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Cassava Biotechnology

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Introduction

Cassava (*Manihot esculenta* Crantz) is a 1–5 m high woody perennial shrub grown for its starchy tuberous roots (*Figure 1*). Cassava belongs to the *Euphorbiaceae*, which also contains other commercially important plants like castor bean (*Ricinus communis* L.) and rubber (*Hevea brazilienesis* L.). The genus *Manihot* comprises 98 species and all the species studied so far have a chromosome number of 2n = 36 (Rogers, 1963; Rogers and Appan, 1973; Nassar, 1978; Hersey, 1983; Bai *et al.*, 1993). The plants contain lactifers and produce latex, and *M. glaziovii* is used as a minor source of rubber (Cock, 1985). Cassava is native to tropical South America, and is one of the oldest cultivated crops (Jennings, 1976) with possibly two centres of origin. Since cassava does not exist in the wild state, and its wild progenitors are not known, the regions of its domestication are disputed (Renvoize, 1972; Rogers and Appan, 1973; Rogers and Flemming, 1973; Nassar, 1978). From Latin America cassava was introduced to Africa in the 16th century and to Asia in the late 17th century, and today it is cultivated world-wide in more than 80 countries between 30° south and 30° north of the equator. Cassava is best suited to warm, humid lowland tropics, but it can be cultivated in most areas where the mean annual temperature exceeds 20°C and the annual precipitation varies between 500 mm and 8000 mm. Cassava roots are usually harvested 6–12 months after planting, but in areas where a cool season, e.g. at high altitudes arrests plant growth and the accumulation of starch in the roots, the growing period can be up to three years.

Cassava is well adapted to poor soils, allowing acceptable harvests even on marginal and eroded soils unable to support any other crop without costly external inputs, and it has the unique advantage over, for example, cereal crops, in that its

Abbreviations: BA, 6-benzylaminopurine; *bar*, phosphinotricin acetyltransferase gene; CIAT, Centro Internacional de Agricultura Tropical; 2,4-D, 2,4-dichlorophenoxyacetic acid; DMSO, dimethyl sulphoxide; GA_3, gibberellic acid; GD, Gresshoff and Doy medium; GUS, β-glucuronidase; *hpt*, hygromycin phosphotransferase gene; IAA, indole acetic acid; IBA, indole-3-butryric acid; MS, Murashige and Skoog medium; NAA, α-naphtalene acetic acid; *nptII*, neomycin phosphotransferase gene; *pat*, phosphinotricin acetyltransferase gene; RAPD, randomly amplified polymorphic DNA; RFLP, restriction fragment length polymorphism; SH, Schenk and Hildebrandt medium.

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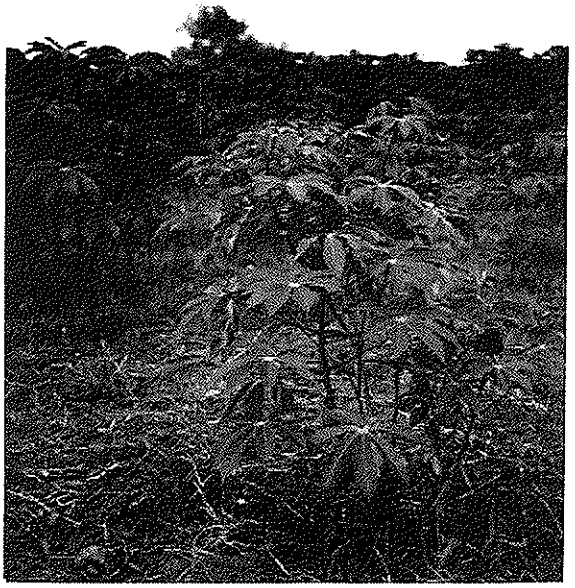
(a)



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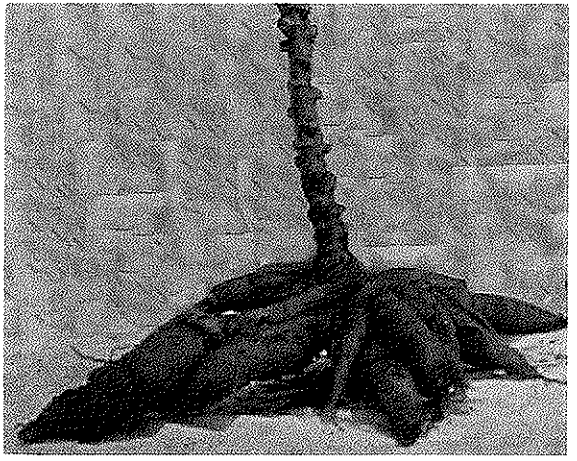

Figure 1. (a) Young cassava plants in the field. (b) Cassava roots

harvesting time is highly flexible. This makes it an excellent famine reserve because the plants can also be partially harvested and left growing in the ground until the roots are needed. After an initial establishment period cassava is able to survive even prolonged seasonal drought and other adverse environmental conditions. Cassava is propagated vegetatively from lignified stem cuttings, 'stakes', which means that none of the yield, roots, needs be set aside to secure planting material for the next season. Most of the harvest is used for human food, either fresh or in various processed forms. The rest is processed to animal feed and industrial products. As raw material, cassava

Table 1. Production, labour requirement and calorie yield estimates of some tropical crops (De Vries *et al.*, 1967; Coursey and Haynes, 1970; Onwueme and Charles, 1994).

	labour energy person days/ha	food energy 10^3 cal/ha/day	yield 10^6 cal/ha	yield 10^6 cal/ha/month
cassava	180	250	12	1.1
yams	300	266	7	0.8
rice	200	176	5	1.0
maize	80	200	8	1.8

can be processed into a wide variety of products for food and industrial uses, such as starches, flours, alcohol, glucose and others. Much of the processing can be done locally, providing rural employment and income (Balagopalan *et al.*, 1988; Carrizales, 1991).

In the tropics, cassava is the most important root crop and the fourth most important calorie source, after rice, maize and sugarcane. Cassava roots contain starch up to 85% of their dry weight and provide basic staple for over 500 billion people. In many developing countries cassava is also the cheapest available calorie source. It produces the highest calorie yields per hectare of all staple crops and has a high efficiency of energy unit per labour input ratio, as its cultivation requires much lower labour input than most tropical crops (*Table 1*) (De Vries *et al.*, 1967; Coursey and Haynes, 1970; Onwueme and Charles, 1994). It is mainly grown by small-scale and subsistence farmers in the poorer regions. World-wide, 65%–80% and in Africa 90% of the harvest is used for human consumption (Balagopalan *et al.*, 1988; Bokanga, 1994a; Onwueme and Charles, 1994), and for example, in 15 sub-Saharan countries, 30 million people get up to 60% of their daily calorie intake from cassava, while in some of the indigenous groups in the Amazonian area over 80% of food energy is derived from cassava (Roca, 1984; Cock, 1985; CIAT, 1994; Dufour, 1994, 1995; Koch *et al.*, 1994). The highest annual cassava consumption per capita on a country basis is 346.6 kg in the Democratic Republic of Congo (Zaire), followed by Congo (254.9 kg) and Ghana (245.7 kg). Of the 12 countries where the annual consumption per capita exceeds 100 kg, 11 are in sub-Saharan Africa, the other one is Paraguay (*Table 2a*) (FAO, 1997). In certain regions cassava leaves are also used as a major component of the diet to provide supplementary protein, minerals and vitamins to complement the carbohydrate rich staple.

The annual production of cassava in 1996 was 162 million metric tonnes (*Table 2a*). The largest cassava producers are Nigeria, Thailand, Indonesia, Brazil and the Democratic Republic of Congo, and the highest yields are obtained on Barbados andin India (27.3 t/ha and 23.5 t/ha, respectively), while the lowest root yields only reach up to 1.8 t/ha in Sudan (FAO, 1997). Cassava production has risen steadily during the past decades, partly due to increased growing area, but also due to introduction of new high-yielding varieties. Much of the increase has been due to the expansion of cassava cultivation in Thailand (*Table 2b*), which exports most of its production as dried chips and pellets to the European Union.

Constraints to cassava use and cultivation

Despite its integral part in food security in developing countries, cassava was long neglected in breeding programmes, and often considered as a hardy crop with little

Table 2a. Cassava production in 1996, consumption in 1995 (FAO, 1997).

	area harvested 10^6 ha	yield t/ha	production 10^6 metric tonnes	consumption kg/pers/year
Africa	9.9	8.4	83.2	
Congo Dem. Rep.	2.1	8.3	17.5	346.8
Mozambique	1.0	4.2	4.2	194.7
Nigeria	2.9	10.7	31.5	172.0
Asia	3.5	13.0	46.3	
India	0.3	23.5	6.0	6.0
Indonesia	1.3	12.1	15.4	54.9
Thailand	1.2	13.3	16.0	3.8
Latin America and Caribbean	2.7	11.8	31.4	
Brazil	1.94	12.6	24.6	52.5
Colombia	0.18	9.8	18.0	36.2
Paraguay	1.75	14.9	2.6	148.8
World	16.3	9.98	162.9	

Table 2b. Development of cassava production during 1970–1999, million metric tonnes (FAO, 1997).

