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# *Agrobacterium*-mediated genetic transformation of Fonio (*Digitaria exilis* (L.) Stapf)

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**Fonio (*Digitaria exilis)* is a crop grown in Africa for its excellent culinary and nutritional properties and is a valuable source of income for smallholder farmers. Its improvement has long been neglected by scientists because the crop was one of the lost crops of Africa. Recent advances in Fonio research are centered on its evolution, nutritional and economic values. The lack of a transformation and other biotechnology protocols developed for Fonio has greatly hindered genetic improvement of this crop. In this study, *Agrobacterium*-mediated genetic transformation was developed for two genotypes of *D. exilis* ('Agyong' and 'Churiwe'). Three-weeks-old callus derived from stem segments were co-cultivated with *Agrobacterium tumefaciens* for 3 days. After selection on Hygromycin B medium, plants were regenerated on Murashige and Skoog (MS) medium supplemented with 0.5 mg $l^{-1}$ BA and 0.1 mg $l^{-1}$ gibberellic acid. Regenerated shoots were rooted on hormone-free MS medium. Histochemical gus assay and polymerase chain reaction (PCR) confirmed the presence of the *gus* gene in transformed tissues. The transformation frequency was 2.1 and 2.7% for "Agyong" and "Churiwe", respectively. The transgenic plants were phenotypically normal. This protocol would provide useful platform for genetic improvement of this crop.**

**Key words:** *Digitaria exilis*. Transfomation efficiency, β-glucuronidase (GUS) assay, *Agrobacterium tumefaciens*.

## INTRODUCTION

Fonio (*Digitaria exilis)* is a C4 annual herb that tillers freely. It is a native of West Africa and belongs to the family Poaceae, sub-family Panicoideae and tribe Paniceae. There are two important cultivated species in the genus, namely, *D. exilis* and *Digitaria iburua*. The inflorescence in both species is a panicle with finger-like projections, 2 to 5 in *D. exilis* and 4 to 10 in *D. iburua*. The grains are extraordinarily tiny, measuring 0.5 to 1 mm in diameter and 0.75 to 2 mm in length. The fruit is a caryopsis firmly secured between two husks (lemma and palea); in *D. exilis* the husk is white, hence the name white Fonio, in contrast to *D. iburua* that has dark-brown husk and is commonly called black Fonio. The most variable of all the species is *D. exilis* which is cultivated in

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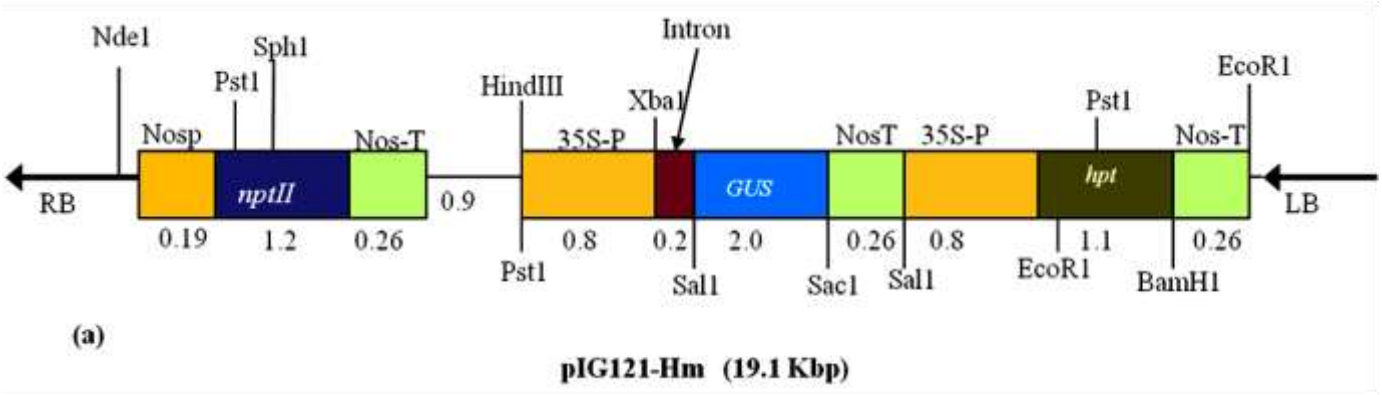


