18. Developing transgenic sweetpotato for resistance to insect pests and diseases

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Introduction

Sweetpotato (*Ipomoea batatas* (L.) Lam.) ranks fifth after rice, wheat, maize, and white potato, among the food crops of the world (Janson and Raman, 1991). Sweetpotato is a staple diet in many tropical and subtropical zones of the developing world (Newell et al, 1995) and is also important as feed and biomass for industrial purposes. In Indonesia, a major constraint in increasing production of sweetpotato is insect damage and plant diseases. Among the major insect problems of sweetpotato, sweetpotato weevil (*Cylas formicarius* F.) is the most destructive one, especially in the tropics (Horton and Ewell, 1991). This insect feeds on roots in the field and in storage, reducing the quality and marketable yield of sweetpotato. Weevil is difficult to control in the tropics because it continuously reproduces throughout the year. Although the sweetpotato weevil damage is the most important constraint, damage brought about by plant diseases such as plant virus caused by Sweetpotato Feathery Mottle Virus (SPFMV) can also be significant.

The most effective management strategies to minimize losses include the use of resistant cultivars, crop rotation, natural enemies, and insecticides. The excessive use of insecticides is harmful to the environment and human health. Recently, several insecticides that were very effective against plant pests and diseases were removed from the market because of safety concerns. The lack of effective yet inexpensive insecticides has made the use of resistant cultivars an attractive option. This so far is the most economical and ecological sound strategy available to farmers. Unfortunately, there is very little source of resistance to weevil and SPFMV in the sweetpotato germplasm. Although there is extensive genetic variability in sweetpotato, it is hexaploid and thus, difficult to improve through conventional breeding (Lowe et al., 1994). Furthermore, there are problems of cross incompatibility, as well as instability in hybrid offspring.

Biotechnology, as a new frontier in agricultural science, has opened new avenues towards the solution of agricultural problems. Genetic modification of plants using recombinant DNA techniques holds the promise of increased crop productivity, product quality, and reduced dependence on chemical inputs for pest control (Asano et al., 1991). Genetic engineering offers a means to introduce desirable traits into sweetpotato, i.e., genes may confer increased resistance to sweetpotato weevil and SPFMV.

The objective of the studies described below is to develop a genotype-independent transformation and regeneration system for sweetpotato, to allow the introduction of genes that would increase resistance to sweetpotato weevil and SPFMV.

Methodology

There have been reports of regeneration from a variety of explant sources and genotypes but with varying degrees of success. Shoot organ genesis has been observed from stem internodes, leaves and roots (Carswell and Locy, 1984). Regeneration has also been induced directly from adventitious roots from shoot cultures (Ozias-Akins and Perera, 1990), and from de novo roots formed in tissue culture (Carswell and Locy, 1984). Somatic embryogenesis has been induced from stem, petiole, leaf, and root tissues (Liu and Cantliffe, 1984, Chee and Cantliffe, 1988). Regeneration from storage roots has also been demonstrated (Hwang et al., 1983, and Newell et al., 1995). It is also possible to regenerate sweetpotato from protoplasts (Sihachakr and Ducreux, 1987).

Approaches to producing transgenic sweetpotato plants include the electroporation of protoplasts (Nishiguchi et al., 1992), particle bombardment (Prakash and Varadarajan, 1992), *Agrobacterium rhizogenes*-mediated transformation (Otani et al., 1993), and *A. tumefaciens*-mediated transformation (Prakash and Varadarajan, 1991). However, these procedures have been very genotype-dependant, and often difficult to reproduce (Lowe et al., 1994). The first report of reproducible transformation and regeneration was achieved using pieces of storage root tissue inoculated with the *A. tumefaciens* strain LBA4404 (Newell et al., 1995). In that report the plasmid vector contained the *uid*A gene encoding -glucuronidase (GUS) (Jefferson, 1987), and the wild-type neomycin phosphotransferase II gene (*npt*II) (Yenofsky et al., 1990).

To develop transgenic plants, it is essential to have reliable methods for efficient production of plants in tissue culture. To meet the objectives, plant regeneration and plant transformation studies have been conducted in the Molecular Biology Research Division of Research Institute for Agricultural Biotechnology and Genetic Resources, the Agency of Agricultural Research and Development, in Bogor.

Plant materials

The sweetpotato cultivars used in the studies were Indonesian and the American cultivar Jewel. The Indonesian cultivars are: Alhamdulillah, Bentoel, BIS 182-81, BIS 183, BIS 192, Borobudur-4, BO68, Jepang, Kunyik, Lokal Majenang, Muara Takus, OP Paopola, SRIS 226, Tamburin Merah, Toweko, and Viola. The Sweetpotato Germplasm Bank in Griffin, Georgia, supplied *in vitro* cultures of sweetpotato cultivar Jewel. Shoot cultures were maintained in magenta boxes (Sigma Chemical Co.) containing micropropagation medium (Murashige and Skoog, 1962). The cultures were maintained under a 16-hour photoperiod at 26^0C.