	1970	1980	1985	1990	1992	1994	1996
Africa	40.5	48.3	58.2	64.1	80.9	82.9	83.2
Nigeria	10.2	11.5	13.5	17.6	29.2	31.0	31.5
Congo Dem. Rep.	10.3	13.1	15.5	17.0	20.2	18.1	17.5
Mozambique	2.9	3.6	3.6	5.0	3.2	3.4	4.2
Asia	23.2	45.8	48.5	52.0	51.4	49.3	46.3
India	5.2	5.8	5.7	5.0	5.8	5.8	6.0
Indonesia	10.6	13.6	14.0	16.3	16.5	15.7	15.4
Thailand	3.4	16.5	19.3	21.9	20.4	19.1	16.0
Latin America and Caribbean	34.7	29.2	29.6	33.7	28.6	31.2	31.4
Brazil	29.5	23.5	23.1	25.4	22.0	24.5	24.6
Colombia	1.2	2.2	1.4	4.0	1.7	1.8	1.8
Paraguay	1.6	2.0	2.9	4.0	2.6	2.5	2.6
World	98.7	124.1	135.7	152.4	161.1	163.5	162.9

problems. Up to 80 t/ha roots can be produced under optimal conditions in a 12 month culture period (CIAT, 1980), but the actual yields are severely reduced due to poor agricultural practice, and infestations by insect pests and diseases (*Table 2a*). On average, various pests and diseases are estimated to cause 20%–50% yield losses world-wide, and locally they can lead to total crop failures. Other important, still unsolved problems are the low protein content of the roots, the poor storability of freshly harvested roots and the cyanogenic nature of cassava.

POST-HARVEST DETERIORATION

The poor storability of fresh cassava roots is one of the main constraints for urban

marketing of cassava. Cassava roots can be kept on plants growing in the soil even for years, but once harvested they must be processed quickly to prevent their deterioration, which in most cultivars takes place within 5–7 days of harvest (for review, see Wenham, 1995). In most varieties the physiological deterioration of the roots commences within 24 hours, and is first characterized by vascular discolouration (streaking), followed by general discolouration of the storage parenchyma and secondary deterioration caused by microbial pathogens, which renders the roots unacceptable for human, animal or industrial use. Although still poorly understood, many of the metabolic changes during the initial physiological deterioration resemble those observed during normal plant wound response reaction, and it has been speculated that the cause of the physiological deterioration could be a sustained wound reaction spreading systemically from the wound site into the whole root (Beeching *et al.*, 1994, 1995). If this is the case, then the regulation of the wound reaction and wound healing in cassava roots, which have no function in plant propagation and are physiologically inactive in comparison to other roots like yams or sweet potato, may be deficient. Increased levels of among others, flavonols and other secondary metabolites are produced during the primary deterioration, and this is accompanied by activation of the enzymes of the phenylpropanoid pathway and also by initiation of *de novo* protein synthesis (Tanaka *et al.*, 1983, Rickard, 1985; Wheatley and Schwabe, 1985; Beeching *et al.*, 1994, 1995).

CYANOGENESIS

Cassava is cyanogenic, i.e. all parts of the plant produce hydrogen cyanide when damaged. Hydrogen cyanide is the breakdown product of cyanogenic glucosides, linamarin and lotaustralin, which are derived from valine and isoleucine, respectively, via three hydroxylation steps followed by glucosylation (Koch *et al.*, 1992). When cassava tissues are disrupted, linamarin and lotaustralin are brought to contact with a β-glucosidase, linamarase, which catalyses their hydrolysis to glucose and cyanohydrins. The breakdown products of cyanohydrins are a ketone and hydrogen cyanide (HCN). This reaction is catalysed by an α-hydroxynitrilase, but can also proceed spontaneously if the pH is higher than 4.0 (for review see Hughes *et al.*, 1995). The cyanogenic potential of cassava is highly variable between individual plants, and even between individual roots of one plant, and it is also influenced by the environment (Bokanga, 1994a; Bokanga *et al.*, 1994). On average, unprocessed fresh cassava roots contain about 150 mg/kg (15–440 mg/kg) cyanide equivalents, while high cyanide cultivars can contain up to 1500 mg/kg (O'Brien *et al.*, 1992). The safety limit for cyanogens for cassava products has been set at 10 mg/kg dry weight (Codex Alimentarius Commission, 1989), and a cyanide dose of 50–100 mg can be lethal within minutes (Rosling *et al.*, 1993). To prevent cyanide poisoning, linamarin and lotaustralin have to be removed by labour-intensive processing, and shortcuts in processing can have fatal consequences (Akintowa *et al.*, 1994). All known cassava cultivars contain cyanogenic glucosides, and despite considerable efforts no acyanogenic variety could be found or produced (Jennings, 1976; Bokanga, 1994a; Dixon *et al.*, 1994). High cyanide varieties are favoured in some areas, as they are considered more high yielding, tolerant to environmental stress and also safer from theft by mammals (Rosling *et al.*, 1993). On the other hand, severe neurological

disorders have been shown to be closely linked to long-term exposure to cyanide (Osuntokun and Monekosso, 1969; Tylleskär, 1994; Tylleskär *et al.*, 1992, 1995). In addition to the health risks of cassava-based food, the waste waters from cassava processing plants often contain toxic amounts of cyanide, and consequently can be serious pollutants (Manilal *et al.*, 1983).

DISEASES AND PESTS

Some of the main diseases of cassava are cassava bacterial blight, superelongation disease, frog skin disease and the African cassava mosaic disease, which can lead up to 100% yield losses (Lozano and Booth, 1974). Cassava bacterial blight caused by *Xanthomonas campestris* pv *manihotis* is one of the main biotic constraints in cassava cultivation world-wide, and heavy infestations of bacterial blight can destroy the whole crop (Lozano, 1979; Boher and Verdier, 1994; Mahungu *et al.*, 1994). The main local dispersal mechanism of the pathogen is rain splashes, and the use of infected planting material and tools can carry the disease considerable distances. By traditional breeding, some resistant varieties have been produced, but so far the resistance obtained appears to be effective only under low infestation pressure (Cooper *et al.*, 1995).

Low root yields in Africa are largely due to heavy disease and pest infestations of cassava plants. African cassava mosaic disease causes losses of up to 40%–50% of total yields throughout the continent, and can locally destroy the whole harvest (Thresh *et al.*, 1994; Otim-Nape, 1995). It is caused by the African cassava mosaic virus (ACMV) which is transmitted by white flies (*Bemisia tabaci*), and also via infected tools and planting material. There have been several epidemics of ACMV in the past, and the occurrence of the disease throughout the continent is very high. The most recent pandemic, possibly caused by a new variety of ACMV, which has caused famine-related deaths and huge losses in cassava production, is spreading from north to south through Uganda and western Kenya at 15–20 km/year (Otim-Nape, 1995; Otim-Nape *et al.*, 1994a,b, 1997; Gibson *et al.*, 1996). So far, no cassava variety completely resistant to ACMV could be produced by traditional breeding (Hong *et al.*, 1996), but in Uganda the use of new varieties and disease-free planting material have showed a positive effect (Bock, 1994; Otim-Nape *et al.*, 1997). On the other hand, as the resistance to ACMV appears to be recessive and polygenic (Hahn *et al.*, 1980), breeding of new varieties may lead to the loss of local land races, and genetic engineering may be necessary to transfer only the desired traits to these cultivars.

Cassava is a long-season crop, often grown by subsistence farmers, for whom the repeated or continual use of pesticides to prevent prolonged attacks of pests is economically prohibitive, in addition to being environmentally unsound. Due to its long growth period, 8–24 months, cassava is susceptible to prolonged and repeated attacks from several insect pests (Bellotti, 1979; Bellotti *et al.*, 1994). White flies can severely damage the plants, in addition to spreading ACMV. Mealybugs and green mites are the main insect pests of cassava in Africa, where green mites cause losses up to 80% (Yaninek, 1994), while leaf consumers like grasshoppers can also cause severe damage locally (Bellotti, 1979, Bellotti *et al.*, 1994; Le Rü and Calatayud, 1994). Nematodes can cause up to 98% losses, and the problem is increasing world-wide in severity in areas of intensive cassava cultivation, where crop rotation and fallow

periods are either shortened or abandoned altogether (Coyne, 1994). Cassava hornworms (*Erinnyis ello*) can completely defoliate the plants and also attack tender stem parts and lateral buds, killing young plants. Defoliation and stem damage reduce the starch quality of the roots and also cause yield losses between 10% and 50%, depending on plant age and the intensity of the attack (Bellotti and Arias, 1979). Recently, stem borers have been identified as one of the main problems in cassava cultivation in Latin America (Roca, pers. comm.)