**Figure 1.** T-DNA map of the construct used for transformation of Fonio. RB: Right border; LB: left border; nptII: neomycin phosphotransferase gene; hpt: hygromycin phosphotransferase; gus: b-glucuronidase gene.

many West African countries where they are called by different names (Ayo and Nkama, 2006; Adoukonou-Sagbadja, 2010).

According to Bennett et al. (2000) and Caponio and Rua (2003), the number of chromosomes in the genus ranges from 2n = 18 (diploid) to 2n = 12x = 108 (dodecaploid).

Fonio seeds have excellent culinary and nutritional properties and are sometimes regarded as the 'grain of life' because they supply food to several millions of people, when the major crops are yet to mature and food is generally scarce. The crop is also used as a valuable source of income, especially for small-scale farmers (Adoukonou-Sagbadja et al., 2007a). According to Kuta et al. (2003), the significant contribution that Fonio could make in resolving food crisis in Africa is expected to rekindle interest in the improvement of this crop. Available literature on the crop centers around genetic characterization of the plant using Random Amplified Polymorphic DNA (RAPD) markers and flow cytometry (Adoukonou-Sagbadja et al., 2007a, b, Adoukonou-Sagbadja, 2010) as well as microsatellite markers (Barnaud et al., 2012). Information is also available on the nutritional and physiochemical property of the crop (Temple and Bassa, 1991; Jideani and Akingbala, 1993; Jideani et al., 1994, 1996; Chukwu and Abdul-Kadir, 2008; Ballogou et al., 2013) as well as cultivation and uses of Fonio (Jideani, 1999).

Although, the use of genetic transformation as a tool for crop improvement and for the development of functional genomics is well documented, literature on *in vitro* cultures or genetic engineering of Fonio is lacking. There is therefore the need to explore the possibility of using genetic transformation for improvement of Fonio so as to increase its productivity. In our previous study, an efficient regeneration protocol for Fonio was developed (Ntui et al., 2010). In the present report, a protocol is given for transformation of *D. exilis* based on *Agrobacterium*-mediated technology.

## MATERIALS AND METHODS

### Seed germination and callus induction

Seeds of two genotypes of *D. exilis*, 'Agyong' and 'Churiwe' were surface sterilized in 70% ethanol for 5 min and 1.5% (v/v) sodium hypochlorite solution for 20 min before rinsing them thrice in sterile distilled water. The seeds were then germinated and maintained for one month on Murashige and Skoog (1962) medium, containing 10 g $l^{-1}$ sucrose and 8 g/l agar in a growth room at 25±1°C, 16 h photoperiod, 30 to 40 µmol $m^{-2}$ $s^{-1}$ cool white fluorescent light. The pH of the medium was adjusted to 5.8 prior to the addition of agar and autoclaving at 121°C for 15 min.

Stem segments (culm) about 5 mm in length were cut from the four week-old seedlings and cultured on callus induction medium (CIM, which was Murashige and Skoog (MS) medium containing 2 mg/l 2,4-D, 1 g/l casamino acid, pH 5.8 and 8 g /l agar). Cultures were placed at 26 ± 1°C in the dark for three weeks. Stem segments were subcultured to fresh medium after two weeks.

