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Short communication

Stable transformation and long-term expression of the *gusA* reporter gene in callus lines of perennial ryegrass (*Lolium perenne* L.)

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Abstract

Stable transformation of perennial ryegrass (*Lolium perenne* L.) was achieved by biolistic bombardment of a non embryogenic cell suspension culture, using the *hpt* and *gusA* gene. The transformation yielded on the average 5 callus lines per bombardment (1.4×10^6 cells). Stable integration of the genes into the plant genome was demonstrated by Southern analysis of DNA, isolated from hygromycin-resistant callus lines. The *gusA* reporter gene, which was regulated by the constitutive promoter of the rice gene *GOS2*, was expressed in both transient and stable transformation assays, indicating that this promoter is suitable for expression of a transferred gene in perennial ryegrass. Long-term GUS expression was observed in ca. 40% of the callus lines, whereas the other callus lines showed instability after 6 months and 1 year of culture.

Using high-velocity microprojectiles, nucleic acids can be delivered into plant cells [14]. Using this biolistic method, transgenic plants have been obtained from soybean [18], maize [9], papaya [7], cotton [6], rice [2], wheat [22] and sugarcane [1]. Biolistic bombardment is with few modifications applicable to many plant species. Therefore, this method was used to obtain stable transformation in perennial ryegrass (*Lolium perenne* L.) to study long term expression of the *gusA* gene regulated by the promoter of the constitutive rice gene *GOS2* [4].

For transformation experiments, a fast-growing non-embryogenic cell suspension culture of perennial ryegrass, a kind gift from Barenbrug Holland BV (Oosterhout, Netherlands), was used. It was originally initiated from mature embryos from the cv. Aurora [3] and was over two years old. The cell suspension was subcultured weekly and grown in the dark at 25 °C in culture medium [3]. Three days after subculture, 0.25 g (fresh weight) log-phase cells (1.4×10^6 cells) were evenly dispersed onto the surface of a 42 mm diameter Whatman filter disc (Schleicher & Schuell) [12]. Subsequently, 0.5 ml of fresh medium was added and the filters were placed onto culture medium solidified with 0.2% (w/v) gelrite and left overnight at 25 °C in the dark. The next

day the filters were used for biolistic bombardment [12] with 0.6 μg plasmid DNA of pORCEHyg [12] (Fig. 1). As a control, filters with suspension material were bombarded using particles without DNA. After bombardment the filters were incubated on culture medium for 24 h at 25 °C in the dark. For histochemical assay of transient GUS expression, 200 μl X-gluc buffer [21] was pipetted onto the filter discs 24 h after bombardment and incubated for 2 h at 37 °C and 48 h at 25 °C. Subsequently, the tissue was scored for blue cell clusters, yielding an average of 36 blue cell clusters per bombarded filter ($n = 4$, SE (standard error) = 15.5), which indicated transient GUS expression. For stable transformation the filters were transferred to selection medium, consisting of culture medium solidified with 0.2% (w/v) gelrite containing 80 or 150 mg/l hygromycin, 24 h after bombardment. On selection medium with 150 mg/l hygromycin growth of control tissue stopped, although it started to proliferate again when retransferred to culture medium after maintenance on selection medium for 4 weeks. On 80 mg/l hygromycin slow growth of control tissue was still detected after one week of culture. Therefore, these cells were transferred to selection medium with 150 mg/l hygromycin one week after bombardment. Four and eight weeks after bombardment, the first and second subculture, actively growing calli, were isolated and maintained separately as hygromycin resistant callus lines on selection medium. With an average of 5.5 transgenic callus lines per bombardment, a total of 60 hygromycin resistant callus lines was isolated from 11 bombarded filters ($n = 11$, SE = 1.5).

GUS expression in selected callus lines was initially determined approximately 2.5 months after bombardment on small pieces of callus tissue (diameter 5 mm), using the same X-gluc buffer and incubation as for the assay of transient GUS expression. Different patterns of blue staining were observed in the assayed callus material and were classified as completely blue (+ +), partially blue (+ −) and no blue staining (−). From the total of 60 selected hygromycin-resistant callus lines, 24 exhibited no blue staining, 26 were partially blue and 10 were totally blue. To analyse the stability of GUS expression upon further culture, the GUS assay was repeated 6 months after bombardment; 5 of the 10 callus lines which had a completely blue GUS phenotype at the first GUS assay showed a partially blue GUS phenotype. One year post bombardment a group of 12 callus lines, consisting of 4 callus lines of each classified GUS phenotype determined at the first GUS assay, was assayed again (Table 1). From the 8 callus lines which exhibited blue staining at the first assay, 5 callus lines showed a decrease of blue staining at the second assay. The 4 callus lines with a negative GUS phenotype at the first X-gluc assay remained negative.

