus was formed. Some other experiments were carried out in Italy, to study the morphogenic response of the local cowpea cultivar "Cornetto" when cultured in vitro in the presence of natural Nigerian coconut water, compared to its commercial counterpart (Sigma C5915, deproteinized) and versus coconut water from coconuts available on the Italian market. The overall frequency of regeneration was, under the conditions used and with the above mentioned genotype, lower than that obtained at IITA with other genotypes. Nigerian coconut water seemed more effective in inducing the production of healthy shoots. However, experiments carried out in Italy have shown that only the basal part of young leaflets are able to produce shoots,

perhaps due to the presence of already formed meristems. The histology of some explants is now being studied, to elucidate the shoot origin (whether regeneration or true differentation).

Multiple bud regeneration. Several experiments have been carried out to induce multiple bud proliferation from highly morphogenic cowpea tissues. The rationale of these experiments was to find a different approach to plant differentiation, in order to obtain transformants by regeneration of transformed tissues. Scientists at Purdue University have tested the effect of media containing a high concentration of Benzyl Amino Purine (BAP) (3–6 mg/L) and a low concentration of auxin on cotyledon segments and embryonic axes from different-age embryos of various cowpea genotypes (e.g., CB5, TARS 36, SUV-2, 283, 1137, 275, TN88-63, B301, 849, and 58-57). When the callus produced on explants grown in a medium with high cytokinin concentration and cultured in darkness was transferred into media with reduced cytokinins and cultured under light conditions, proliferation of shoots occurred from regenerated buds. Since mature seed explants gave, on average, the same regeneration frequency as those of immature seeds, the former were chosen for genetic transformation experiments. Cotyledon explants developed shoots at a frequency of 50% at best after 3 weeks of in vitro culture.

Recently, the herbicide thidiazuron has been used in some grain legumes (Malik and Saxena 1992) as a growth regulator to induce multiple bud proliferation from cotyledonary and apex nodes. At the University of Naples, the effect of three different concentrations of thidiazuron (5, 10, and 20 mM) on seed germination, and on apical and lateral bud proliferation, has been studied on the local cultivar "Cornetto" and on three lines selected at IITA (TVu 9062, VITA3, and VITA4). The cultivar Cornetto and the line TVu 9062 gave, on average, the best results in terms of frequency of multiple bud proliferation from apices, with an average of 87% and 85%, respectively. Shoots from these buds produced roots only when transferred into a basal medium without the presence of thidiazuron. The results confirmed that this in vitro regeneration protocol is still genotype-dependent.

On the basis of these experiments, differentiation of new shoots in the presence of coconut water is, at present, a cumbersome protocol: it requires tissues in a particular stage of growth, it is strictly genotype-dependent, and it is slow. Finally, the efficiency of this protocol in producing new buds is very low in comparison with those applied to other crops, which is the major drawback to applying it in genetic transformation experiments. Instead, multiple bud proliferation is a less demanding task, which can be accomplished by using some well-defined cytokinins. The protocol involving the herbicide thidiazuron showed the best performance on more than one genotype: it is fast, highly reproducible, and explant handling is relatively easy for subsequent manipulation for gene transfer experiments, with either physical or *Agrobacterium*-mediated protocols.

Genetic transformation

In the absence of a reliable regeneration protocol for cowpea, scientists working with this species have been forced to seek a different approach for plant genetic transformation. All the methodologies developed in the past few years are based on the rationale that highly morphogenic tissues, i.e., meristems, can be transformed in the same way as other tissues. Therefore, the main goal has been to rescue shoots developed from a bud regenerated from

a previously transformed tissue. Hence, studies were, at first, focused on the ability of meristematic cells to be genetically transformed. Two explant sources were tested: apical vegetative meristems and lateral (cotyledonary) meristems. Moreover, two different gene transfer methodologies were tested: *Agrobacterium*-mediated DNA transfer and direct plasmid DNA transfer into meristematic cells.

***Agrobacterium*-mediated gene transfer.** *Agrobacterium*-mediated plant genetic transformation involves interaction between plant and bacterial genotypes (Lurquin 1987). This requirement is more stringent when meristems are involved in the gene transfer. Scientists at Purdue University and the University of Naples have tested several *Agrobacterium* strains, having various degrees of virulence: the highest was the A281 strain (Hood et al. 1986), a hypervirulent oncogenic strain, followed by the EHA101 strain, a hypervirulent cured strain (Hood et al. 1986), while the lowest was the LBA4404 strain (Hood et al. 1986), a cured strain. Binary vectors carrying multiple *vir*-gene copies, kindly supplied by S. Gelvin of Purdue University, have also been tested.

Frequencies of explants espressing *gus* reporter gene varied between 2% and 84%, depending on the kind of transformed explant (apical or lateral meristems performed poorly in comparison with other tested plant tissues) and the presence of *vir*-gene enhancer (bacteria conditioned with acetosyringone were more efficient in gene transfer than those not conditioned). However, no transformed shoots were obtained from these cultures, even though some evidence of chimeric shoot generation was produced at Purdue University. In all, no experiments in these laboratories ruled out the possibility of obtaining transformed meristems and, thus, transformed shoots. All experiments showed, however, that considerable work and a higher number of explants are needed to pursue our goal of genetic transformation in cowpea.