### *Agrobacterium* culture, co-cultivation, selection and plant regeneration

In this study, *Agrobacterium* strain EHA101 (Hood et al., 1986) was used. The *Agrobacterium* carries the plasmid pIG121-Hm (Ohta et al., 1990) which contains hygromycin phosphotransferase (hpt), neomycin phosphotransferase II (nptII), and β-glucuronidase (gus) as reporter genes in T-DNA region (Figure 1). The *Agrobacterium* was cultured overnight in a reciprocal shaker (120 cycles/min) at 28°C in 50 ml liquid LB medium containing 50 mg/l kanamycin sulphate, 25 mg/l hygromycin B and 25 mg/l chloramphenicol. After centrifugation, the bacteria were resuspended to final density of $OD_{600}$ = 0.5 in inoculation medium, which was MS medium containing 68 g/l sucrose, 36 g/l glucose, 3 g/l KCl, 4 g/l $MgCl_2$, pH 5.2 and 100 µM acetosyringone (3,5-dimethoxy-4-hydroxy-acetophenone; Sigma-Aldrich, St. Louis, MO, USA).

Three-week-old calli were dipped in the bacterial suspension for 10 min with gentle shaking. Thereafter, the calli were dried on a Whatman filter paper and co-cultivated in the dark on CIM, pH 5.2 and supplemented with 100 µM acetosyringone for three days.

After co-cultivation, the calli were washed three times with sterilized distilled water containing 10 mg/l meropenem trihydrate (Meropen; Dainippon Sumitomo Pharma, Osaka, Japan) (Ogawa and Mii, 2007) and cultured for 4 weeks on CIM supplemented with 20 mg/l meropenem to kill bacterial carryover and 50 mg/l

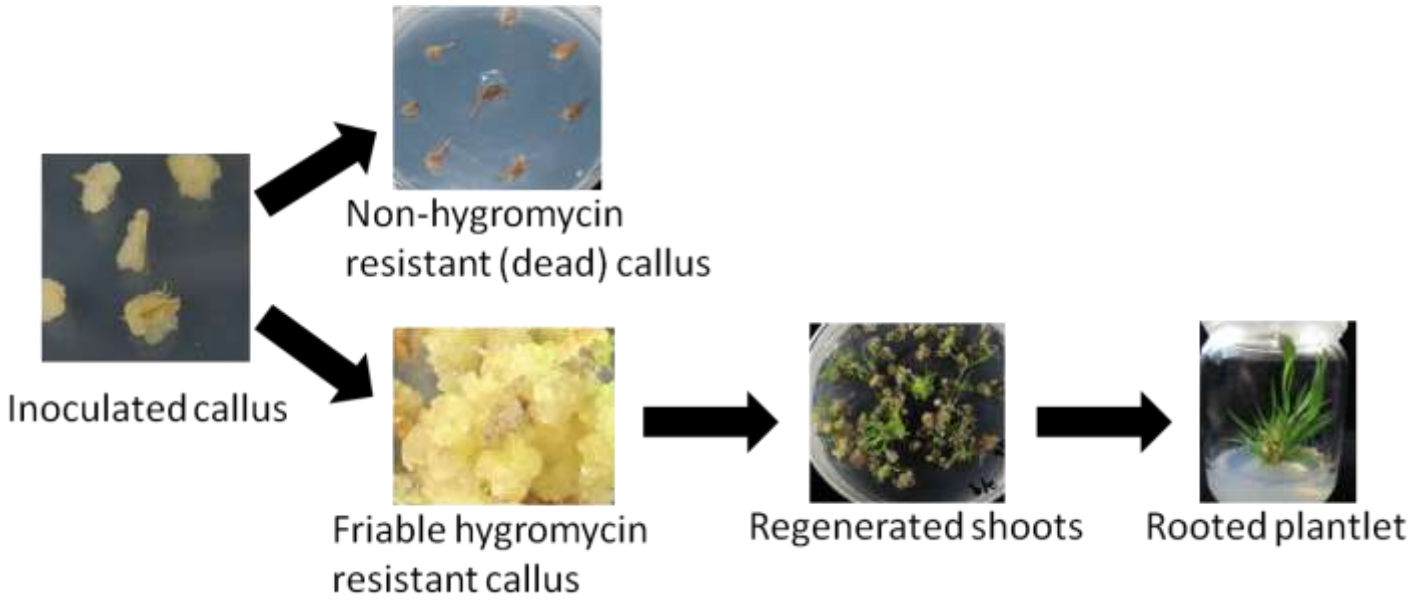


**Figure 2.** Production of transgenic plants from three month old callus after inoculation with *Agrobacterium* strain EHA 101pIG121-Hm.