Plasmid DNA and bacterial strains

Two plasmids of pTwa and pRQ6 were used for co-transformation via particle bombardment. The pTwa carries 0.6 kb insect resistance gene, protease inhibitor (pin II) gene, the 35 S promoters from the cauliflower mosaic virus (CaMV), and *bar* gene for selection marker. Whereas, pRQ6 contains the reporter gene, B-glucuronidase (*gus*) and selectable marker gene, and *hpt* for hygromycin resistance. The pin II gene used for insect resistance was obtained from Dr. Ray Wu's laboratory at Cornell University, Ithaca, New York. The pMON10574 and pMON10575 plasmids used for plant transformation for resistance to SPFMV were constructed by Dr. Florence Wambugu at Monsanto (unpublished). Both constructs contained nptII, uidA and the SPFMV coat protein gene. *Agrobacterium tumefaciens* strain LBA 4404 was used as plant transformation vector.

Plant regeneration study

Leaf and petiole explants of sweetpotato cultivars were used in the study. The sweetpotato cultivars or genotypes were: Alhamdulillah, Bentoel, BIS 182-81, BIS 183, BIS 192, Borobudur-4, BO68, Jepang, Jewel, Kunyik, Lokal Majenang, Muara Takus, OP Paopola, SRIS 226, Tamburin Merah, Toweko, and Viola. Three methods were used in the plant regeneration study: Newell et al. (1995), Gosukonda et al. (1995), and Wang et al. (1998). The procedure of Newell et al. (1995) involved placing of explants (leaf and petiole) on three steps of media, which first stimulated callus development on MS + 0.5 mg/l 2,4-D + 0.1 mg/l kinetin, followed by second step media of MS + 1 mg/l NAA + 0.1 mg/l BAP for pro embryo induction, and the third step media of MS + Staba vitamin for plant regeneration. Gokusonda et al. (1995) used MS + 0.4 mg/l tiamin HCl + 0.2 mg/l 2,4-D for callus induction, and MS + 0.4 mg/l tiamin HCl + 0.2 mg/l kinetin for plant regeneration. Whereas, Wang et al. (1998) used MS + 0.05 mg/l 2,4-D + 0.5 mg/l kinetin for callus induction and MS + 3 mg/l BAP or MS + Staba vitamin for plant regeneration. All cultures were maintained under a 16-hour photoperiod at 26^0C. The tissue was assessed at 3-4 weeks for percent embryogenesis. After 5-8 weeks, the number of explants regenerating shoots on a particular medium was recorded.

Plant transformation study

The regeneration study demonstrated that only the Jewel cultivar was able to regenerate well. Consequently, leaf and petiole tissues of Jewel were used for plant transformation study. The studies were performed to attempt to genetically transform sweetpotato using an *Agrobacterium*-based approach, in comparison to using particle bombardment.

A. tumefaciens

Transformation procedure was used in the studies based on Newell et al. (1995) and Otani et al. (1998) methods. The leaf or petiole were inoculated for 30-60 minutes with the disarmed *A. tumefaciens* strain LBA4404, containing genes either pin II or SPFMV coat protein. The leaf or petiole explants were placed on co-culture medium containing MS + 0.4 mg/l tiamin HCl + 0.2 mg/l kinetin and 10 mg/l of acetosyringone for 3-4 days. The explants were than transferred to medium containing MS + 0.4 mg/l tiamin HCl + 0.2 mg/l kinetin, MS + 1 mg/l NAA + 0.1 mg/l BAP, or MS + Staba vitamin containing 250 mg/l of cefotaxime and 50 mg/l of kanamycin.

Particle bombardment

The plasmid Twa and pRQ6 in 2 to 1 ratio were adsorbed on gold particle microcarriers as described by Klein et al. (1988). Leaf and petiole explants were incubated on the medium containing high osmotic solution with 45 g/l of manitol and sorbitol. The explants were bombarded at 100 psi by the microcarrier coated with both DNA plasmids. The explants were grown in the callus induction medium MS + 0.5 mg/l 2,4-D + 0.1 mg/l kinetin or MS + 0.4 mg/l tiamin HCl + 0.2 mg/l 2,4-D containing 25 mg/l of hygromycin or 0.5 mg/l basta. The selected explants were transferred into medium MS + 0.4 mg/l tiamin HCl + 0.2 mg/l kinetin or MS + 1 mg/l NAA + 0.1 mg/l BAP and MS + Staba vitamin containing 25 mg/l of hygromycin or 0.5 mg/l basta for plant regeneration.

