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IMPROVING SWEET POTATO THROUGH BIOTECHNOLOGYError! Reference source not found.

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Abstract

Sweet potato (*Ipomoea batatas*) cultivars with novel traits such as resistance to diseases and pests and enhanced nutritional quality can be rapidly developed by using biotechnology. Genetic engineering is being used to understand the sweet potato's genomic biology and improve its productivity. To develop transgenic plants, a technique for regenerating plants *in vitro* in high frequencies is essential. The authors recently developed a two-stage regeneration protocol, employing petiole explants cultured on a medium with 2,4-D in the first stage and thidiazuron in the second. To develop an optimal transformation system, *Agrobacterium* vectors and particle bombardment approaches are being employed to introduce marker genes (*GusA, npt II,* and *hpt*) into sweet potato. The aim is to develop transgenic sweet potato cultivars with multiple 'value-added' traits. Genes for disease resistance and improved protein quality are being introduced into sweet potato. The disease resistance gene (*Shiva-1*) encodes for a lytic peptide that is highly anti-microbial; and the artificial storage protein (*asp-1*) gene encodes for essential amino acids such as lysine, leucine, isoleucine, methionine, and threonine. The expression of this gene is being selectively targeted to the storage roots by fusing the sporamin promoter to the *asp-1* coding sequence. The capsid protein and anti-sense RNA genes of sweet potato feathery mottle virus are being introduced to develop resistance to this disease. The success of this gene transfer research may also facilitate similar research on engineering resistance to sweet potato weevil by introducing delta-endotoxin genes from *Bacillus thuringiensis*. Finally, the DNA amplification fingerprinting approach was employed to identify complex polymorphisms in sweet potato genotypes in a consistent and reproducible manner. This approach is useful to rapidly and precisely identify sweet potato accessions and to characterize the genetic diversity in the germ plasm collection. It also has potential, in phylogenetic studies, to identify the progenitors of *Ipomoea batatas* and in developing a genetic linkage map of sweet potato.

Introduction

Agricultural research has been recently undergoing an exciting phase of invigoration, especially in developed countries, because of genetic engineering. Research in molecular and cellular genetics has made available many novel approaches such as gene transfer and gene mapping, which have

considerable potential to contribute to improved agricultural productivity. Biotechnological research is most intense in commercially important crops such as tomatoes, potatoes, tobacco, cotton, rice, and maize. Transgenic plants with value-added traits, including resistance to diseases and pests and tolerance of herbicides, have been developed in these crops by introducing genes from other organisms. Furthermore, several genes controlling traits critical to plant biology have been identified, cloned, and characterized in model crops such as tobacco and *Arabidopsis*, contributing significantly to our understanding of the fundamental processes in the plant. DNA markers are being increasingly employed to develop genetic maps that aid in the indirect selection of complex traits such as disease resistance and quality characters in crop plants. DNA markers are also contributing to germ plasm identification and phylogenetic studies.

Tropical root and tuber crops such as sweet potatoes, cassava, and yams—despite their importance—have not been the subject of intensive biotechnological research. However, this may change as several biotechnology laboratories around the world are beginning to target these crops for their research. Molecular genetic approaches such as gene transfer help breeders circumvent the arduous sexual hybridization strategies and yet complement existing breeding programmes by facilitating rapid introduction of precise traits into adapted cultivars, such as resistance to a new strain of pathogen or insect. DNA molecular markers, relatively easy to detect, enable the construction of genetic maps, which assist in the indirect selection of complex traits such as yield or pest resistance. DNA fingerprinting can have a significant impact on germ plasm studies as it facilitates the positive identification of accessions, elimination of duplicates, and estimation of genetic distances.