Role of biotechnology in cassava improvement

Traditional breeding of cassava is difficult due to the fact that the highly heterozygous plants do not reproduce true to type via seeds. In addition, the low fertility and outcrossing nature with strong inbreeding depression of the plants prevent inbreeding the plants to homozygosity; and the limited gene availability in the sexually compatible germplasm further restricts the use of traditional breeding in cassava improvement. Many of the available resistance traits are polygenic and/or recessive, which makes breeding for such characteristics complicated. Thus introgression of the desired characteristics to the numerous land races (as cassava is vegetatively propagated, every cultivar is basically a clone), adapted to specific environments will be complicated and slow. Biotechnology is a powerful tool to complement traditional breeding, and can extend the genetic pool for useful gene sources over species barriers. It also offers the possibility of engineering single traits precisely, without the problems encountered in traditional breeding that arise from the introduction of additional, often undesirable genetic material. The transfer of multigenic traits also lies within the scope of biotechnology, once the essential basic techniques have been established. These techniques comprise reliable *in vitro* culture and regeneration methods compatible with regeneration methods, which allow production of transgenic plants, combined into a routine and efficient system easily transferable between different laboratories. Characterisation of cassava genome, gene isolation and the development of molecular maps are prerequisites for the development of marker-assisted breeding programmes and map-based gene cloning, as well as for further studying and manipulating metabolic pathways in cassava.

Tissue culture (*Figure 2*) is used routinely for production of disease-free planting material and for mass propagation of selected cassava lines (for reviews see Roca, 1984; Raemakers *et al.*, 1997a), but until lately, cassava was considered recalcitrant to genetic engineering. A recent breakthrough was achieved, when first reports on regeneration of transgenic cassava plants were published (Li *et al.*, 1996; Raemakers *et al.*, 1996; Schöpke *et al.*, 1996). Besides genetic engineering technology, great advances have also been made in developing methods for *in vitro* conservation, molecular mapping, gene isolation and microbial processing techniques.

Tissue culture and regeneration of cassava

MERISTEM CULTURE AND DISEASE ELIMINATION

Cassava is propagated vegetatively, and hence it is susceptible to diseases that are carried from one generation to the next through infected planting material. Especially

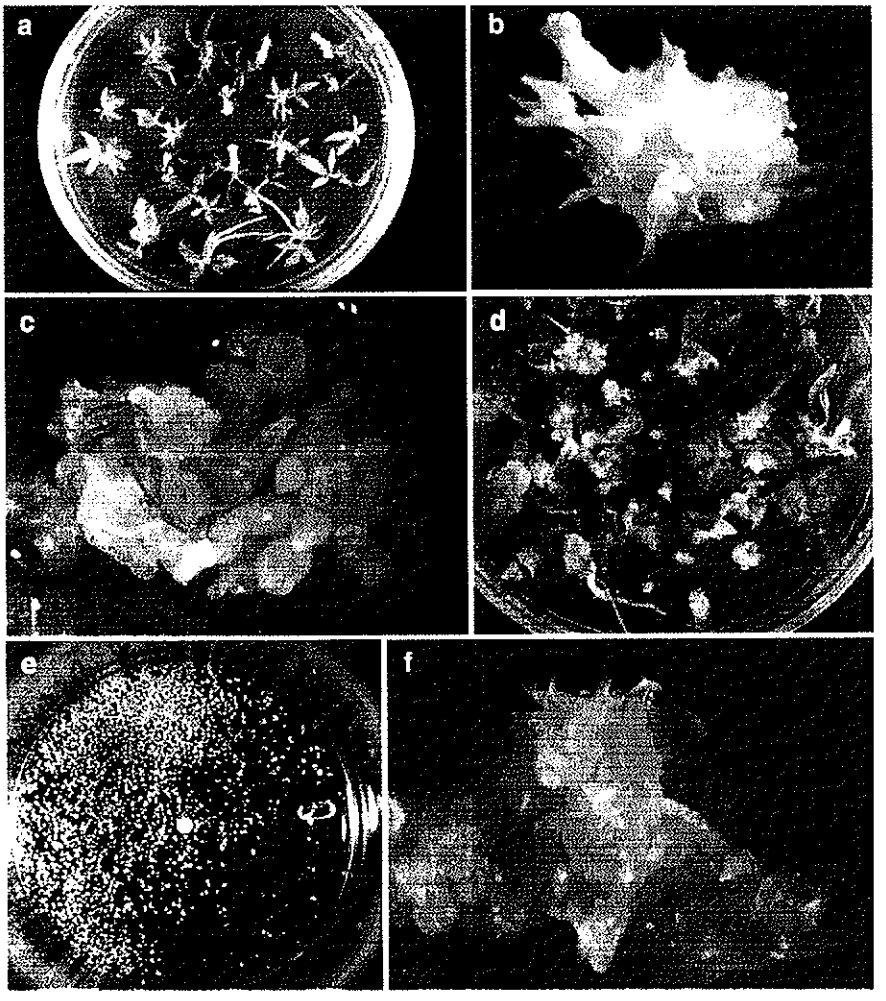

Figure 2. *In vitro* culture and regeneration methods in cassava. (a) Shoot regeneration from node culture. (b) Multiple shoot regeneration from meristem. (c) Torpedo stage and maturing somatic embryos. (d) Germinating somatic embryos. (e) Embryogenic suspension. (f) Shoot regeneration via organogenesis

international exchange of plant material, but also the maintenance of plant germplasm require the use of disease-free stocks (Roca *et al.*, 1982; Schilde-Rentschler and Roca, 1987). Meristem cultures can be used to maintain and propagate cassava cultivars *in vitro* and to produce clean planting material free of viruses and other diseases. *In vitro* culture of cassava meristems was first reported in 1974 by Kartha, who could regenerate shoots from five cassava varieties, and showed that the growth of and shoot regeneration from the meristems is promoted by addition of 0.1 mg/l BA, 0.04 mg/l GA_3 and 0.2 mg/l NAA in the culture medium (Kartha, 1974). Meristem cultures were used to produce plants free from cassava mosaic virus, when explants smaller than 0.4 mm were used (Kartha and Gamborg, 1975). Meristem culture can be combined with thermotherapy to improve the elimination rate of a number of viruses and bacteria, and

it also allows the use of larger meristem explants up to 0.8 mm (Kartha and Gamborg, 1975). For disease elimination of field grown material, cassava stakes are grown in the greenhouse at 35°C or 40°C/35C day/night temperatures for 3–4 weeks, after which the meristems of the sprouting shoots are isolated and cultured *in vitro* for 3–6 weeks. The shoots formed by the meristems are then tested for the presence of infectious agents, and if clean can be used for *in vitro* storage, micropropagation and for germplasm exchange. The meristems from still infected shoots can be isolated again and subjected to thermotherapy *in vitro* (Roca, 1984; Ng *et al.*, 1990; Roca *et al.*, 1991; Frison, 1994; Escobar *et al.*, 1995). By use of meristem culture and thermotherapy 90%–100% success has been reported in eliminating among others cassava bacterial blight, frog skin disease (Schilde-Rentschler and Roca, 1987), cassava brown streak virus (Kaiser and Teemba, 1979) and African cassava mosaic virus (Adejare and Coutts, 1981). Cleaning the material of pathogens has been shown to increase the productivity of cassava 50%–100% in the farmers' fields (Mabanza *et al.*, 1995).

MICROPROPAGATION

Multiplication and delivery of improved cassava lines and disease-free plants to the farmers is essential to ensure sustained cassava cultivation. Propagation of cassava via stakes is slow, as one plant can produce only 10–20 stakes per year (Roca, 1984). New techniques based on single leaf-bud cuttings increase the multiplication rate considerably (Roca *et al.*, 1980), with a potential up to 300 000 new stakes per plant per year. Micropropagation *in vitro* based on meristem culture has even greater potential. Meristems cultured *in vitro* can be induced to form multiple shoots on cytokinin-containing medium. BA at concentrations between 0.5 and 10 mg/l alone or in combination with low amounts of auxins can be used to break down the apical dominance of axillary or apical meristems, which allows the proliferation of multiple shoots that can be harvested until several months after culture initiation. Culturing cassava shoot tips on a medium containing 1 mg/l BA led to the formation of rosette cultures, from which up to 20 shoots per original explant could be harvested weekly (Roca, 1984). In theory, the potential of the most efficient multiple shoot induction protocols has been estimated to be up to 1.2×10^{20} new shoots in one year (Smith *et al.*, 1987), and in China 800 000 transplantable plantlets of a high-yielding genotype Nan-Zhi 188 have been produced for distribution to the farmers in two years using an efficient low-cost *in vitro* propagation system (Guo and Liu, 1995).

