Stability of the ß-Glucuronidase Gene in a RO Population of Grape *(Vitis ruspestris S.)* *

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A study on the stability of the ß-glucuronidase gene has been conducted in a population of transgenic plants regenerated from somatic embryos of *Vitis rupestris* S. Molecular tests demonstrated no loss of the inserted gene following either the long-term embryo culture and the plant regeneration events. Dot-Blot analysis proved to be a useful assay for a simultaneous assay of the exogenous gene in the population, and Southern Blot analysis showed the marker gene in the inserted form with a agreeable efficiency (92%).

Recombinant DNA techniques offer interesting possibilities for the genus *Vitis*. Recently, promising results have been obtained with various approaches in different laboratories (Le Gall *et al.*, 1994; Martinelli & Mandolino, 1994; Krastanova *et al.*, 1995; Scorza *el al.*, 1995; Kikkert et al., 1996). *Agrobacterium*- mediated transformation has proved to be an applicable technique for grape; however, relevant problems such as unstable gene insertion, chimeric tissue production and difficult plant regeneration (Colby *et al.*, 1991) have been described as the most important factors affecting transgenic grapevines. However, we have established a suitable protocol for genetic transformation and transgenic plant regeneration of *Vitis rupestris* S. with the marker gene for ß-glucuronidase (GUS) (Martinelli & Mandolino, 1994). The present paper is a study of the stability of the marker gene and its insertion efficiency in a plant population regenerated from transgenic somatic embryos.

MATERIALS AND METHODS

Transformation of somatic embryos: Genetic transformation of isolated somatic embryos of *Vitis rupestris* Scheele was performed as previously described (Martinelli *et al.*, 1993). The embryos were infected with *Agrobacterium tumefaciens* strain LBA4404 which contained the plasmid pBI121 (Jefferson *et al.*, 1987) carrying the neomycin-phosphotransferase and the ß-glucuronidase genes. Eight transgenic somatic embryos developed into transformed cellular lines and were labelled A, B, F, J, K, M, N and Z. These lines were competent for recurrent secondary somatic embryogenesis as well as for plant regeneration and as a result eight families of transgenic plants were obtained. Moreover, many sister plants were yielded within each family since every secondary somatic embryo regenerated numerous shoots *via* organogenesis (Fig. 1).

The molecular assays for the demonstration of the GUS gene insertion into the genomes were carried out with fully developed micropropagated plants, each derived from a different regeneration event. The plantlets used for Southern Blot experiments were 6-8 cm tall. Tables 1 and 2 summarize the 8 families used and the number of the regenerated plants assayed within each family.

Molecular Analysis

Dot-Blot Analysis: In order to determine the presence of the GUS gene within the genome of a group of 66 transformed and 2 non-transformed plants, genomic DNA was extracted from 0.5-1.0 g of leaf tissue, following the CTAB protocol (Rogers & Bendich, 1985). Genomic DNAs (5 µg each) were blotted onto nylon membranes (Hybond-N, Amersham) using a Bio-Dot apparatus (BioRad). Filter treatment, hybridization and washing conditions were similar to those described for the Southern Blot analysis.

Southern Blot analysis: The genomic DNA extracted from 47 transgenic and one non-transformed plant (Rogers & Bendich, 1985) was restricted with *Eco*RI at a concentration of about 5 units/µg of DNA, and the digestion was carried out for 6 h under the conditions specified by the supplier. The restricted DNA was run in 1% agarose gels at a voltage of 5 V/cm. The gels were subsequently stained to check for complete digestion, depurinated for 15 min in HC1 0.25 M, denatured for 45 min in NaOH 0.5 M/NaC1 1.5 M, neutralized for 45 min in Tris-C1 0.5 M (pH 7.2)/NaC1 1.5 M/EDTA, and capillary blotted overnight onto nylon Hybond-N (Amersham) filters. The filters were dried at 80°C for 10 min followed by cross-linked for 3 min on a 305 nm transilluminator. The filters were prehybridized at 65°C in 5x SSC (NaC1 0.75 M, Sodium citrate 0.51 M, pH 7.0) supplemented with 5x Denhardt's solution and 0.5% SDS (Sodium dodecyl sulphate). Denatured herring test DNA was added at a concentration of 20 µg/ml.

