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*Full Length Research Paper*

# Direct genetic transformation of *Hibiscus sabdariffa* L.

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**Transgenic *Hibiscus sabdariffa* plants have been produced by a tissue culture independent method using *Agrobacterium tumefasciens* transformation procedure. Embryo axes of mature seeds with one cotyledon excised were infected by immersion in a suspension of *Agrobacterium* LBA 4404 strain culture that carries pBal plasmid with β-glucuronidase p35SGUSINT and plant selectable marker Neomycin Phospho-Transferase gene (*npt*II). Following a 24 h co-cultivation with *Agrobacterium* strain and decontamination with cefotaxime, embryos were grown on soil rite containing MS medium added with a killer concentration of kanamycin (100 µg/ml) during 4 weeks at room conditions and thereafter transferred to greenhouse. 54.3% of the seedlings grew well on the selective medium; 68% of the explants excised from putative transformed plants were found to be GUS positive. After 60 days evaluation point, the assessment of the transformation by PCR revealed that *H. sabdariffa* line tested, carried the *npt*II gene.**

**Key words:** *Hibiscus sabdariffa*, genetic transformation.

## INTRODUCTION

*Hibiscus sabdariffa* is a crop widely cultivated in Sub Saharan Africa, growing on sandy soils after the harvest of the main crop (groundnut or millet) for additional income in rural areas. The crop is now cultivated in peri-urban areas due to the great demand. The economic importance of the species for African farmers in income generation is more and more emphasized.

The non dehiscent and fleshy fruit containing anthocyanic pigments is largely used as tea and refreshing beverage but also as natural dye. The calices (Bricage, 1985) contain high content of anthocyanic pigments, cyanidin (29%) and delphinidin (70.9%). For this reason the crop has a great economic potential in sub-Saharan Africa (TDC, 1997).

The crop is subject to attacks by *Phytophtora parasitica* (Follin, 1966), which is the main cause of disease in early stages of development. *H. sabdariffa* has been regenerated through axillary branching (Sadio, 2000). Callus culture for secondary metabolites production showed that only cyanidin can be produced through young explant callus (Mizukami et al., 1988). *Hibiscus* improvement programs aimed at the development of varieties resistant to insect and viral diseases or with a high content of anthocyanic pigments. Modification of *Hibiscus* genome using genetic engineering methods would facilitate rapid development of new varieties with traits that confer resistance to virus and insects or new floral varieties for production of natural dye. An efficient *in vitro* plant regeneration method is often considered as a prerequisite before transformation. A potential limitation of this tissue culture method is the availability of a regeneration protocol by callus; in addition somaclonal variation events generated through tissue culture may induce unwanted mutation (Venkatachalam et al., 2000). Direct transformation protocol targeting meristems and embryos without an *in vitro* phase is fast and advantageous by reducing the effort needed to obtain a reasonable number of transformed plants (3 months instead of 9 months).

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We report here a rapid and efficient method for producing transgenic plants from *H. sabdariffa* embryos, which does not require *in vitro* regeneration.

## MATERIALS AND METHODS

### Plant material

Seeds of *H. sabdariffa* were soaked in Bavistin (0.1%) for 30 min, rinsed with distilled water, then soaked again in $HgCl_2$ (0.1%) for 6-7 min and thoroughly rinsed with sterile distilled water. After an imbibition period (8 h), the seed coat were removed, and the two cotyledons kept. The embryonic axes were also used for inoculation with *Agrobacterium* strain.

### Bacterial strains and vectors

*Agrobacterium* strain LBA 4404 strain containing pBal plasmid with p35SGUSINT and plant selectable marker *npt*II was used for inoculation. 200 to 500 µl of an *Agrobacterium* culture taken from the stock were suspended in 25 µl of 2YT medium. The *Agrobacterium* culture medium was added with 50 µg/ml Kanamycin and 25 µg/ml of rifampicin and grown overnight on the shaker (150 rpm) at 28°C until the bacterial culture reach exponential phase of growth.