GERMPLASM CONSERVATION

Because cassava does not breed true to type, the collected lines cannot be stored as seeds. Cassava gene banks throughout the world conserve cassava germplasm either as field collections or *in vitro*. Field collections are maintained as continuous clonal cultivations, which are bulky and require large areas. Also, they expose the plants to environmental hazards, genetic erosion, diseases and pests. *In vitro* gene banks are an alternative for field collections. To reduce the amount of work involved and to minimize the risk of infections through frequent subculturings in maintenance of *in vitro* banks, slow growth shoot culture techniques have been developed. The shoots are maintained under low light conditions on modified MS (Murashige and Skoog, 1962) medium supplemented either with no growth regulators or with 0.01 mg/l NAA,

0.02 mg/l BA and 0.1 mg/l GA_3 (Roca *et al.*, 1989). CIAT (Centro Internacional de Agricultura Tropical) in Cali, Colombia, has the world mandate for cassava, and maintains the largest *in vitro* collection of cassava containing almost 6000 accessions, requiring about 50 m^2 space (Escobar *et al.*, 1993, 1997). The suitability of *in vitro* collections for maintaining genetic stability in cassava has been recently confirmed on the molecular level in plants retrieved from cultures kept under slow growth conditions for ten years (Angel *et al.*, 1996).

Even slow growth cultures may, however, be subject to genetic and physiological changes, and the most stable form of germplasm conservation is cryopreservation, where the tissues are frozen and stored under liquid nitrogen at –196°C. Early studies showed that cryopreservation of cassava meristems, seeds and somatic embryos is possible (Bajaj, 1977; Karthaet *al.*, 1982; Marinet *al.*, 1990; Mycocket *al.*, 1995), and recently an improved protocol for cryopreservation of cassava shoot tips allowing 50%–70% plant recovery was reported (Escobar *et al.*, 1997). Shoot tips were isolated from *in vitro* grown shoot cultures, pre-cultured on a medium containing 182.2 g/l sorbitol and 0.78% DMSO, treated with cryoprotectant (182.2 g/l sorbitol and 10% DMSO, 4% sucrose), for 2 hours and dehydrated for 1 hour on filter paper, after which they were cooled stepwise to–40°C, and finally transferred to liquid nitrogen. Regrowth of shoots from cryopreserved shoot tips was initiated on shoot culture medium after a recovery period of two days first on 25.7% sucrose and 0.25% activated charcoal and then on one-third strength MS medium with 12.8% sucrose and 1 g/l inositol in the dark. Originally 11 out of 15 tested varieties could be successfully regenerated after cryopreservation, but later modifications of the protocol allowed also some of the initially recalcitrant cultivars to be regenerated at high frequency.

EMBRYO RESCUE

The survival and germination rates of cassava seeds are low, which can severely constrain breeding programmes (Byrne, 1984; Bai, 1987; Ogburia and Adachi, 1995). Plant germination from seeds could be improved from 1%–15% to 41%–80% by isolating and culturing embryonic axes from mature seeds at 30°C on a medium containing 0.25 mg/l IBA under continuous light (Biggs *et al.*, 1986). Newly matured seeds had the highest germination rate, and in ageing seeds the germination rate reduced rapidly. Both mature and immature embryos of cassava and wild *Manihot* species could be induced to germinate *in vitro* on a medium containing half strength MS salts. Up to 100% plantlet growth could be obtained when mature embryos were cultured on a medium supplemented with 3% sucrose, while immature embryos responded best on a medium supplemented with 4% sucrose, 15% coconut water and 5 mg/l IAA (Ng, 1992). When cultured on a medium supplemented with 2% sucrose and 1 mg/l GA_3, immature embryos isolated 35 days after pollination showed the highest germination rate (Catano *et al.*, 1993).

DE NOVO REGENERATION

Multiple shoot induction

Meristems cultured *in vitro* on shoot culture medium containing no, or only low amounts of, growth regulators produce one shoot, but they can be induced to form

multiple shoots on cytokinin-containing medium. Most of the shoots are derived from pre-existing axillary meristems (see section on micropropagation), but also *de novo* formation of new meristems and shoots has been described (Konan *et al.*, 1994a, 1995, 1997). Axillary buds could be induced to swell to bulb-like structures on media containing 2–10 mg/l BA, and transfer of such structures to media supplemented with either 0.1 mg/l NAA, 1 mg/l BA, 0.1 mg/l GA_3, or with 1–2 mg/l BA led to production of up to 14 multiple shoots per explant (Konan *et al.*, 1994a; Frey, 1996; Puonti-Kaerlas *et al.*, 1997a). Bhagwat *et al.* (1996) showed that a pre-treatment in liquid culture medium containing 0.025–0.05 mg/l thidiazuron increased the frequency of multiple shoot formation from nodal explants in several cultivars on solid culture medium containing 0.5 mg/l BA and 0.5 mg/l GA_3. Addition of the surfactant Pluronic-F68 in the medium for a two-week period during the multiple shoot induction on 10 mg/l BA also increased the shoot proliferation in cultivars responding poorly to BA treatment alone, and in the best responding cultivars allowed up to 25 shoots to be harvested per explant (Konan *et al.*, 1997). Using a similar protocol, multiple shoots could also be induced from apical meristems, but the number of shoots was lower than when axillary buds were used (Frey, 1996; Puonti-Kaerlas *et al.*, 1997a).

Somatic embryogenesis

Production of somatic embryos was first reported from cotyledons and embryonic axes of cassava zygotic embryos in 1982 (Stamp and Henshaw, 1982), and somatic embryogenesis is now the most commonly used regeneration method for cassava (*Figure 3*). The competence for somatic embryogenesis in cassava is restricted to

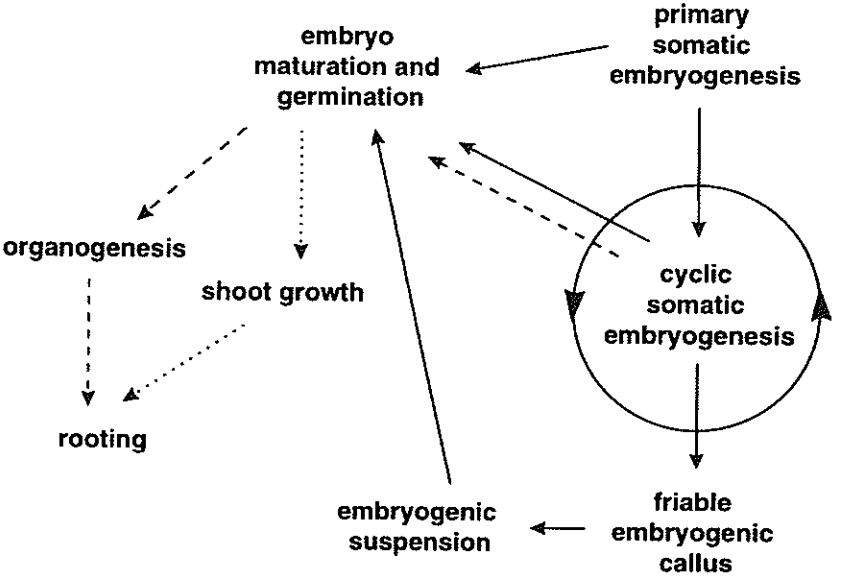

Figure 3. Regeneration schemes based on somatic embryogenesis in cassava. Dashed lines show regeneration via organogenesis, dotted lines regeneration from somatic embryos not directly regenerable to plantlets via germination.

meristematic and embryonic tissues, and somatic embryos can only be induced on a limited number of explants, e.g. cotyledons or embryonic axes from zygotic embryos (Stamp and Henshaw, 1982, Konan *et al.*, 1994a,b), immature leaf lobes (Stamp and Henshaw, 1987a; Szabados *et al.*, 1987; Mathews *et al.*, 1993; Raemakers, 1993; Raemakers *et al.*, 1993a; Li *et al.*, 1995, 1996, 1998), meristems and shoot tips (Szabados *et al.*, 1987; Narayanaswami *et al.*, 1995; Puonti-Kaerlas *et al.*, 1997a), and immature inflorescences (Mukherjee, 1995; Puonti-Kaerlas and Woodward unpublished data) on auxin-containing media. Picloram at 1–12 mg/l, dicamba at 1–66 mg/l and 2,4-D at 1–16 mg/l have been used to induce primary somatic embryogenesis, the efficacy of the different growth regulators depending on the explant and the cultivar used (Ng, 1992; Mroginsky and Scocchi, 1993; Sudarmonowati and Henshaw, 1993; Taylor *et al.*, 1993; Li *et al.*, 1995, 1998). The embryogenic potential in general in cassava is highly genotype dependent, and there are some cultivars from which, despite repeated efforts, no somatic embryos could be obtained so far. Pretreatment of the donor plants with 2,4-D (Matsumoto *et al.*, 1991; Raemakers, 1993), and the addition of ABA (Konan *et al.*, 1994b), or supplementary cupric sulphate to the embryo induction medium (Schöpke *et al.*, 1993), have been shown to improve the embryo induction frequency in some cassava cultivars. Often it is possible to induce proembryonic structures, or even globular embryos on explants from recalcitrant cultivars, but these do not develop further to torpedo-shaped or maturing embryos (Sudarmonowati and Bachtiar, 1995; Raemakers *et al.*, 1997b; Puonti-Kaerlas unpublished data).