One year after bombardment, stable integration of the transferred *hpt* and *gusA* genes was confirmed by Southern analysis for the 12 callus lines, which were also tested for long term GUS expression. All the 12 callus lines analysed displayed individual integration patterns, when the band patterns visualized by the HPT and GUS probe are combined, indicating independent transformation events. *Eco* RI digestion yielded the expected bands of 1.0 and 1.8 kb, hybridizing with the HPT probe, suggesting the presence of

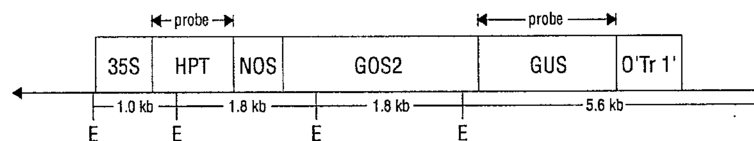

Fig. 1. The plasmid pORCEHyg contains the *hpt* gene [10], which confers hygromycin resistance, as a selection gene and the *gusA* gene [13] as a reporter gene. The *hpt* gene is under control of the 35S CaMV promoter and the nopaline synthase polyadenylation signal [11]. The expression of the *gusA* gene is regulated by the promoter of the constitutively expressed rice gene *GOS2* [4] and the terminator of the Tr mannopine synthase 0 gene. The chimeric gene construct has been subcloned in pBluescript. *Eco* RI digestion of pORCEHyg plasmid DNA yields the fragments as indicated in the physical map. The sequences corresponding to the *hpt* and *gusA* genes that were used as probes in Southern analyses are indicated above the map.

Table 1. GUS expression in transgenic callus lines of perennial ryegrass, 2.5 months and 1 year after bombardment.

Callus lines	2.5 months	1 year
1	–	–
2	–	–
3	–	–
4	–	–
5	+	–
6	+	+
7	+	–
8	+	–
9	++	+
10	++	+
11	++	++
12	++	++

intact *hpt* copies (Fig. 1). Using the GUS probe always a 5.6 kb band was detected (Fig. 1). Figure 2a and c show integration patterns of the *hpt* and *gusA* gene of 2 representatives of each classified GUS phenotype (Table 1). Callus lines 2 and 3 both show multiple copy integrations of both the *hpt* and the *gusA* gene. Numbers 6, 7 and 12 only display the 1.0 and 1.8 kb bands of the *hpt* gene. The integration patterns of the *gusA* gene of these callus lines show the 5.6 kb fragment and larger molecular weight fragments. Callus line 11 shows the 1.0 and 1.8 kb fragment and a high-molecular-weight band with the HPT probe, whereas with the GUS probe, lower-