Our laboratory's primary goal is to use novel molecular and cellular genetic approaches to understand the fundamental genomic biology of sweet potatoes and employ this knowledge to develop improved cultivars. Most of our effort is directed towards developing a system for introducing foreign genes into sweet potatoes and regenerating transgenic sweet potato plants with improved traits such as disease resistance and higher protein quality. Another project concerns the use of DNA amplification fingerprint (DAF) markers to characterize the sweet potato germ plasm. Here, we summarize some recent research findings and provide an outline of ongoing studies in tissue culture, vector construction, and development of transgenic plants and DAF in sweet potatoes.

Sweet Potato Tissue Culture

As Ritchie and Hodges (1993) have stated, the regeneration system must be compatible with the chosen gene delivery system to develop transgenic plants successfully. We developed a rapid method for regenerating adventitious sweet potato plants at a very high rate after examining various factors such as genotypes, explant types, developmental stage of the explant, media conditions, and media additives such as auxin and cytokinin (Porobo Dessai et al. 1993).

The optimized protocol uses petiole explants from *in vitro* grown plants of responsive genotypes such as PI 318846-3. Explants are cultured in a MS medium (Murashige and Skoog 1962) with 2,4-D (0.2 mg/L) for 3-4 days until the base of the petiole begins to swell. They are then transferred to a medium containing thidiazuron (Prakash et al. 1993).

We routinely observed that 80%-90% of the petiole explants regenerated shoots in genotype PI 318846-3. In contrast, when leaf lamina were used as explants, levels of regeneration were considerably lower (Table 1). Within 14 days, shoot primordia arose from the base of the petioles and, by 28 days, most explants showed such shoot primordia and developing shoots when cultured on a medium with thidiazuron at a concentration of 0.2 mg/L (Table 2). The use of thidiazuron alone in the second-stage medium produced successful regeneration in genotype PI 318846-3, but not in 20 others tested.

To extend the regeneration protocol to other genotypes, cytokinin N^6 (delta^2-isopentenyl)-adenine (2iP) was tested in combination with thidiazuron (0.05 mg/L). Use of 2iP at 0.05 mg/L elicited regeneration from 8 of the 13 cultivars tested. In addition to genotype Pl 318846-3, three others (PI 531143, PI 508507, and PI 318846) produced satisfactory levels of shoot regeneration. Genotype Pl 318846-3, an accession from Timor but imported to the USDA sweet potato germ plasm centre in Griffin, Georgia, from New Zealand, has consistently produced very high levels of regeneration in all our studies. When five other similar accessions from Timor were tested, none, except PI 318846, produced shoots.

Although the use of responsive genotypes such as PI 318846-3 and the two-stage media with 2,4-D (stage I) and thidiazuron (0.2 mg/L) with or without 2iP (0.05 mg/L) (stage II) were crucial, several additional factors account for successful regeneration in sweet potato. The development stage of the explant is critical, particularly as the younger leaves (second and third from the apex of the stem) yielded the most regenerable petioles (Porobo Dessai et al. 1993). Explants must be transferred from the 2,4-D medium to the thidiazuron medium soon after the base of the petiole starts to swell. If they are incubated longer, they exhibit decreased shoot organogenesis.

The placement of the explant on the nutrient medium during incubation also appears to critically influence the number of shoots regenerated per explant. Normally, a petiole piece is placed horizontally on the medium during the first stage (2,4-D) and vertically (upright) with its base in the medium during the second stage (thidiazuron). However, in a study aimed at testing the effect of explant placement on regenerating efficiency of sweet potatoes, we observed a two- to three-fold increase in the number of shoots per explant when explant placement is altered. Petiole explants placed on the thidiazuron medium in a horizontal or vertically inverted manner (so that the apex of the petiole is in the medium) exhibited a higher number of shoots per explant than those placed vertically upright.

Gene transfer to sweet potatoes

Foreign genes can now be delivered into plant cells in a variety of ways, including the *Agrobacterium* vector, particle bombardment, protoplast uptake, and tissue electrophoresis. *Agrobacterium tumefaciens* is a soil-borne pathogen that causes crown gall disease in plants by transferring a piece of DNA (T-DNA) from its Ti plasmid to the plant chromosome. Being a dicotyledon, the sweet potato is susceptible to *A. tumefaciens*. Disarmed vectors of this bacterium have been successfully employed to transform sweet potato explants (Prakash and Varadarajan 1991; 1992a, b) and develop transgenic plants.