When maintained on auxin-containing medium, some of the somatic embryos spontaneously start developing to torpedo-shaped and eventually mature embryos with greening cotyledons. This process is enhanced if the embryos are transferred to hormone-free medium or to a medium containing low amounts of BA and auxin (Stamp and Henshaw, 1982,1987a). The frequency of germination of mature somatic embryos, however, is usually low, and the root development is incomplete. A two-step protocol is therefore normally applied first to allow shoot elongation of the mature somatic embryos on a medium containing 0.1–1.0 mg/l BA and 0.01 mg/ 2,4-D and then to root the shoots on a hormone-free medium or a medium containing low amounts of auxin. A new method including a culture step on hormone-free medium containing 0.5% activated charcoal followed by desiccation before transfer to hormone-free medium allowed up to 83% plant regeneration from germinating embryos with normal root development (Matthews *et al.*, 1993). Likewise, additional GA_3 was shown to be beneficial for the further development of shoots from somatic embryos (Matsumoto *et al.*, 1991; Szabados *et al.*, 1987).

Primary somatic embryos can be induced to produce secondary somatic embryos by further subculturing on auxin-containing medium (Stamp and Henshaw, 1987b). In contrast to primary embryogenesis, also NAA can be used to induce secondary embryogenesis (Raemakers *et al.*, 1995a; Sofiari *et al.*, 1997). By constant subculturing of somatic embryos, a cyclic embryogenesis system can be established either in liquid or solid medium, where the embryos rarely pass the torpedo stage, until transferred to germination medium. Paclobutrazol used in combination with 2,4-D has been shown to increase the frequency of secondary somatic embryogenesis, and substituting maltose for sucrose in the embryo induction medium increases the rate of somatic embryogenesis, germination and plant regeneration, allowing up to 24 plants to be

regenerated per original explant (Li *et al.*, 1995, 1998). The efficiency of secondary somatic embryogenesis has been shown to be higher in liquid cultures than on solid cultures (Raemakers *et al.*, 1993b,c; Li *et al.*, 1995), and the use of NAA allows efficient production of somatic embryos with high germination capacity. To obtain maturing embryos with complete shoot and root poles from NAA induced embryo cultures, desiccation and further culture on a medium containing 0.1–4 mg/l BA is necessary, but then the efficiency is higher than when embryos maintained on 2,4-D are used (Raemakers *et al.*, 1997c; Sofiari *et al.*, 1997). In most tested cultivars the BA treatment enhanced both germination and root formation of NAA induced embryos, while in 2,4-D induced embryos with one exception only the root formation frequency was improved. Controlled desiccation experiments showed that the highest germination rate is obtained when embryos were allowed to dry to 60% of their original weight (Raemakers *et al.*, 1997c).

Friable embryogenic callus and embryogenic suspensions

A fraction of the cycling somatic embryos maintained on a MS medium supplemented with 12 mg/l picloram is able to produce a new tissue type which consists mainly of small globular embryo-like structures. When isolated, it will produce a highly friable embryogenic callus (FEC) (Taylor *et al.*, 1996). The frequency of FEC production can be increased by substituting the MS medium with GD medium (Gresshoff and Doy, 1974). Once pure FEC has been obtained it can be easily transferred to liquid culture to establish a rapidly proliferating embryogenic suspension in liquid SH (Schenk and Hildebrandt, 1972) medium supplemented with 6% sucrose and 12 mg/l picloram. By transfer to hormone-free medium, development of maturing embryos is induced. The efficiency of the maturation step could be improved by transferring the cultures maintained on 10 mg/l picloram to a medium supplemented either with 1 mg/l picloram or 1 mg/l NAA, which allows the globular embryos to continue their development to torpedo stage and germinating embryos. The conversion of globular embryos is still the limiting step of the regeneration protocol, as only a small fraction of the embryos is able to develop into torpedo stage embryos, and the variation in the cultures is high (Raemakers *et al.*, 1997c).

Organogenesis

Early results of shoot regeneration from cassava callus (Tilquin, 1979) could never be repeated, and only recently an efficient alternative regeneration system via organogenesis has been developed in order to circumvent the problems encountered when regenerating plants via germinating somatic embryos (Li *et al.*, 1995,1996,1998). Shoot primordia were induced directly on cotyledon explants from germinating cycling somatic embryos. A cycling system where the secondary somatic embryos were induced on cotyledon explants from maturing somatic embryos was established. To induce shoot organogenesis, cycling somatic embryos were transferred to maturation medium containing 0.1 mg/l BA, and the developing young green cotyledons were harvested, cut to pieces, transferred to organogenesis medium containing 1 mg/l BA and 0.5 mg/l IBA and cultured for 20 days in the darkness. After a passage on elongation medium containing 0.4 mg/l BA, the regenerating shoots could be easily

rooted on hormone free medium and transplanted into soil in the greenhouse. Shoot induction frequency of different cassava cultivars varied between 42% and 67% and shoot primordia could be induced on cotyledons from cycling embryos maintained either on 2,4-D or picloram. Cotyledon explants derived from cycling somatic embryos showed the highest competence for organogenesis, while those from primary somatic embryos responded very poorly. Compared to regeneration via germination of embryos derived from suspensions, shoot regeneration via organogenesis is faster, thus requiring less time in tissue culture. Using organogenesis, transplantable shoots can be regenerated from cotyledon explants within 60–65 days. In addition, the germination/maturation steps in the protocol ensure the selection for highly regeneration competent embryos, hence minimizing the risk of producing embryogenic cultures that will be arrested in their development.

Genetic transformation

Production of stably transformed plants requires several factors to be fulfilled (Potrykus, 1991). First, an efficient *in vitro* culture system that allows regeneration of plants has to be developed. Plant cells are generally considered to be totipotent, thus being able to regenerate whole plants from single cells *in vitro*. The ability to react to external stimuli and to regenerate *in vitro* is, however, often limited to certain tissues and developmental stages, as discussed in the previous chapter on cassava regeneration, and the requirements for transformation and regeneration competence may not always be compatible. Secondly, a method for efficient transfer and stable integration of the transgenes into the plant genomic DNA is essential; this also includes means for identifying and selecting for transformed cells. Finally, the introduced genes must be correctly expressed in the primary transgenic plants and stably transmitted to their progeny. In the case of cassava, which is vegetatively propagated, the transgenes can be fixed already at the level of the primary transgenic plants, and stable inheritance is of concern only when the transgenic plants are to be incorporated in breeding programmes.

There are several methods for delivering foreign DNA into plant cells (for a set of protocols, see Potrykus and Spangenberg, 1995). The most commonly used transformation method is *Agrobacterium*-mediated gene transfer. Not all plant species, however, are amenable to *Agrobacterium*-mediated gene transfer, and for these, direct gene transfer methods have to be developed. Of the vectorless methods, particle bombardment is the most frequently used, other techniques include among others chemically induced DNA uptake, electroporation, microinjection and electrophoresis.

AGROBACTERIUM-MEDIATED GENE TRANSFER

In nature, the crown gall disease on plants is caused by virulent strains of *Agrobacterium tumefaciens* (for review, see Kado, 1991). These strains contain a large plasmid (Ti-plasmid), from which a sequence (T-DNA) is transferred into the nucleus of the plant cells and integrated stably into their genomic DNA. Expression of the genes encoded by the T-DNA cause, among other things, uncontrolled proliferation of the transformed cells, which leads to gall formation. T-DNA is delimited by borders, 25 base pair long direct repeats, which are the only T-DNA sequences essential for T-DNA

recognition, excision and transfer. The whole DNA sequence inside the borders can be replaced by any passenger sequence, which will subsequently be transferred into plant cells by the engineered *Agrobacterium*. Non-oncogenic vectors, which no longer carry the genes leading to tumour development, and allow regeneration of normal plants, have been developed (e.g. Zambryski *et al.*, 1983) and are widely used for transformation of a number of plant species. Cassava has been shown to be susceptible to *Agrobacterium* (Calderon-Urrea, 1988), but the efficiency of different strains is highly variable and genotype-dependent (Chavarriaga-Aguirre *et al.*, 1993; Sarria *et al.*, 1993a; Li *et al.*, 1996; Puonti-Kaerlas *et al.*, 1997b,c).

VECTORLESS SYSTEMS

'Naked' DNA was first delivered into plant protoplasts by chemically induced DNA uptake (Davey *et al.*, 1980; Krens *et al.*, 1982). Transformation of protoplasts by electroporation was first reported in 1985 (Fromm *et al.*, 1985, Shillito *et al.*, 1985). The disadvantage of protoplast techniques is that the regeneration of whole plants from protoplasts is still limited to a few plant species. The use of complex explants can circumvent the need for protoplast regeneration, but the cell wall was long considered a barrier to DNA transfer into intact plant cells. The development of particle guns and tissue electroporation methods made it possible to transfer DNA also into plant cells surrounded by cell wall and to whole tissues. First transgenic plants were regenerated from electroporated immature embryos of maize with partially digested cell walls and from maize embryogenic callus that had been mechanically wounded (D'Halluin *et al.*, 1992), and later it was shown that no wounding is required for DNA transfer (Klöti *et al.*, 1993). Although transgenic rice plants could be produced from electroporated embryo tissues (Xu and Li, 1994), the transformation frequencies obtained using tissue electroporation are relatively low, and the technique is not widely used.