**Table 1.** Transformation efficiencies of two cultivars of *D. exilis* inoculated with *Agrobacterium tumafaciens* EHA101pIG121-Hm.

| Cultivar | No. of calli inoculated | No. of plants regenerated | PCR+Plants | Transformation efficiency (%) |
|---|---|---|---|---|
| Agyong | 234 | 31 (13.25) | 5 (16.13) | 2.14 |
| Churiwe | 219 | 26 (11.87) | 6 (23.1) | 2.74 |

Values in brackets are percentages.

hygromycin B for selecting transformed tissues in the dark. The calli were subcultured bi-weekly into fresh medium of same composition.

After selection, brown or black calli were removed and only creamish healthy calli were transferred to MS medium containing 30 g/l sucrose, 0.5 mg/l BA, 0.1 mg/l gibberellic acid ($GA_3$), 20 mg/l hygromycin B and 10 mg/l meropenem for shoot regeneration. Regenerated shoots were transferred to hormone-free MS medium containing 20 mg/l hygromycin B and 10 mg/l meropenem.

### Gus assay and polymerase chain reaction

Histochemical gus assay (Jefferson et al., 1987) was used to assess gus staining of putative transformed plants using 5-bromo-4-chloro-3-indolyl beta-D-glucuronide (X-Gluc) as the substrate. After selection, a few creamish healthy calli were subjected to gus assay by incubation in sodium phosphate buffer containing X-Gluc for 18 h at 37°C.

For polymerase chain reaction (PCR) analysis, genomic DNA was extracted from leaves of regenerated seedlings using the modified cetyltrimethylammonium bromide (CTAB) method as reported by Rogers and Bendich (1988). PCR was done for the *gus* gene using the following pair of primers: GUS-F: 5′-GGTGGG AAAGCGTTACAAG-3′; GUS-R: 5′-GTTTACGCGTTGCTTCCGCCA-3′.

Transformation efficiency was calculated as the number of PCR positive plants × 100/number of calli inoculated.

## RESULTS AND DISCUSSION

### Overview of the transformation and regeneration protocol

Two genotypes, ‘Agyong’ and ‘Churiwe’ were selected to develop transformation protocol for Fonio. These genotypes were selected because of their high regeneration efficiency based on our previous study (Ntui et al., 2010). Culm segments were used as the explants, as these were the only tissues which developed calli based on our previous study (Ntui et al., 2010).

Approximately, 80% of the culms developed calli within 3 weeks of culture on callus induction medium. After inoculation with *Agrobacterium* and upon selection on hygromycin medium, some calli turned brownish and died while others remained creamish and produced friable callus (Figure 2). These friable calli together with other hygromycin resistant calli were transferred to regeneration medium. Of the calli that were transferred to regeneration medium, only 13.25 and 11.87%, respectively for Agyong and Churiwe, produced shoots (Table 1). This regeneration frequency was very low and was produced only by friable calli. Regenerated shoots were successfully