To achieve gene transfer, sweet potato explants were co-cultivated with disarmed *A. tumefaciens* containing a binary plasmid with *gusA* and *npt II* genes. Successful transformation was observed in both leaf and petiole cells as revealed by GUS histochemical analysis (Prakash and Varadarajan 1992a, b). Antibiotic-resistant calli were regenerated when transformed calli were moved to a kanamycin medium, and these subsequently developed shoots and roots. These plantlets were positive for both *gusA* and *npt II* gene expression, as evidenced by an ELISA assay for neomycin phosphotransferase ll enzyme encoded by the *npt II* gene. Polymerase chain reaction (PCR) amplification of the *npt II* gene was performed on the genomic DNA of transgenic shoots to confirm the presence of the introduced DNA in sweet potato cells.

Several variables such as length of co-cultivation, stage of selection, and presence of *vir*-gene-inducing chemicals were investigated to further improve sweet potato transformation rates with *Agrobacterium*. The presence of the *vir*-inducing substances such as acetosyringone and ß-galacturonic acid in the medium influenced the extent of explant area transformed but did not appreciably affect the frequency of transformation. Length of co-cultivation of sweet potato explants with *Agrobacterium* culture also had an impact on transformation efficiency, 1-3 days being the most effective. When various sweet potato organs were tested, the petiole was always the most competent tissue for transformation, compared with leaves, shoots, and roots (Blay et al. 1992). We also screened nearly 40 sweet potato genotypes and identified some that are more competent for transformation than others.

Several different plasmid constructs were compared for their efficiency in transforming sweet potato: (1) LBA 4404/pB1 121 (with *gusA* gene with CaMV 35S promoter); (2) LBA 4404/pBCCS1 (with *gusA* gene under the control of double CaMV 35S promoter); (3) EHA 101/pGUS-Intron which has intron sequences located at the 5' end of the *gusA*-coding region; and (4) C58/GUS::*npt II* fusion (*gusA* and *npt II* genes fused under the control of enhanced CaMV 35S promoter and with AMV translational enhancer). The *Agrobacterium* strains containing the enhanced or doubled CaMV 35S promoter (C58/gus::*npt II* fusion and LBA 4404/pBCCS1) resulted in larger transformed areas, compared with other constructs. The EHA strain with the pGUS-Intron also resulted in transformation, suggesting that the intron from the castor catalase gene spliced efficiently in sweet

potato cells.

Antibiotics have a dual role in plant transformation research. Selective antibiotics such as kanamycin and hygromycin are used to select transformed plant cells expressing genes resistant to these antibiotics (*npt II* and *hpt*). We thus determined the tolerance limits of the sweet potato explants for these antibiotics *in vitro*. The minimum inhibitory concentration was 50 mg/L for kanamycin, 10 mg/L for hygromycin, and 10 mg/L for geneticin of G418. The antibiotics cefotaxime and carbenicillin are used to eliminate *Agrobacterium* from the explants after co-cultivation. Both antibiotics promoted callus proliferation in sweet potato explants, thus exhibiting a growth regulator-like activity, and did not appear to have an adverse effect on organogenesis.

The biolistic approach is a novel means of directly introducing foreign genes into plants. Foreign genes were successfully introduced into intact sweet potato cells, which were bombarded with tungsten microprojectiles, using gunpowder acceleration (Prakash and Varadarajan 1992a, b). Callus and root isolates of two cultivars ('Jewel' and 'TIS-70357'), with signs of stable transformation, were recovered. Plasmid pBI 221 with the *gusA* gene, which encodes for ß-glucuronidase (GUS), controlled by a promoter from cauliflower mosaic virus (CaMV), was employed. Tungsten microprojectiles, coated with plasmid DNA, were shot at high velocity into targeted sweet potato tissues. A histochemical examination of bombarded leaf and petiole tissues for expression of *gusA* gene revealed that most explants had some transformed cells.