Biolistic methods are based on particle guns which use microprojectiles that are accelerated into plant cells through the cell walls. The transforming DNA is bound to the particles that carry it into the cells. Particle acceleration is based either on gunpowder explosion (Klein *et al.*, 1987; Sanford *et al.*, 1987), instantaneous water vaporisation (McCabe and Christou, 1993) or on gas bursts (Finer *et al.*, 1992). The advantages of biolistics are that cell layers situated under the surface can be reached, and little tissue specificity in the efficiency of particle delivery has been observed so far. Bombardment parameters can be easily optimized using transient expression studies of visual markers, but high transient expression after particle bombardment does not always correlate with high stable transformation frequency. On the other hand, both electroporation and particle bombardment require access to highly developed equipment, whereas transformation via *Agrobacterium* only necessitates use of standard laboratory facilities.

SELECTABLE AND SCREENABLE MARKER GENES

Stable transformation frequencies are low, and the use of different marker genes is necessary to allow the identification and selection of the stably transformed cells (for review, see Schrott, 1995). Of the visual markers, GUS encoded by the *uidA* gene (Jefferson, 1987; Jefferson *et al.*, 1986) is the most commonly used. In a histochemi-

cal assay a blue indigo precipitate indicates the activity of the *uidA* gene in transformed plant cells. The disadvantage of the GUS assays is that they are lethal, and in only few cases can be used to select for and to produce transgenic plants (Christou, 1990; Christou and McCabe, 1992). Other visual non-lethal marker genes include the *C1* and *Bperu* that can be used to induce biosynthesis of anthocyanin in transformed cells (Ludwig *et al.*, 1990; Bilang *et al.*, 1993; Klöti *et al.*, 1993), the green fluorescent protein gene (Chalfie *et al.*, 1994), and the luciferase genes from the firefly *Photinus pyralis* (Ow *et al.*, 1986) and from the soft coral *Renilla reniformis* (Mayerhofer *et al.*, 1995). Transformed plant cells expressing the luciferase genes emit light when supplied with an appropriate substrate, and transgenic *Dendrobium* plants could be produced using luciferase as the sole marker system for identifying and isolating transgenic tissues (Chia *et al.*, 1994). Luciferase is an efficient, sensitive non-invasive detection method, but its use requires access to costly equipment like a charged-coupled device camera for detection and localization of the bioluminescence from transformed cells.

Selectable marker genes render plant cells resistant to an antibiotic, a metabolic analogue or a herbicide, thus allowing the cells containing and expressing the transgene to survive and proliferate, while the wild-type cells are either arrested in their growth or killed. The most commonly used selectable marker genes include *nptII*, which encodes resistance to aminoglycoside antibiotics like kanamycin, neomycin, geneticin and paromomycin (Bevan *et al.*, 1983; Fraley *et al.*, 1983; Herrera-Estrella *et al.*, 1983; Potrykus *et al.*, 1985), *hpt*, which encodes resistance against hygromycin (van den Elzen *et al.*, 1985; Waldron *et al.*, 1985), and *pat* and *bar* which encode resistance against phosphinotricin, the active ingredient in many herbicides (Murakami *et al.*, 1986; De Block *et al.*, 1987; Thompson *et al.*, 1987; Wohllenben *et al.*, 1988).

Transformation of cassava

MERISTEMS

Transient and stable expression of both GUS and luciferase have been demonstrated in meristems and meristem-derived somatic embryos and multiple shoot clusters after particle bombardment (Puonti-Kaerlas *et al.*, 1997a) or cocultivation with *Agrobacterium* (Konan *et al.*, 1995; Puonti-Kaerlas *et al.*, 1997a). Transgenic sectors could be detected in the developing shoots, but so far no fully transgenic plants have been regenerated.

SOMATIC EMBRYOS

Both primary and secondary somatic embryos develop from groups of cells, usually located at or near the vascular tissue (Stamp, 1987; Raemakers *et al.*, 1995b). The multicellular origin of the somatic embryos makes them poorly suited for genetic engineering, and their location under the plant epidermis also limits their accessibility to *Agrobacterium*. Transgenic sectors were detected in somatic embryos after electroporation, but up till now no transgenic plants have been regenerated from the electroporated embryos (Luong *et al.*, 1995). Particle bombardment of embryogenic

clusters has led to high transient expression of visible marker genes in several laboratories, but at best only sectorial transgenic embryos could be regenerated from bombarded embryos. Transgenic callus can be easily obtained from explants from cycling somatic embryos, but as a rule the competence for embryogenesis is lost when the cultures are treated with antibiotics to select for transformed cells (Chavarriaga-Aguirre *et al.*, 1993; Schöpke *et al.*, 1993; Puonti-Kaerlas unpublished data).

There is only one report on successful regeneration of transgenic cassava plants via secondary somatic embryogenesis with PCR data to show the presence of transgenes in the regenerants (Sarria *et al.*, 1995). In this case, secondary somatic embryos were induced on cotyledon explants from primary somatic embryos of cultivar MPeru183 after cocultivation with the wild-type *Agrobacterium* strain CIAT 1182 carrying the genes encoding GUS and resistance to the herbicide Basta. Selection on 16–32 mg/l Basta allowed the regeneration of putative transgenic secondary embryos that could be germinated to plants. The cultivar MPeru183 and the *Agrobacterium* strain CIAT 1182 were selected after a screen for the most efficient combination to ensure high transformation frequency (Sarria *et al.*, 1993a). The reproducibility of this method is still to be tested, and its main current disadvantage is that the CIAT 1182 is still oncogenic and thus its use for production of transgenic plants on routine basis is limited. Disarming of the vector should in the future offer great potential for further improvement of *Agrobacterium*-mediated transformation methods.

EMBRYOGENIC SUSPENSIONS

In contrast to the primary or secondary somatic embryos, the new embryogenic units in friable embryogenic callus develop from the surface cells of the globular embryo clusters, and appear to be of single cell origin, which makes them good targets for transformation (Taylor *et al.*, 1996). Particle bombardment of embryogenic suspensions of cultivar TMS 60444 allowed regeneration of transgenic cassava plants via two different approaches. One was based on selection for resistance to paromomycin (Schöpke *et al.*, 1996), the other on visual selection using firefly luciferase (Raemakers *et al.*, 1996). When antibiotic selection was used, the bombarded tissues were first grown for two weeks after transformation in liquid medium containing 12 mg/l picloram, after which paromomycin was added to the liquid culture at 15 mg/l. After 4–5 weeks of liquid selection the developing embryogenic units were transferred to solid culture medium under the same selective conditions and cultured further for another 4 weeks. Regeneration of plants was only possible, when no selection was applied. Therefore, after the transgenic units were multiplied as friable embryogenic callus, shoot regeneration was initiated by sequential transfer and culture on a series of media to induce the differentiation of globular and torpedo stage embryos (1.2 mg/l picloram), development of cotyledons (0.93 mg/l NAA) and maturation (0.5% activated charcoal, no growth regulators) of embryos. Before attempting to root the regenerants, a multiplication step to induce multiple shoot formation from the apical meristem of the germinating embryos was applied (1 mg/l BA). The regeneration capacity differed greatly between the selected lines, and in some cases no plants could be regenerated. Southern blot data were published to prove the presence of the *UidA* gene in one regenerated shoot.

When firefly luciferase was used to identify and select for transgenic tissues, the

embryogenic suspensions were cultured for one day after bombardment on solid medium containing 6% sucrose, and then transferred either to liquid medium containing 6% sucrose or to solid medium on which the sucrose concentration was reduced from 6% to 2% in two 3-day subculture steps. Two weeks after bombardment the cultures were monitored for the first time for luciferase activity, and clusters of embryogenic units at and around the luciferase positive spots were isolated and cultured further. The luciferase screen and tissue selection was repeated at 2-week intervals, until 2 months after bombardment the friable embryogenic calli clusters containing at least 1% luciferase positive units were transferred to maturation medium containing 1 mg/l picloram for development of somatic embryos. The maturing luciferase positive embryos were further multiplied via secondary somatic embryogenesis on media containing either 10 mg/l NAA or 8 mg/l 2,4-D, and in subsequent steps of cyclic somatic embryogenesis 10 mg/l NAA. After desiccation, the somatic embryos were induced to germinate on a medium containing 1 mg/l BA, and then multiplied as shoot cultures by nodal cuttings. The efficiency of the secondary embryo formation from selected embryos was 83%, but only 1–15% of the embryos could be germinated to transplantable shoots. Southern blot data confirmed the transgenic nature of three plants. A method combining antibiotic selection using phosphinotricin and luciferase showed that use of 20 mg/l phosphinotricin allowed the development of both transformed and non-transformed maturing embryos from bombarded friable embryogenic callus, but the luciferase screening could be efficiently used to exclude escapes, and inclusion of 20 mg/l phosphinotricin in the maturation medium efficiently blocked the maturation of non-transgenic embryos (Snepvangers *et al.*, 1997).