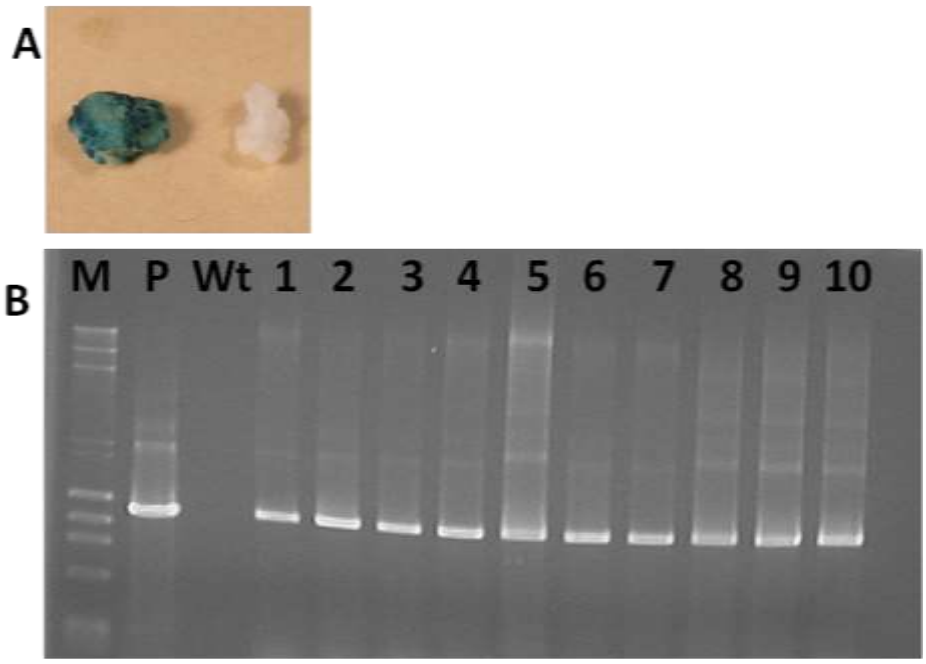


**Figure 3.** Molecular analysis of transgenic tissues. (A) Detection of Gus activity in callus. (B) PCR amplification of the *gus* gene. M: Molecular size marker (ø174HaeIII digests); P: positive control (plasmid DNA); Wt: wild-type (untransformed control). Lanes 1-10, independent transgenic lines of 'Agyong' (1-5) and 'Churiwe' (6-10).

rooted in hormone-free medium (Figure 2).

## Gus assay and PCR analysis

Gus activity was only detected in transgenic tissues but not in the untransformed tissues after using X-Gluc (Figure 3A). From PCR analysis done, it was found that a DNA fragment corresponding to the *gus* gene was amplified from genomic DNA isolated from leaves of putative transformed plants (Figure 3B). The transformation efficiencies obtained were 2.14 and 2.74, respectively for cultivars 'Agyong' and 'Churiwe' (Table 1), which are quite low. The low transformation efficiencies may have resulted from the high number of escapes probably because of insufficient selection regime and also due to the low concentration of hygromycin used in the regeneration medium. In some cereals, hygromycin concentration of up to 100 mg/l has been used to select transgenic plants. In some rice genotypes, 50 mg/l hygromycin is sufficient to select transgenic tissues (Sahoo et al., 2011). Since the sensitivity of callus to hygromycin B was not checked, possibly the right concentration of the antibiotic was not used for the selection. Checking the sensitivity of callus to hygromycin is important to minimize escape. However, as this is not the only factor that affects transformation efficiency of plants, other factors such as *Agrobacterium* strain and concentration, pH, inoculation and co-cultivation time amongst others should be optimized to increase the transformation efficiency of this plant and subsequently facilitate its functional genomics.

Genetic transformation provides a greater potential for overcoming breeding barriers in crop improvement (Wambugu, 1999, 2001; Machuka, 2001). The transformation protocol developed above could prove useful for genetic engineering of Fonio for increased grain size, pest and disease resistance, herbicide tolerance and ability to withstand lodging among others. A number of gene constructs for many of these traits and which are routinely used for cereal transformation are available and can be explored for Fonio genetic engineering by adopting the transformation protocol developed in this study.

## Conclusion

In this protocol, it was observed that it took about 10 weeks (the essential steps given in Figure 4) to produce transgenic plants from co-cultivation with *Agrobacterium*. Although, the transformation efficiency is low, this is however, the first report of *Agrobacterium*-mediated genetic transformation of *Digitaria*. The protocol described here will provide useful platform for developing high efficient transformation protocol and for genetic improvement of this important cereal.