### Infection

75-100 embryos were incubated in *Agrobacterium* culture on rotary shaker (150 rpm) for 6 h, co-cultivated for 24 h in agro-culture then blotted dry on paper filter and cultivated on autoclaved soil rite containing M-S/2 mineral elements (Murashige and Skoog, 1962) with a killer concentration of 50µg/ml kanamycin. After 25 days on selection medium containing antibiotics, green and healthy plants were transferred in pots and let grown in greenhouse with the same selection pressure. Plants were assessed by histochemical GUS test and PCR.

### Histochemical Assay

*Hibiscus* explants inoculated with pBal containing GUS intron were tested. To identify GUS positive tissues from plants growing on selection medium, hypocotyls (1.5 cm) and apex were excised. The tissues were incubated overnight at 37°C in 2 ml of an aliquot from 5 mg Xgluc, 250 µl DMFO, 8.94 ml of 0.1 M phosphate buffer pH 7, 500 µl of potassium ferricyanide, 500 µl of potassium ferrocyanide (Jefferson et al., 1987). After staining, explants were soaked in 70% ethanol for bleaching and stored in methanol.

### PCR

Three to four youngest and upper leaves were isolated from one month old putative transformed plant grown in greenhouse and ground in liquid nitrogen in a mortar using a pestle; genomic DNA was extracted according to a modified CTAB procedure (Saghai-Maroof et al., 1984). DNA extracted from fresh tissues was then dissolved in 20 µl of TE buffer containing RNAse. After 30 min, the DNA was completely dissolved and store at –20°C.

For PCR analysis of *npt*II gene in the transformants, 2 primers were used: 5′-GAG GCT ATT CGG CTA TGA CTG-3′ and 5′-ATC GGG AGG GGC GAT ACC GTA-3′. PCR were performed in a total volume of 25 µl. The PCR products were later analyzed on an agarose gel (1%) dissolved in 1X TAE buffer. Bands are viewed on UV transilluminator.

## RESULTS

### Efficiency of the transformation protocol

After 3 days of co cultivation in darkness, mature *Hibiscus* embryos isolated from germinated seedlings co-cultivated with *Agrobacterium* started to germinate but on selection medium, they lacked chlorophyll, staying etiolated for one week. The transformed plants started turning green after 10 days on selection medium and developed thereafter in green healthy plants. The cultivated embryos produced plants in 25 days culture on selection medium containing 50 µg/ml of kanamycin. Only putative transformants grew well on the selective medium containing lethal dose of kanamycin. Non-transformed embryos did not germinate, becoming bleached rapidly and dying. The rate of putative transformation in *Hibiscus* was 60.46%.

### Expression of GUSINT

Immunological essay was performed for *Hibiscus* inoculated with pBal containing GUS intron. The analysis of the presence and expression of *uidA gene* was done 10 days after co-cultivation on different parts of the seedlings (hypocotyls, cotyledons and apex). Hypocotyls tissues showed a dark blue color and were GUS positive. Histochemical activity was also localized both in apex and cotyledonary meristems. On 25 putative transformed plants analyzed, 17 were found GUS positive.

### Detection of *npt*II gene in plant genomic DNA by PCR

*Npt*II expression in transformed *Hibiscus* lines were tested by PCR. Analysis carried on 5 *Hibiscus* randomly selected plants, showed amplification of *npt*II gene on 3 lines (Figure 1). The plants grew very well and set flowers and fruits.

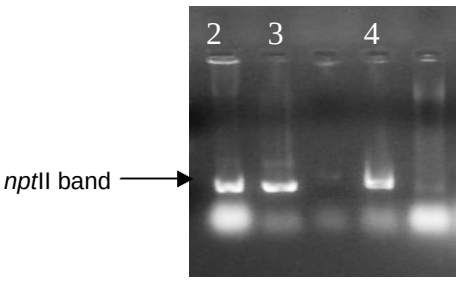


**Figure 1.** *H. sabdariffa* PCR showing 3 lines with *npt*II positive bands out of 5 lines tested.

## DISCUSSION

The development of a fast procedure of direct (or *in planta*) plant transformation is the main result achieved in these experiments. We have shown that this procedure is efficient in *H. sabdariffa* tested with interesting rates of transformation. In *Arachis hypogea*, Rohini and Rao (2000) reported a direct transformation after wounding with 5 lines transformed on 150 tested. Other reports dealing mainly on *Arabidopsis* (Bechtold and Pelletier, 1998*), Medicago* and soybean *in planta* transformation using vacuum infiltration and flower dipping pointed out the low rate of transformation and the variability of the results (Birch, 1997).