When cultured on *in vitro* medium, calli and roots developed in most bombarded tissues. Similar results, but with a lower frequency of transformation, were observed when plasmid pBl 121 (with *gusA* and antibiotic resistance *npt II* genes) was employed and bombarded explants cultured on an antibiotic selection medium. Subcultured roots and calli were positive for *gusA* expression when tested after more than 15 cycles of transfer. Foreign gene expression therefore appears to be fairly stable (Prakash and Varadarajan 1992a, b).

Genetic engineering for disease resistance

Sweet potatoes are subject to attack by many fungal, bacterial, and viral diseases that often cause substantial economic damage (Clark and Moyer 1988). Genetic engineering offers a means to incorporate resistance into some of these pathogens.

A class of proteins (lytic peptides) have potent anti-microbial properties by disrupting the cell membranes of bacteria and fungi (Boman and Steiner 1981). The lytic peptides such as cecropin A are thus relatively non-toxic to humans, animals, and plants but are highly toxic to bacteria, fungi, and other micro-organisms. Very low concentrations of cecropin A were found to be lethal to bacterial plant pathogens such as *Pseudomonas, Erwinia,* and *Xanthomonas* in *vitro* tests (Jaynes et al. 1993).

Synthetic substitution analogues of these peptides were developed with improved native sequences to facilitate high expression in plants. Two such proteins (SB-37 and *Shiva-1*) were found to be more potent than cecropin A (Jaynes et al. 1993). Synthetic chimeric genes that encode for these two proteins were introduced into tobacco plants, using an *Agrobacterium* vector. The resulting plants were challenged with pathogenic *Pseudomonas solacearum*. Transgenic plants expressing the *Shiva-1* gene exhibited a delayed appearance of symptoms and a dramatic reduction in mortality, compared with control plants (Jaynes et al. 1993). The *Shiva-1* and SB-37 gene constructs, with a promoter from the proteinase inhibitor II gene, are being used to achieve a pathogen-inducible defence response to disease in transformed sweet potato plants.

Transgenic sweet potato plants with these genes will be developed on a larger scale by the *Agrobacterium* and particle bombardment approaches, and by the use of vectors containing not only these two genes but also selectable and screenable marker genes. Disease-screening studies will be conducted on the transgenic and control plants of sweet potato to assess the effectiveness of lytic peptide genes in conferring resistance to sweet potato pathogens.

Sweet potato feathery mottle virus (SPFMV) causes 'russet crack', a major production constraint, particularly in Africa. Recently, the coat protein genes were isolated and cloned, and the SPFMV anti-sense RNA genes developed. We are now attempting to introduce these genes into sweet potato cells. We will then inoculate transgenic sweet potato plants expressing these genes to test for resistance to the virus.

Genetic engineering to improve protein quality

Most plant proteins, including those of sweet potatoes, cassava, and yams, are deficient in certain essential amino acids so that sole dietary reliance on such proteins can lead to a malnourished state in humans. The difference in quality of plant and animal proteins is striking: a child weighing 20 kg could obtain 100% of his or her daily essential amino acid equivalent by consuming either 170 g of meat or eggs or 2.3 kg of sweet potatoes (Jaynes et al. 1986). An artificial storage protein (*asp-1*), which codes for a protein rich in essential amino acids and with an extremely high degree of stability and aggregation, has been designed *de novo*. The nutritional quality of *asp-1* is higher than that of milk or egg protein, and this gene has been expressed in potatoes with very encouraging results (Destéfano-Beltrán et al. 1991).