The probe corresponding to the GUS coding region was prepared by digesting 2 µg of the plasmid pBI221

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FIGURE 1

Plant regeneration *via* organogenesis from a transgenic somatic embryo. Morphogenesis occurred after embryo deformation and proliferation, and numerous shoots were yielded from each embryo (Scale: 4:1).

TABLE 1
Results of Dot-Blot analysis for the presence of the ß-glucuronidase gene in a population of 66 transgenic and two non-transgenic plants. Letters label the eight transgenic families assayed; within each family the number of plants tested is proportional to the number of plants regenerated.

Family code	No. of plant tested	No. of plants with exogenous gene presence
A	8	7
N	4	4
F	4	2
K	18	17
M	8	6
B	8	7
Z	9	8
J	7	6
Tot.:	66	57 (86,4%)
Control	2	---

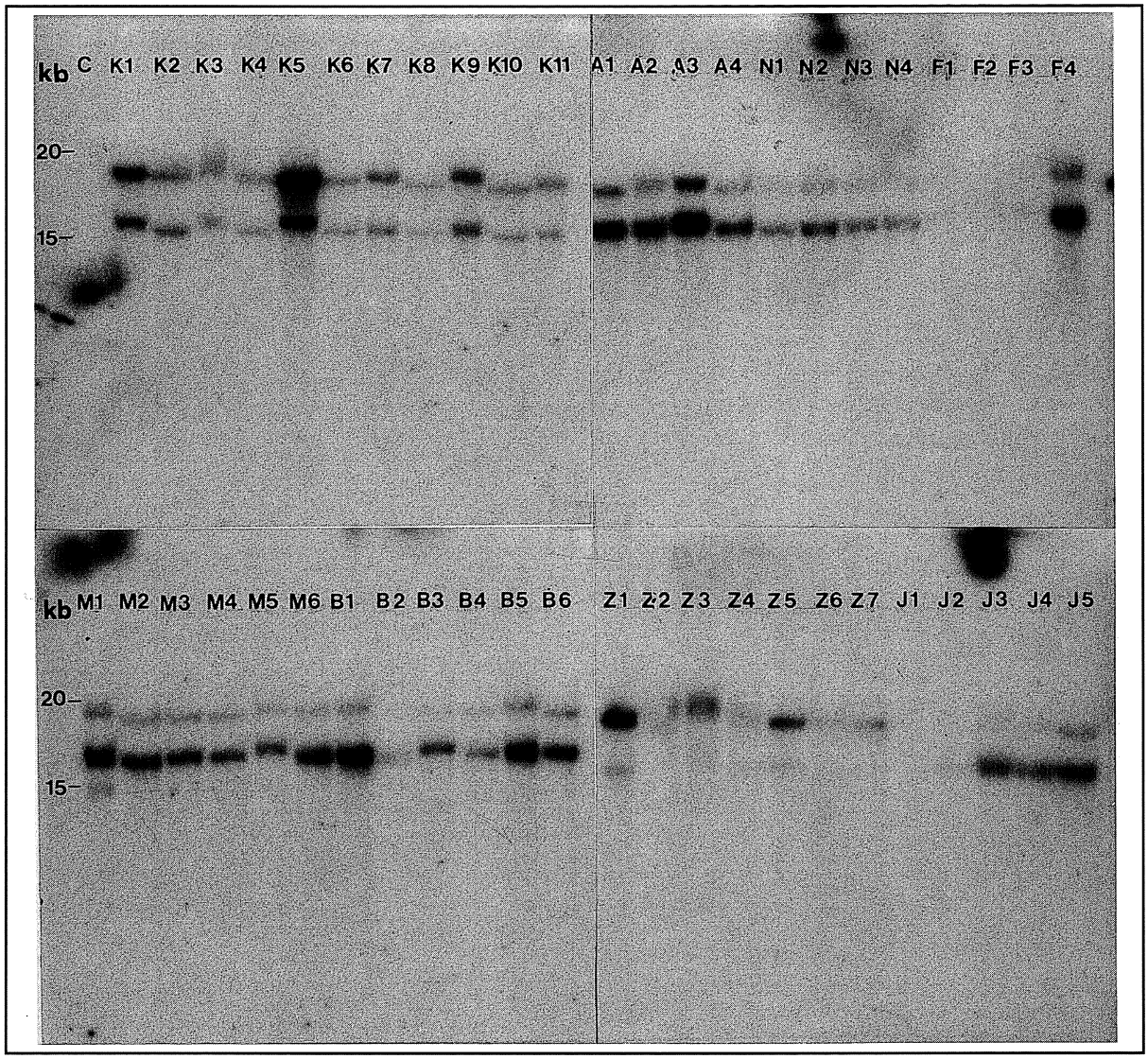

FIGURE 2

Southern Blot analysis of 47 transformed plants (lettered as K, A, N, F, M, B, Z, J) and one non-transformed plant (lane C). The probe used was the 3.1 kb GUS fragment from pBI221 (Clontech).

TABLE 2

Results of Southern Blot analysis for the presence of the integrated ß-glucuronidase gene in a population of 47 transgenic plants and one non-transgenic plant. Letters label the eight transgenic families assayed; within each family the number of plants tested is proportional to the number of plants regenerated.

Family code	No. of plant tested	No. of plants with exogenous gene integration
A	4	4
N	4	4
F	4	1
K	11	11
M	6	6
B	6	6
Z	7	7
J	5	4
Tot.:	47	43 (92%)
Control	1	---

(Clonthech) and gel-purifying the 3.1 kb fragment containing the GUS gene using the GeneClean (Bio 101) procedure. The purified DNA fragment was labelled to high specific activity with γ-^{32}P-dCTP using the random priming protocol (Feinberg & Vogelstein, 1983). The labelled probe was added directly to the prehybridization solution. After a 16-hour hybridization at 65°C, the filters were washed once in 3x SSC at 65°C and twice in 2x SSC at the same temperature. After the final wash the filters were exposed to autoradiographic cassettes with Kodak X-Omat AR 5 type films for the required period.

RESULTS AND DISCUSSION

***Dot-Blot analysis*:** In order to evaluate a wide population of transformed plants, dot-blot analysis was carried out to assess the presence of the GUS sequence in the genome of 66 individual regenerated plants. Although in dot-blot evaluation the foreign gene integration into the genome is not assessed, this technique appears to be reliable for a rapid screening of several plants in a simultaneous test. Table I summarizes the results of this assay. The percentage of GUS sequences found in a group of 66 leaf DNA preparations of individual plants within the eight families reached an average of 86,4% with some differences between distinct families.