In *H. sabdariffa* this is the first report on genetic transformation *in planta*, with several of the plants tested expressing the transgene. The rate of transformation may suggest that there is a strong interaction between the plant genotype and the strain (Hooykaas and Schilperoot, 1992).

This study has shown for the first time that a recombinant gene can be integrated in *H. sabdariffa* following *A. tumefasciens* mediated transformation. Further optimization of the protocol is necessary to obtain a high yield of transgenic lines in the progeny. This strategy of direct transformation through embryonic axes, established in peanut (Rohini and Rao, 2000), is simple fast and can contribute significantly to genetic improvement of *Hibiscus* cultivars in sub-Saharan Africa.

### REFERENCES


Bechtold N, Pelletier G (1998). *In planta Agrobacterium* mediated transformation of adult *Arabidopsis thaliana* plants by vacuum infiltration. Methods Mol. Biol. 82:259-266.

Birch RG (1997). Plant transformation: problems and strategies for practical application. Annu. Rev. Plant Physiol. Plant Mol. Biol. 48:297- 326

Bricage P (1985). Etude des phénotypes pigmentaires du bissap, *Hibiscus sabdariffa* L., Malvacées. IV. Influence des phénotypes parentaux et des conditions stationnelles sur la germination et le développement des individus: compétition entre individus. Bulletin de l'I.F.A.N. $T_{46}$, série A, n° 1-2: 140-166.

Du CT, Francis FJ (1973). Anthocyanins of Roselle (*Hibiscus sabdariffa* L.). J. Food Sci. 38: 810- 812.

Follin JC (1966). Le Chancre du collet de la Roselle (*Hibiscus sabdariffa* var.*altissima* Hort.). Influence de quelques facteurs agronomiques sur le développement de la maladie. Coton et Fibres tropicales 4: 459-463.

Geetha N, Venkatachalam P, Prakash V, Lakshmi Sita G (1999). *Agrobacterium* mediated transformation in pigeon pea (*Cajanus cajan* L). Plant Biotechnol. 16(3):213-218.

Hooykaas PJ and Shilperoort RA (1992) . *Agrobacterium* and plant genetic engeneering. Plant Mol. Biol. 19: 15-38.

Jefferson RA, Kavanagh TA, Bevan M (1987). GUS fusions: beta glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6:3901-3907.

Mizukami H, Tomita K, Ohashi H, Hiraoka N (1988). Anthocyanin production in callus cultures of roselle (*Hibiscus sabdariffa* L.). Plant Cell Rep. 7:553-556.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

Rohini VK, Sankara Rao (2000). Transformation of peanut (*Arachis hypogea* L.): a non tissue culture based approach for generating transgenic plants. Plant Sci. 150: 41-49.

Sadio M (2000). Etude des potentialités de clonage *in vitro* chez *Hibiscus sabdariffa* L.. Mémoire de DEA, Université Cheikh Anta Diop, Dakar, Sénégal, 52 p.

Saghai-Marof M.A, Soliman K, Jorgensen RA, Allard RW (1984). Ribosomal DAN spacer length polymorphisms in barley mendelian inheritance, chromosomal location and population dynamics. Proc. Natl. Acad. Sci. USA 81: 8014-8018.

TDC Sénégal (1997). Etude technico-économique pour la valorisation du Bissap *Hibiscus sabdariffa* L. Rapport Provisoire 87 p.

Venkatachalam P, Gheeta N, Khandelwal A, Shaila MS, Lakshmi Sita G (2000). *Agrobacterium* mediated genetic transformation from cotyledonary explants of groundnut (*Arachis hypogeae* L) via somatic embryogenesis . Curr. Sci. 78: 1130-1136.