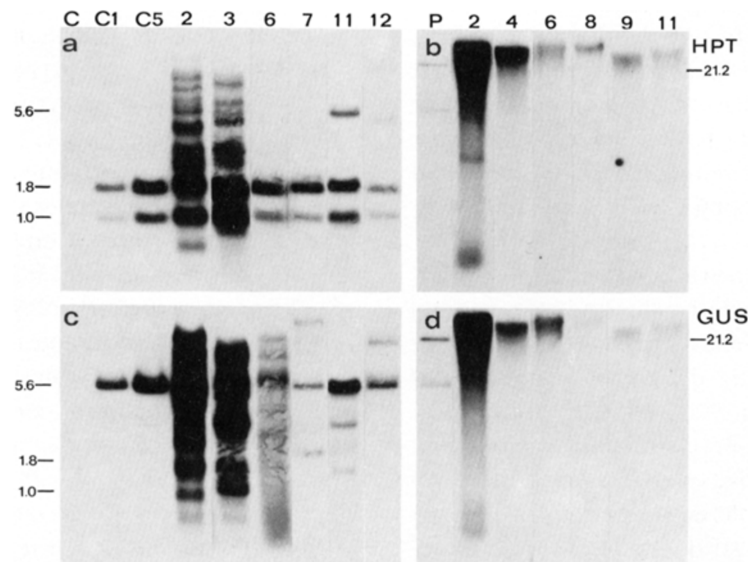

Fig. 2. Southern blot analysis of *Eco* RI-digested (a, c) and undigested DNA (b, d) from hygromycin-resistant callus lines of perennial ryegrass, one year after bombardment. Total DNA was isolated from selected hygromycin-resistant and control callus lines [20]. The control callus originated from bombarded control tissue unable to grow on selection medium and for DNA isolation subsequently maintained on culture medium. DNA was digested with a five-old excess of *Eco* RI under conditions recommended by the supplier (Amersham). 15 μg DNA was size-separated by electrophoresis on a 0.8% (w/v) agarose gel and transferred to GeneScreen Plus membranes, according to conditions recommended by the supplier (Dupont). Prehybridization was performed for 1 h at 65 °C in 1% (w/v) SDS, 1 M NaCl, 10% (w/v) dextran sulphate and 100 μg/ml sheared herring sperm DNA. The membranes were hybridized in a hybridization mixture identical to the prehybridization mixture supplemented with a random-prime-labelled [5]^{32}P-probe at 65 °C for 24–48 hours. The membranes were washed in 2, 0.5 and 0.2 × SSC, 1% (w/v) SDS for 5, 15 and 15 min, respectively, at 65 °C. Hybridization patterns were visualized by autoradiography using X-Omat AR film (Kodak). For rehybridization, the membranes are stripped with 0.1 × SSC, 1% (w/v) SDS for 30 min at 100 °C. As probes the 1.0 kb *Bam* HI fragment of plasmid pLG90 carrying the *hpt* gene [10] and the 1.8 kb *Bam* HI fragment containing the *gusA* gene from the plasmid pCal1Ga, a kind gift from the Walbot Laboratory (Stanford University), were used (Fig. 1). Lanes of a and b, hybridized with the HPT probe, are the same lanes as in c and d, respectively, hybridized with the GUS probe. C, C1 and C5 represent control DNA and control DNA mixed with 1 (35 pg) and 5 (175 pg) copy standards of *Eco* RI-digested plasmid pORCEHyg per diploid genome (15 μg). The figures above the lanes correspond to the callus lines in Table 1. The lane indicated with 'P' represents intact pORCEHyg plasmid.

molecular-weight bands are displayed in addition to the 5.6 kb fragment. In Fig. 2b and d, high-molecular-weight bands of undigested DNA of two representatives of each classified GUS phenotype (Table 1), hybridized with the two probes, are displayed, which indicates integration of plasmid DNA. The fact that as well as higher and lower molecular weight bands the 1.0 and 1.8 kb fragments using the HPT probe, and the 5.6 kb fragment using the GUS probe, were always observed, possibly indicates multiple insertions of tandem copies or partial copies. For an estimation of the copy number of the *hpt* and *gusA* genes present in the transgenic callus lines, the band pattern of the *Eco* RI digested DNA was used together with 1 and 5 copy standards per diploid genome (see legend Fig. 2). In the 4 callus lines which had a negative GUS phenotype at the first and second GUS assay, integration patterns of a very high number of *gusA* and *hpt* copies were detected, whereas the remaining 8 callus lines had 1 to about 5 *gusA* copies, one year after bombardment.

The variation in blue staining patterns of GUS expression in the selected callus lines of perennial ryegrass is in agreement with results obtained for maize [15] and, using the same plasmid under comparable conditions as used here, for rice [12]. For maize and rice, levels of GUS expression, assayed by quantitative analysis, generally corresponded to the degree of staining from the histochemical GUS assay [12, 15]. Therefore, decline of blue staining in ca. 60% of the tested callus lines upon further culture could be interpreted as a decrease of GUS expression. This decline is not the result of disappearance of the *gusA* gene from the callus DNA, as was proven by Southern analysis one year after bombardment. A similar decrease of GUS expression was found in transgenic rice callus lines [12] and cell suspensions [19]. These results show that expression of a delivered gene can decrease or be suppressed in time. Absence of expression after multiple copy integration, as was found for the *gusA* gene in transgenic callus lines of perennial ryegrass, has also been reported for, amongst others, transgenic plants of *Petunia* [16], and tobacco after additional integration of a second gene construct [8, 17]. This suppression of expression was either caused by methylation of promoter sequences [16, 17] or remained unexplained [8].

This is the first report on stable transformation of perennial ryegrass. Earlier stable direct gene transfer to protoplasts of Italian ryegrass, a species of the same *genus*, has been reported [23]. The promoter from the constitutive rice gene *GOS2* [4], which was active in transient and stable expression of the *gusA* gene, will be suitable for expression of an introduced gene in perennial ryegrass. In ca. 40% of the callus lines long term expression of the *gusA* reporter gene was observed, whereas the other callus lines showed instability after 6 months and 1 year of culture.