***Southern Blot analysis*:** The insertion of the GUS sequence within the plant genome was assessed in a group of 47 transformed plants by single digestions of total genomic DNA extracted form individual plantlets with *Eco*RI. Under these conditions the 3.1 kb fragment used as hybridization probe detected one or more DNA fragments abobve 15 kb in length (Fig. 2, lanes A14, N1-4, K1-11, M1-6, B1-6, Z1-7 and J1-5), confirming the integration of the homologous sequence in the plant genome. In comparison, no hybridization of the labelled GUS coding region to non-transformed plant tissue was observed (Fig. 2, lane C.) Of 47 transformed plants tested, belonging to eight families derived from eight different transformed embryos, only four gave no detectable hybridization signals in genomic Southern Blots (Table 2 and Fig. 2, lanes F1-3, and J1). In the other cases (92%), one to four bands were detectable at dimensions all above 15 kb. These results confirm, at the level of a wide plant population, the gene integration in the genome as previously described by Martinelli & Mandolino (1994) with research on transformed somatic embryos and young regenerated plantlets. The occurrence of multiple bands in several cases suggests the presence of more than one copy of the inserted gene *per* genome. Since small polymorphisms, both in the insertion process and in the copy number, are not associated with different transformation events, this variability could have occurred after the GUS cassette insertion, i.e. during regeneration. Besides, the basic homogeneity of the fragments' number and size across all the transgenic lines suggests the possibility of constraints in the insertion site(s) of the exogenous construct. Such a limited variety of insertion sites might be the result of reduced vitality and/or regeneration competence of cells incorporating the foreign cassete at other sites in the genome.

The present work demonstrates theat *Agrobacterium*-mediated transformation of somatic embryos is a promising strategy for *Vitis* and offers possibilities for further transgenic experiments with useful genes in the genus *Vitis*. In this regard, attention should be focused on regeneration capability, which could be a limiting step in the overall transformation protocol.

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