We are trying to develop transgenic sweet potato cultivars that express the *asp-1* gene in their storage roots. We have constructed new vectors that contain the *asp-1* gene, interrupted by an intron and driven by the sporamin A gene promoter. This promoter enables the expression of the new protein specifically in the storage roots of sweet potatoes (Hattori and Nakamura 1988). The presence of intron ensures that *asp-1* is not expressed in the *Agrobacterium* vector. Transgenic plantlets will be regenerated, using antibiotic selection, and analysed for their protein quality to test

whether the expression of *asp-1* contributes to improved protein quality.

DNA amplification fingerprinting of sweet potato genetic resources

We have employed the DAF technique, a powerful and rapid approach to detect genetic polymorphisms (Caetano-Anollés et al. 1991; Williams et al. 1990), in sweet potatoes. The technique is simple, cost effective, involves less labour, requires no radioactivity, and is well suited to the analysis of a large number of samples. The procedure requires very small amounts of DNA, uses universal primers, and does not require cloning or prior knowledge of DNA sequences.

DNA amplification was tolerant of wide variations in both template and primer concentration, whereas an increased concentration of Mg was critical to obtaining the maximum number of amplification products. Informative, reliable, and consistent results were obtained when amplifications were conducted, using 6.4-66.0 ng/μL of template DNA, 1.90-11.25 μM octamer primer, 5 mM $MgCl_2$, deoxy nucleotildes (200 μM each), 5 units of truncated AmpluTaq DNA polymerase (Stoffel fragment; Perkin-Elmer Cetus), with Cetus-supplied reaction buffer in a 25-μL reaction volume. Amplified products were resolved on PCR Purity Plus, a novel gel matrix (Biochem, Malvern, PA), and visualized by silver staining (Caetano-Anollés et al. 1991).

The Stoffel fragment produced a larger number of amplification products than regular AmpliTaq. DAF profiles were highly reproducible; five independent amplifications showed no variation in banding patterns. Neither were differences found between banding patterns from amplification reactions performed on two thermal cyclers. A single octamer primer could generate DNA banding patterns that were individual-specific and which unambiguously fingerprinted many sweet potato genotypes.

The DAF approach was also very useful in cultivar identification studies and for characterizing the genetic diversity of sweet potato germ plasm. Seven of the 28 octamer primers screened were highly informative and detected high polymorphism in sweet potato genotypes. These seven primers were used individually to develop DAF profiles of 70 sweet potato accessions collected from around the world and 30 accessions that represented U.S. cultivars and their progenitors.

Very high genetic variability was evident in the global sample; in contrast, the U.S. cultivars were relatively uniform with most bands being monomorphic, suggesting a narrow genetic base of sweet potatoes in the USA. Use of certain primers resulted in individual-specific DAF profiles, enabling clear discrimination of the accessions. DAF profiles were highly reproducible as no significant variations were found in banding patterns in replicate runs.

DNA fingerprinting technique can thus be usefully employed to assess genetic variation in

sweet potatoes. It also facilitates the collection of improved germ plasm by identifying those geographic areas with greatest genetic diversity. DNA fingerprints are valuable to breeders by enabling them to identify divergent parental lines for hybridization and to monitor somatic hybrids and somaclonal variation.

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Table 1. Effect of various levels of thidiazuron (TDZ) (during second stage) on the regenerating frequency of petiole and lamina explants of sweet potato genotypes PI 318846-3[a].

Error! Reference source not found.TDZ concn (mg/L)	Petiole	Lamina
0.0	23.4	0.0
0.1	66.6	26.3
0.2	100.0	25.0
0.3	67.5	8.3
0.4	49.7	0.0

a. Values represent percentage of explants regenerating shoots; first-stage medium consisted of MS, with 2,4-D (0.2 mg/L).

Table 2. Percentages of explants regenerating shoots. First-stage medium consisted of MS, with 2,4-D (0.2 mg/L).

Error! Reference source not found. Time (days)	Concentration of thidiazuron (mg/L)				
	0.0	0.1	0.2	0.3	0.4
14	6.7	26.7	45.0	11.7	0.0
21	16.7	50.0	68.3	55.0	0.0
28	23.3	66.7	85.0	65.0	0.0