The use of the protocols described above depends on the availability of embryogenic suspensions, the establishment of which is highly genotype dependent and labour intensive. The low regeneration rates and the regeneration of possibly abnormal plants reduce the transformation efficiencies obtained after bombardment of embryogenic suspensions (Taylor *et al.*, 1996; Raemakers *et al.*, 1997b,c; Snepvangers *et al.*, 1997). In all the protocols where embryogenesis is used as a way of regeneration, the use of antibiotic selection, with the possible exception of phosphinotricin, reduces the regenerative potential of the transgenic material. To avoid this, relatively complicated, time-consuming and labour-intensive regeneration schemes must be followed, which may increase the risk of somaclonal variation in the transgenic plants.

ORGANOGENESIS

The shoots regenerated via organogenesis develop from cells at or close to the cut edges of the cotyledon explants, which makes them good targets for *Agrobacterium*-mediated gene transfer. In contrast to the protocols based on somatic embryo production, both callus and shoot development were inhibited by similar amounts of antibiotics, and both geneticin, hygromycin and phosphinotricin could be used as selective agents (Li *et al.*, 1996; Puonti-Kaerlas *et al.*, 1997a,b). In compatibility tests conducted with four different *Agrobacterium* strains carrying an intron-interrupted *uidA* gene (Vancanneyt *et al.*, 1990), cassava cotyledon explants cocultivated with LBA4404 (pTOK233) (Hoekema *et al.*, 1984; Hiei *et al.*, 1994) and LBA4404(pBin9GusInt) (Holtorf *et al.*, 1995) showed the highest transient transformation frequencies. Preinduction of the *Agrobacteria* with acetosyringone increased the transformation

frequency considerably, and a two-hour preinduction time was found to be optimal. Extending the cocultivation time to four days resulted in the highest transient transformation rates without excessive bacterial contamination. The developmental state of the explants was also found to be a critical factor in the transformation procedure. Cotyledons from newly germinated embryos were very sensitive to *Agrobacteria*, and survived the cocultivation procedure poorly, which resulted in low transformation rates. Cotyledon explants from older embryos survived better, but explants from germinating embryos older than 20 days regenerated less efficiently. The highest regeneration and transformation frequencies were obtained by using cotyledon explants from somatic embryos cultured for 15 days on maturation medium.

Following cocultivation, callus and small resistant shoot primordia developed on selection medium containing 15 mg/l hygromycin or 20 mg/l geneticin from the cotyledon explants cocultured with LBA4404(pBin9GusInt) or LBA4404(pTOK233), respectively. In GUS assays three out of 27 regenerated geneticin resistant shoot primordia and six out of 30 hygromycin resistant shoots stained blue. After rooting, the putative transgenic shoots could be transferred to soil in the greenhouse (Li *et al.*, 1996). Cloned plant material was stained for GUS activity in order to asses the expression of the 35S promoter in different cassava tissues. In contrast to earlier reports based on transient assays after particle bombardment (Arias-Garzón and Sayre, 1993), the 35S promoter was shown to be highly expressed in all cassava tissues, including all parts of the roots. The highest expression levels, as determined by the intensity of the blue colour, were in the youngest tissues, including apical and axillary meristems and root tips (Puonti-Kaerlas *et al.*, 1997b,c). The stable integration of the transgenes into cassava nuclear DNA was demonstrated in five transgenic plants and Northern data to prove the transcriptional activity of the transgenes were presented. The selection system still needs further improvement, as some escapes could be found, but by use of PCR the escapes can be easily identified and discarded.

Potential of genetic engineering in cassava improvement

DISEASE RESISTANCE

African cassava mosaic virus (ACMV) has been ranked the most serious vector-borne plant disease in Africa (Geddes, 1990), and it is a severe constraint to cassava cultivation. There are few means to protect the plants in the field from being infected with the virus, except by trying to control the spread of the vector, whitefly, by agrochemicals, which, for the subsistence farmers, is unviable economically and also unsound environmentally (Fishpool and Burbank, 1994). Virus resistance has been introduced to over 20 plant species by genetic engineering (for review, see Kahl and Winter, 1995), and there are indications from heterologous species (Frischmuth and Stanley, 1991; Hong and Stanley, 1996; Hong *et al.*, 1996; Stanley *et al.*, 1990; von Arnim and Stanley, 1992) that resistance against ACMV could also be transferred to cassava. Genetically engineered resistance against bacteria has been achieved, e.g. in tobacco and beans by either using genes rendering the plants resistant to bacterial toxins or expressing plant or non-plant defence genes or genes regulating cell death in infected tissues (for review, see Herrera-Estrella and Simpson, 1995). Such an

approach could also be used to enhance resistance of cassava against bacterial blight in the future.

PEST RESISTANCE

Cassava is a long-season crop, often grown by subsistence farmers, for whom the repeated or continual use of pesticides to prevent prolonged attacks of pests is economically prohibitive, in addition to being environmentally unsound (Bellotti *et al.*, 1994). The soil bacterium *Bacillus thuringiensis* carries a set of *cry* genes encoding insect specific ð-endotoxins (Bt toxins), which are efficient against a variety of insects. Spraying *Bacillus thuringiensis* has been shown to be efficient in biological control of cassava hornworm (Bellotti and Arias, 1979), and the spraying of Bt toxins has been recommended as a biological control method for cassava hornworm (Anon, 1978). Bt toxins have been shown to be very efficient in rendering transgenic plants resistant against several lepidopteran, dipteran and coleopteran species, and promising results are also emerging on nematode and mite control (DeWald, 1995; Kahl and Winter, 1995). An attractive alternative to spraying of commercial Bt products would be the production of the toxin in the plants themselves. Transgenic tobacco (Barton *et al.*, 1987; Vaeck *et al.*, 1987), tomato (Fischhoff *et al.*, 1987), cotton (Perlak *et al.*, 1990), potato (Chen *et al.*, 1992) and rice (Wünn *et al.*, 1996) expressing transferred *cry* genes have been shown to be effectively protected from pest attacks. Expression of the *cry* genes in transgenic cassava would complement the available methods for pest control in an environmentally and economically sustainable way.

CYANOGENESIS

The cyanogenic nature of cassava makes it necessary to process the roots or leaves before use, which is time consuming and laborious, and if not conducted properly also presents a health hazard to consumers.

By manipulating the key enzymes in linamarin synthesis, e.g. the cytochrome P-450 oxidase (Koch *et al.*, 1994), by downregulation via antisense technology, the levels of cyanogenic glucosides in cassava roots could be reduced. As the fear of possible poisoning is an obstacle to selling fresh cassava roots directly from the farms in certain parts of Africa, production of acyanogenic cassava would also contribute to the household income in these regions. Alternatively, increased expression of linamarase and α-hydroxynitrilase, the enzymes breaking down linamarin (Hughes *et al.*, 1995), would offer a way to enhance the rate of cyanide release during cassava processing. This would reduce the time and effort required for processing, which is the most labour-intensive phase of cassava utilisation, and also help to save firewood, at premium in many sub-Saharan countries. The residual cyanohydrins in processed cassava have been shown to be the main source of dietary cyanide (Tylleskär *et al.*, 1992), and thus maintaining a high activity of the α-hydroxynitrilase during cassava processing would be of high interest.

DIETARY SUPPLY OF PROTEIN AND VITAMINS

One of the problems of cassava consumption is the low protein content of the roots,

which can lead to qualitative protein malnutrition in areas where the diet is based mainly on cassava. Also, protein deficiency has been shown to aggravate symptoms related to cassava toxicity (Rosling, 1988). Introduction of a storage protein gene into cassava roots would improve the nutrient balance of cassava, and provide a cheap protein source. Cassava leaves contain valuable protein and provide a reliable, cheap source of vitamins, minerals and proteins (Balagopalan *et al.*, 1988; Bokanga, 1994b; Dahniya, 1994). However, frequent leaf harvesting reduces root production, and therefore leaves can only be harvested every two months in order to minimize losses in root yields (Bokanga, 1994b). Prolonging the life of individual leaves could help to maintain a satisfactory photosynthetic area while allowing more frequent harvesting of leaves. As the market value of leaves is often higher than that of the roots (Lutaladio and Ezumah, 1981), this could also contribute to household economies. Prolongation of leaf life has already been demonstrated in transgenic tobacco using a senescence-regulated promoter to control cytokinin production in ageing leaves (Gan and Amasino, 1995).