Other nontissue-culture approaches involving *Agrobacterium*-mediated genetic transformation have been successfully set up on *Arabidopsis thaliana*. These methods are based on seed co-cultivation with bacteria (Feldmann and Marks 1987), in planta *Agrobacterium* infiltration (Chang et al. 1994), and in planta *Agrobacterium* inoculation. All of these techniques produced stably transformed progenies, as verified by Southern analysis. Frequencies of transformation ranged from 0.3% using the seed imbibition technique to ~ 30% using in planta *Agrobacterium* inoculation in buds. This latter method was applied to cowpea in Portici, using a binary vector, harboring NPT II, GUS and the α-amylase inhibitor genes, kindly supplied by Prof. M. Chrispeels (UC San Diego, USA), as part of the joint project with IITA. In the project, more than 2700 T_1 seeds were collected; after a preliminary screening based on NPT II and GUS assays, the material is at present under evaluation at IITA for *C. maculatus* resistance.

Direct plasmid DNA transfer into meristematic cells. Physically mediated plasmid DNA transfer into cells can be accomplished using different methods: microprojectile bombardment, electroporation, DNA adsorption by dry tissues, etc. (Potrykus 1990). All these methods have been tested by scientists involved in the Cowpea Biotechnology Project. It has been shown that using these methods, plasmid DNA can be expressed in the cells. However, results indicate that > 90% of this expression is transient, and that there is a decline in activity a few days after the experiment; only a small percentage of cells

transformed in this way have been found to be stable subsequent to transformation after at least 2 weeks of culture. In all experiments, the *gus* (β-glucuronidase) gene from *Escherichia coli* was used as the reporter gene, in order to verify all transformation events.

A helium-driven gene-gun apparatus, used to shoot cells using tungsten or gold microprojectiles covered by a DNA solution, was tested by scientists at Purdue University and at the University of Naples. The shooting vector was also supplied by M. Chrispeels and contained the same genes mentioned earlier. Several shooting parameters were evaluated: helium pressure, distance of carrier disk from the stopping plate, distance of the stopping plate from tissues, developmental stage of seeds, and different genotypes. Transient expression was first studied by testing GUS activity in explants 3 days after microprojectile bombardment. Even transient expression was found to be genotype-dependent. Scientists at Purdue University showed that among 7 genotypes, the mean number of single transient transformed cell of cotyledonary nodes ranged from 5.4±1.3 for the cowpea line 1137 to 45.5±6.9 for the CB5 genotype. At the University of Naples, some parameters were tested to improve the frequency of transformed explants and the mean number of blue-stained cells per explant. Apical and lateral thidiazuron-induced multiple meristems gave the same results when helium pressure was set to 1100 p.s.i. and the microcarrier was placed 9 cm away from the stopping plate. However, the number of transformed leaf cells steadily decreased from the maximum, an average of 150 per explant 24 h after shooting, to 5 per explant after 14 days, due to DNA transient expression. This value is still too low to be useful for genetic transformation experiments.

Other experiments were performed combining direct with indirect genetic transformation systems: plain microprojectiles bombardment followed by co-culture with *Agrobacterium tumefaciens*. In some pilot tests, ~ 100% of explants showed stable DNA integration in cells very close to the meristematic ones, as revealed by GUS-istochemical assays.

Recently, a novel method for plant transformation, useful for recalcitrant species like cowpea, has been set up by Paul Lurquin's group at Washington State University, USA (Chowrira et al. 1994). This method is based on electroinjection of plasmid DNA directly into meristematic cells of in vivo cultured plants, hence avoiding all in vitro procedures, meristem regeneration, and somaclonal variation. About 8–10% of the electroinjected plants rescued were stably expressing the *gus* gene. Pea, lentil, and soybean plants were obtained from in vivo treatment, and some of their T_2 progenies showed the presence of the introduced gene by Southern analysis. Moreover, cowpea plants stably expressing the *gus* gene were obtained. Some of their progenies were analyzed for the presence of the introduced gene. The presence of the GUS-INT gene, revealed by Southern analysis, confirmed the possibility of transforming cowpea using this system (M.G. Chowrira et al., personal communication). This technique, successful in Pullman, Washington State University, has been applied at the University of Naples to introduce the α-amylase inhibitor gene isolated from bean. The obtained progenies are now under screening at IITA.

Conclusions

It seems clear that very important progress has been achieved in the past few years in the use of biotechnologies in cowpea, mainly in genome mapping through molecular markers and in obtaining transgenic plants. This last achievement opens the road to apply genetic

engineering to this species, with very good prospects of success because the methods are set up and some useful genes are available.

Research should now concentrate on problems related to the step from a transgenic plant to a transgenic crop, which implies the study of the expression level of the inserted genes and the interaction of the new genotypes with different environments.

Globally, we know that cowpea is not a major cash crop and that, therefore, no private biotechnology industry will invest in it, but we have also seen that it can benefit from information and material used for other, better researched crops. This is why an international collaborative action involving leading laboratories should be maintained for making further progress in this effort.

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