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References

1. Bower R, Birch RG: Transgenic sugarcane plants via microprojectile bombardment. Plant J 2: 409–416 (1992).
2. Christou P, Ford TL, Kofron M: Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important Indica and Japonica varieties via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos. Bio/technology 9: 957–962 (1991).
3. Creemers-Molenaar J, van der Valk P, Loeffen JPM, Zaal MACM: Plant regeneration from suspension cultures and protoplasts of *Lolium perenne* L. Plant Sci 63: 167–176 (1989).
4. de Pater BS, van der Mark F, Rueb S, Katagiri F, Chua NH, Schilperoort RA, Hensgens LAM: The promoter of the rice gene *GOS2* is active in various different monocot tissues and binds rice nuclear factor ASF-1. Plant J 2: 837–844 (1992).

5. Feinberg AP, Vogelstein B: A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132: 6–13 (1983).
6. Finer JJ, McMullen MD: Transformation of cotton (*Gossypium hirsutum* L.) via particle bombardment. Plant Cell Rep. 8: 586–589 (1990).
7. Fitch MMM, Manshardt RM, Gonsalves D, Slightom JL, Sanford JC: Stable transformation of papaya via microprojectile bombardment. Plant Cell Rep 9: 189–194 (1990).
8. Fujiwara T, Lessard PA, Beachy RN: Inactivation of the nopaline synthase gene by double transformation: reactivation by segregation of the induced DNA. Plant Cell Rep. 12: 133–138 (1993).
9. Gordon-Kamm WJ, Spencer TM, Mangano ML, Adams TR, Daines RJ, Start WG, O'Brien JV, Chambers SA, Adams WR Jr, Willets NG, Rice TB, Mackey CJ, Krueger RW, Kausch AP, Lemaux PG: Transformation of maize cells and regeneration of fertile transgenic plants. Plant Cell 2: 603–618 (1990).
10. Gritz L, Davies J: Plasmid-encoded hygromycin B resistance: the sequence of hygromycin B phototransferase gene and its expression in *Escherichia coli* and *Saccharomyces cerevisiae*. Gene 25: 179–188 (1983).
11. Hensgens LAM, Fornerod MWJ, Rueb S, Winkler AA, van der Veen S, Schilperoort RA: Translation controls the expression level of a chimeric reporter gene. Plant Mol Biol 20: 921–928 (1992).
12. Hensgens LAM, Rueb S, de Bakker EPHM, van Os-Ruygrok EP, Rueb S, van der Mark F, van der Maas HM, van der Veen S, Kooman-Gersmann M, 't Hart L, Schilperoort RA: Transient and stable expression of *gusA* fusions with rice genes in rice, barley and perennial expression. Plant Mol Biol, in press.
13. Jefferson RA, Kavanagh TA, Bevan MW: GUS fusions: β-glucuronidase as sensitive and versatile gene fusion marker in higher plants. EMBO J 6: 3901–3907 (1987).
14. Klein TM, Wolf ED, Wu R, Sanford JC: High velocity microprojectiles for delivering nucleic acids into living cells. Nature 327: 70–73 (1987).
15. Klein TM, Kornstein L, Sanford JC, Fromm ME: Genetic transformation of maize cells by particle bombardment. Plant Physiol 91: 440–444 (1989).
16. Linn F, Heidmann I, Saedler H, Meyer P: Epigenetic changes in the expression of the maize *A1* gene in *Petunia hybrida*: role of numbers of integrated gene copies and state of methylation. Mol Gen Genet 222: 329–336 (1990).
17. Matzke MA, Primig M, Trnovsky J, Matzke AJM: Reversible methylation and inactivation of marker genes in sequentially transformed tobacco plants. EMBO J 8: 643–649 (1989).
18. McCabe DE, Swain WF, Martinell BJ, Christou P: Stable transformation of soybean (*Glycine max*) by particle acceleration. Bio/technology 6: 923–926 (1988).
19. Meijer EGM, Schilperoort RA, Rueb S, van Os-Ruygrok EP, Hensgens LAM: Transgenic rice cell lines and plants: expression of transferred chimeric genes. Plant Mol Biol 16: 807–820 (1991).
20. Mettler IJ: A simple and rapid method for minipreparation of DNA from tissue cultured plant cells. Plant Mol Biol Rep 5: 346–349 (1987).
21. Rueb S, Hensgens LAM: Improved histochemical staining for β-D-glucuronidase activity in monocotyledonous plants. Rice Genet Newsl 6: 168–169 (1989).
22. Vasil V, Castillo AM, Fromm ME, Vasil IK: Herbicide resistant fertile transgenic wheat plants obtained by microprojectile bombardment of regenerable embryogenic callus. Bio/technology 10: 667–674 (1992).
23. Potrykus IP, Saul MW, Petruska J, Paszkowski J, Shillito RD: Direct gene transfer to cells of a gramineous monocot. Mol Gen Genet 199: 183–188 (1985).