Vitamin A is vital to normal development in humans, and the consequences of vitamin A deficiency range from night blindness through total blindness to reduced resistance to various terminal diseases (Sommer, 1988; West Jr. *et al.*, 1989). According to UNICEF, approximately 124 million children in the world suffer from vitamin A deficiency (Sommer, 1988, Humphrey *et al.*, 1992). Expressing the corresponding genes from plants producing β-carotene, the precursor for the synthesis of vitamin A, in cassava roots would provide a step towards alleviating this problem. Cassava roots have a basic capacity for β-carotene synthesis, as shown by the identification of cassava cultivars with yellow roots containing carotenoids (Moorthy *et al.*, 1990; Adewusi and Bradbury, 1993). Thus the expression of the first enzyme in the pathway, phytoene synthase might be sufficient to produce yellow cassava roots. Burkhardt *et al.* (1997) have recently shown that it is possible to engineer this pathway in plants.

Genomic mapping, molecular markers and gene cloning in cassava

Cassava is a highly heterozygous species, and the results from cytogenetic studies have led to the proposition that cassava is either a segmental allotetraploid (Magoon *et al.*, 1969) or an allopolyploid (Umannah and Hartman, 1973). Isozyme studies, however, have revealed a predominantly disomic inheritance pattern (Levefre and Charrier, 1993; Sarria *et al.*, 1993b; Fregene *et al.*, 1995), which would indicate that cassava now behaves like a diploid. There is no classical genetic map of cassava, as the use of the traditional linkage analysis is constrained by the paucity of morphological markers in cassava. A genetic linkage map, which should greatly help in understanding the complexity of cassava genome, has recently been constructed in cassava (Fregene *et al.*, 1997). A set of RFLP markers was developed (Angel *et al.*, 1993) in order to select those showing the highest polymorphism for the construction of a framework molecular map, and RFLP and RAPD markers were developed to identify the intraspecific cross to produce the F1 mapping population (Gomez *et al.*, 1996). As the parents used to produce the intraspecific F1 mapping population were heterozygous, the framework map consists of female and male parent maps, and covers approximately 60% (931.6 cM) of the cassava genome. The female parent map

contains 132 RFLP, 30 RAPD, 3 microsatellite and 3 isoenzyme markers, and consist of 20 linkage groups spanning about 60% of the cassava genome, with an average marker density of 1 marker per 7.9 cM. The second map consisting of 107 RFLP, 50 RAPD, 1 microsatellite and 1 isoenzyme markers was constructed for the male parent. The maps revealed a significantly higher recombination rate in the gametes of the male parent than in the female parent; also the pattern of marker distribution shows that recombination does not occur uniformly in the cassava genome. The joining of the female and male maps and the addition of more markers to the map to produce a highly saturated map with tightly linked markers will allow its use in marker assisted breeding, map-based gene isolation and in more detailed characterization of the genome structure in cassava.

Genomic and cDNA libraries from leaves, roots, and cotyledons of cassava have been constructed (Hughes *et al.*, 1992; Salehuzzaman *et al.*, 1992; Tenjo and Mayer, 1993; Bohl *et al.*, 1997). These can be used to isolate genes and promoters from cassava, and also to characterize cassava germplasm (Beeching *et al.*, 1993). Branching enzyme (Salehuzzaman *et al.*, 1992) and granule-bound starch synthase (Salehuzzaman *et al.*, 1993) genes have been cloned as cDNAs from cassava root libraries. Expression studies showed that both enzymes are highly expressed in roots, but the branching enzyme signal is also highly abundant in the stems, while that of the granule-bound starch synthase is abundant in leaves of *in vitro* grown plants (Salehuzzaman *et al.*, 1994). Both the granule-bound starch synthase and ADP glucose pyrophosphorylase B gene, the key enzyme in starch synthesis, have been shown to be low copy number genes, while the branching enzyme is a single copy gene (Salehuzzaman *et al.*, 1992, 1993; Munyikwa *et al.*, 1995). The genes involved in the breakdown of cyanogenic glucosides, linamarase (Hughes *et al.*, 1992), and α-hydroxynitrilase (Hughes *et al.*, 1994) as well as the gene encoding the last enzyme in the linamarin synthesis pathway UDPG-glucosyltransferase (Hughes *et al.*, 1993) have been cloned as cDNAs. The linamarase gene appears to belong to a small gene family, while the α-hydroxynitrilase may be a single-copy gene (Hughes *et al.*, 1995). Using *in situ* hybridisation it has been shown that in cassava leaves the expression of the linamarase is restricted to the latex vessels (Pancoro and Hughes, 1992). Isolation of a genomic clone of cassava PEP carboxylase has also been reported (Tenjo and Mayer, 1993).

Processing technology

The cyanogens in cassava roots as well as the perishability and bulkiness of the roots constrain their use. Processing removes the cyanogens, and processed products have a longer shelf-life, are easier to transport, and also offer a wider variety of dishes for consumption. Microbial fermentation has been used in cassava processing traditionally in preparing fermented cassava products like farinha in Latin America, gari in Africa and tape in Indonesia. Bacteria, mostly of the genus *Lactobacillus* and *Corynebacterium*, and fungi, including *Aspergillus* and *Saccharomyces*, are used to detoxify cassava and to modify the taste, nutritional value and properties of cassava-based meal. A number of microbial strains and species involved in this process have been studied in order to develop more efficient methods for producing high quality products with high consumer acceptance, and also for developing new food and non-

food products (Balagopalan *et al.*, 1988; Bokanga, 1993, 1995; Oguntimein *et al.*, 1995; Padmaja and George, 1995; Suismono *et al.*, 1995; Zakhia *et al.*, 1995). Programmes directed towards products like protein-enriched animal feed (Dung *et al.*, 1995; Pham *et al.*, 1995), and recycling of effluents from starch factories, containing high amounts of organic matter and cyanide to produce e.g. microbial protein enriched animal feed (Balagopalan and Ray, 1993; Balagopalan *et al.*, 1995; Soccol *et al.*, 1995a,b) also show a great promise. New value-added products based on cassava include among other things food colourings, ethanol, citric acid, sweeteners and vitamin C (Balagopalan and Ray, 1993; Balagopalan *et al.*, 1995; Sriroth, 1995; Zakhia *et al.*, 1995). Fermentation of cassava to produce sour starch dried in the sun has been shown to be essential for the production of cassava flour that can be used for bread-making without the addition of gluten or rising agents (Westley and Cereda, 1994; Zakhia *et al.*, 1995; Figueroa Jr., 1997).

Conclusions

The development of new techniques allowing more efficient use and improvement of cassava has proceeded rapidly in the past few years. The new processing technologies provide possibilities for more diversification in the cassava industry, and for more efficient treatment of effluents. Genetic characterization and map construction of cassava are progressing, and will be a great asset for marker-assisted breeding, and for map-based gene isolation. Isolation of promoters and genes as cDNAs and genomic clones from cassava will contribute to the increased knowledge of metabolic pathways, their regulation, and eventually, their genetic engineering. Some examples of future projects, in addition to those discussed before, are the manipulation of starch composition in cassava and the production of biodegradable plastics. It has been shown that a transgenic potato line producing amylose-free starch could be obtained by expressing an antisense construct of the cassava granule-bound starch synthase gene in potato (Salehuzzaman *et al.*, 1993). As a first step towards production of biodegradable plastics in plants, accumulation of polyhydroxyalkanoates has been demonstrated in transgenic *Arabidopsis* plants expressing bacterial PHA genes (Poirier *et al.*, 1995). As soon as more information is available about post-harvest deterioration of cassava roots, means to control this problem may also be developed, possibly by manipulating the regulation of the key enzymes involved in the wound response reaction in cassava roots.

It is now possible to regenerate transgenic cassava plants, but so far there is little information on the transferability of the current protocols to other cultivars beyond the model cultivars used, or to other laboratories, and genetic improvement of cassava via biotechnology is still constrained by the lack of routine, efficient and genotype independent transformation methods. Despite the recent breakthroughs, transformation technology will still be one of the greatest bottlenecks in applying genetic engineering to cassava improvement, until a reliable routine technique, compatible with as many cultivars as possible has been developed. Different transformation systems may be needed for different cassava cultivars, and thus both direct gene transfer methods as well as those based on *Agrobacterium* should be developed further. The high proliferation rate of embryogenic suspensions will make the multiplication of transgenic tissues efficient, and embryogenic suspensions also

appear to have some potential as sources of regeneration competent protoplasts (Sofiari, 1996; Raemakers *et al.*, 1997c). There is preliminary evidence suggesting that the embryogenic suspensions are compatible with *Agrobacterium*-mediated transformation systems (Puonti-Kaerlas unpublished data), which would make this system less dependent on use of costly equipment. Likewise, the use of green fluorescent protein instead of luciferase might dispose of the need for the expensive detection system needed for luciferase assays. On the other hand, organogenesis may be a less genotype-dependent regeneration mode for cassava than germination of somatic embryos, and it will also allow more flexibility in the choice of selectable marker genes. The compatibility of the organogenesis-based regeneration system with particle bombardment is currently being tested, in order to overcome potential compatibility problems between cassava cultivars and *Agrobacterium*. The main limitation to the rapid development of new techniques for cassava is, and has been, the lack of funding for research in this area. It is to be hoped, that the progress achieved so far with limited resources will help to increase the interest in this important crop.

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