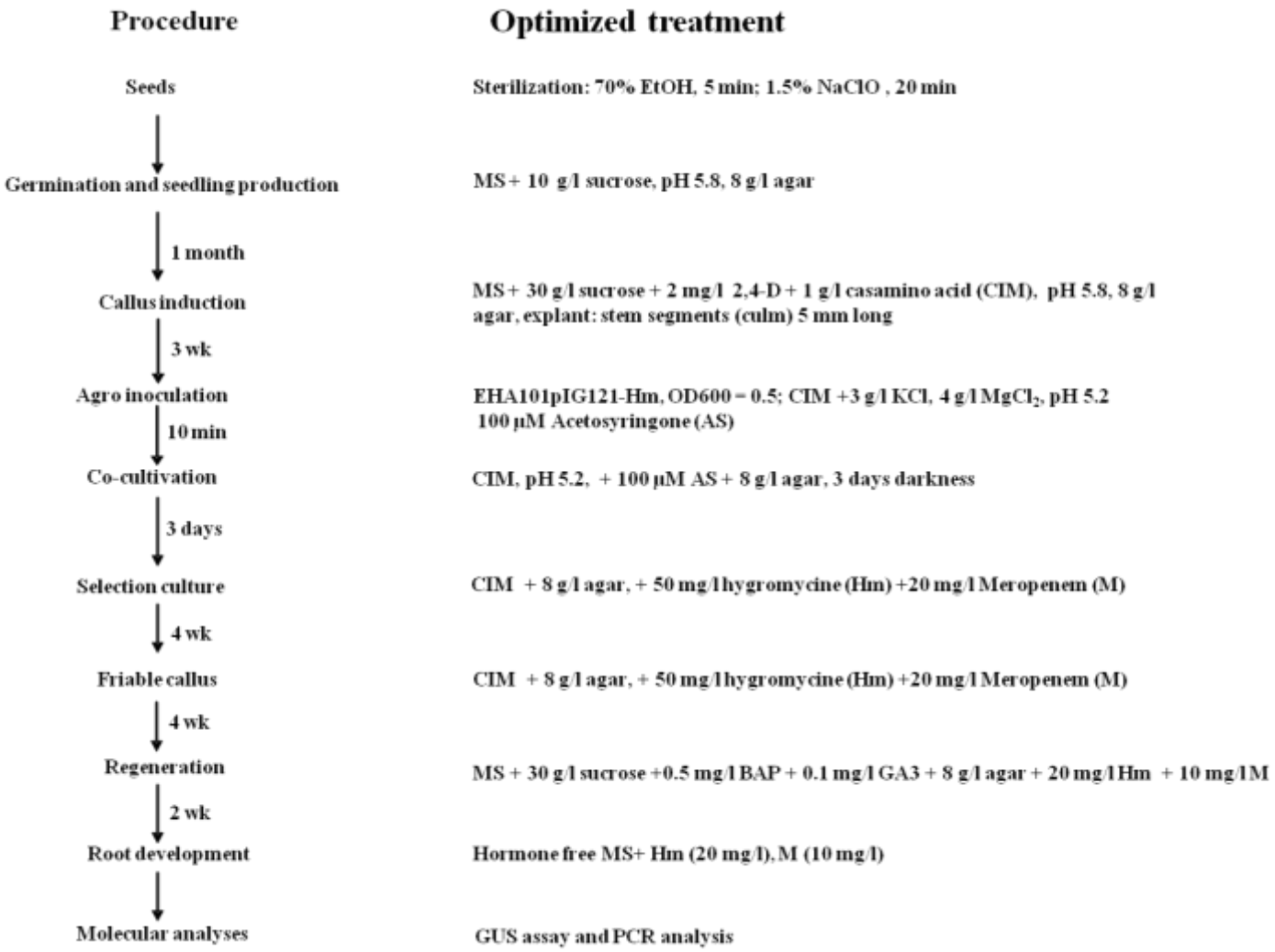


**Figure 4.** Flow chart for the transformation of Fonio.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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