-Glucuronidase assay

Three days after particle bombardment, pieces of leaf and petiole tissues were assayed for -glucuronidase activity based on the method of Jefferson (1987). The explants were soaked in

100 mM sodium phosphate, 5 mM ferrycyanide, 5 mM potassium ferrocyanide, and 0.5% triton X-100 for overnight at 37^0C.

Results

Plant regeneration study

From this study, it was found that among 17 genotypes tested, only 2 genotypes produced plantlets through embryogenesis and 6 genotypes produced plants through organogenesis.

Embryogenesis study

The study indicated that regeneration was genotype dependent. Only Jewel and Kunyik genotype showed plantlet formation (Table 1). Jewel can be said to be a highly responsive genotype among the 17 used in the study. Both methods of Newell et al. (1995) and Wang et al. (1998) demonstrated that leaf and petiole explants of Jewel could regenerate into plantlets (Table 1).

Organogenesis study

Shoot regeneration has been achieved on Alhamdulillah, BIS 192, BIS 182-81, Jewel, Kunyik, and Toweko. Jewel also showed to be very responsive genotype for plant regeneration (Table 2).

Plant Transformation Study

After three days particle bombardment, the explants were treated with X-Gluc, to detect -glucuronidase activity in transiently transformed cell. When explants were treated after 3 days, it was found that 87.9 % showed blue spots or positive gus on leaf explant, whereas 72% showed positive on petiole (Table 3).

It was found that plant transformation via *Agrobacterium* using pin II gene was a better method compared with particle bombardment. The results showed that there was 5.1 percent plantlet formation derived from petiole explant and 0.7 percent from leaf through *Agrobacterium* (Table 4) versus 0.3 percent derived from petiole explants via particle bombardment (Table 5). Plant transformation through *Agrobacterium* using SPFMV coat protein gene demonstrated that only 1.3 percent plantlet formation can be derived from petiole explant (Table 6).

Conclusions

1. Plant regeneration of sweetpotato is genotype-dependent.
2. Jewel was the best genotype of sweetpotato for *in vitro* regeneration and plant transformation
3. .For sweetpotato, *Agrobacterium*-based transformation is a better system than particle bombardment.

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Table 1. **Plant regeneration through embryogenesis**

Methods	Genotypes	Explants	Embryogenic callus (%)	Embryo somatic	Plantlets
Newel (1995)	Jewel	Petioles	41.3	369	312
		Leaves	39.8	1036	600
	Kunyik	Petioles	32.3	6	0
		Leaves	14.6	3	3
Wang (1998)	Jewel	Petioles	20.0	99	37
		Leaves	3.3	25	9

Table 2. **Plant regeneration through organogenesis**

Methods	Genotypes	Explants	Responsive callus (%)	Shoots	Plantlets
Newel (1995)	Alhamdulillah	Petioles	13	4	4
		Leaves	33	0	0
	BIS 192	Petioles	15	1	1
		Leaves	31	0	0
Gosukonda (1995)	Jewel	Petioles	35	32	25
		Leaves	0	0	0
	Kunyik	Petioles	5	2	2
		Leaves	5	1	1
	Toweko	Petioles	10	1	1
		Leaves	8	5	5
	BIS 182-81	Petioles	35	10	10
		Leaves	2	1	1
Wang (1998)	Jewel	Petioles	20	27	19
		Leaves	10	21	11

Table 3. **Gus expression of transformed explants** *

Explant	Number of explant	Explant with positive gus (%)
Petioles	23	87.9
Leaves	25	72.0

* Cultivar: Jewel
Transformation system: *particle bombardment*
Gene: pin II
Selection: kanamycin 50 mg/l.

Table 4. **Plant regeneration of sweetpotato transformants** *

Explant	Number of explant	Selected callus II (%)	Selected callus I (%)	Shoot (%)	Plant (%)
Petioles	549	72.9	52.0	9.1	5.1
Leaves	309	83.2	36.2	1.3	0.7

* Cultivar: Jewel
Transformation system: *Agrobacterium tumefaciens*
Gene: pin II
Selection: kanamycin 50 mg/l

Table 5. **Plant regeneration of sweetpotato transformants** *

Explant	Number of explant	Selected callus II (%)	Selected callus I (%)	Plant (%)
Petioles	325	74.2	39.4	0.3
Leaves	164	87.2	55.5	-

* Cultivar: Jewel
Transformation system: *particle bombardment*
Gene: pin II
Selection: kanamycin 50 mg/l

Table 6. **Plant regeneration of sweetpotato transformants** *

Explant	Number of explant	Selected callus II (%)	Selected callus I (%)	Plant (%)
Petioles	217	78.3	51.6	1.8
Leaves	88	86.4	34.1	-

* Cultivar: Jewel
Transformation system: *Agrobacterium tumefaciens*
Gene: CP-SPFMV
Selection: kanamycin 50 mg/l