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Luciferase as a reporter gene for transformation studies in rice (*Oryza sativa* L.)

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Abstract Transformed rice plants of var 'TN1' were regenerated from immature embryos following particle bombardment with a construct containing the firefly luciferase gene as a reporter gene and the hygromycin resistance gene as a selectable marker. Expression of the luciferase gene in the presence of the substrate luciferin was visualised in the calli derived from bombarded immature embryos and in the leaves and roots of the regenerated transformed plants using a low light imaging system (luminograph). Embryogenic callus proliferation and plant regeneration were unaffected by luciferin treatment and luminograph screening. The quantitative Luc assay using samples of leaf tissue from the segregating generations gave early information about the homozygous and hemizygous state of the *luc* transgene.

Key words *Oryza sativa* · Luciferase gene · Transient expression · *luc* · Luminograph · Luminometer

Introduction

In plant transformation studies, reporter genes are necessary for the rapid detection of DNA introduction. The β-glucuronidase (*gus*) gene is the most widely used reporter gene in cereal transformation. Although it has some useful properties, one of the disadvantages of using this as a reporter gene is that histochemical staining for *gus* expression is destructive and transgenic tissue cannot be recovered for further callus proliferation and regeneration of plants after its identification. An alternative reporter gene, the firefly luciferase gene (*luc*), provides the opportunity to recover the putative transformed tissue after its identification (Chia et al. 1994). A controversial aspect of plant transformation studies is the use of antibiotics as selective agents to inhibit the growth of non-transformed tissues. These often also inhibit the growth and development of transformed tissues, especially when the starting material is a delicate explant such as the protocorm-like bodies of orchids (Chia et al. 1994) or haploid microspores of cereals. Moreover, there is also the problem of field release of transgenic plants containing antibiotic resistance genes. It is possible that an efficient reporter gene could eliminate the use of selectable markers altogether.

Several reports exist concerning the detection of luciferase gene activity in a non-destructive way using imaging techniques (luminograph), either after infiltrating or treating the living plant or plant parts with the substrate luciferin and then visualising light production either by contact photography (Ow et al. 1986; Howell et al. 1989; Gallie et al. 1989; Schneider et al. 1990) or by an image-enhancing video system (Wick 1989; Robinson 1989; Millar et al. 1992; Chia et al. 1994; Kay et al. 1994). In cereal transformation studies where the luciferase gene was used as a reporter, its activity was measured in tissue extracts in terms of relative light units (RLUs) per amount of protein (*luc*-assay; Cornejo et al. 1993; Sadasivam and Gallie 1994). This is, however, a destructive way of identifying the transformants, although it does give a quantitative estimation of luciferase activity in transformed tissue.

The expression patterns of the luciferase gene in different plant parts at different stages of growth in cereal crops has not been reported. The experiments described here were conducted to investigate the behaviour and inheritance pattern of the luciferase gene and its activity in transformants, throughout the growth phase, using rice (*Oryza sativa* L.) as a model cereal crop. Thus, we examined whether embryogenic callus could proliferate and regenerate after luciferin treatment, this being an essential first step for video imaging of transformed tissue. Luciferase expression was imaged (luminograph) successively in putative transformed callus during the tissue culture phase

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and in different plant tissues at different stages of growth in first, second and third generations of a transformed rice line generated from an immature embryo via particle bombardment using an electrical discharge gun. For this purpose, we used the luminograph LB 980, a high-performance luminescence imaging system. Simultaneously, quantitative *luc*-assays were performed using a luminometer (LB 9501) to analyse whether there was any variation in luciferase activity between different plant parts at different stages of growth, and between transgenic homozygote and hemizygote plants. Gene integration in the first- and second-generation transformed plants was confirmed by Southern blotting.

Materials and methods

Reporter gene constructs

A pBluescript-based plasmid pAL52 containing an improved firefly luciferase (*luc*$^+$) reporter gene (Sherf and Wood 1994) and hygromycin resistance (*hmr*) gene, each driven by the *ubi1* promoter and *ubi1* intron and terminated by the nopaline synthase (*nos*) polyadenylation site was used for the transformation studies.

Plant material, preparation of explants and bombardment

'TN1', a variety frequently used as a parental source of indica rice (*Oryza sativa* L.), was used for the transformation studies. Fifteen days after anthesis, immature seeds were harvested from glasshouse grown plants, dehusked, sterilised for 30 min in 50% sodium hypochlorite and a few drops of Tween 20 and washed thoroughly in distilled water. Fifty immature embryos were excised aseptically from the dehusked seeds, plated scutellum side up in two 6-cm petri dishes containing CC medium (Potrykus et al. 1979) supplemented with 2 mg l^{-1} 2,4-D. Two days after culture, the scutella of the immature embryos were bombarded using at an accelerating voltage of 15 kV an electric discharge gun, following the protocol of Christou et al. (1991).

Selection of transformants and regeneration

Two days after bombardment, the tissues were transferred to 9-cm petri dishes containing a similar medium supplemented with 50 mg l^{-1} hygromycin. Every 10–12 days each callus clump was split into several pieces, and this subculture was repeated three to five times. This was to facilitate subsequently selection of transformed tissue. Cultures were kept in the dark at 28 °C. Finally, the fast growing tissues were again subdivided and plated onto regeneration media (CC medium without hormones) and exposed to a 16-h light and 8-h dark cycle at 28 °C. After the appearance of shoots they were split further and transferred to a hormone-free MS medium (Murashige and Skoog 1962), still with 50 mg l^{-1} hygromycin, for rooting. When the plantlets attained a height of 7–10 cm they were transferred to soil in 9-cm^3 plastic pots and put in a propagator to maintain humidity. After a week they were removed and the plantlets raised in a glasshouse under similar conditions as the donor plants.

Video imaging of luciferase activity

A low-light imaging system, luminograph LB 980 (EG&G Berthold), was used for imaging the luciferase activity in the plant tissues. Transient expression of the luciferase gene 2 days after bombardment as well as expression in transformed callus and plant tissues at the T_0, T_1 and T_2 generations was visualised by first treating the samples with 1 mM beetle luciferin potassium salt (Promega) dissolved in simple MS liquid medium and then examining the samples with the luminograph. Before every subculture, this procedure was performed to localise the transformed parts of the callus or the whole transformed callus by overlaying the luminograph after imaging on top of a photograph of the same culture plate. In the case of leaves, uniform wetting with luciferin was a problem. This was overcome by washing the leaves with 5% Tween 20 followed by washing with water twice, and blotting off excess water, before the luciferin treatment.

Quantitative luciferase assay (Luc assay)

Quantitative luciferase activity was measured following the protocol of Ow et al. (1986) in extracts of calli, shoots and roots of regenerating plantlets and in roots and leaves of mature plants using a luminometer (Lumat LB 9501). Results were expressed in terms of relative light units (RLU) per milligram of protein. For this, plant tissues, either a piece of callus, or three- to four-cm-long leaf or root sections, were collected in 1.5-ml Eppendorf tubes, frozen immediately in liquid nitrogen, ground in the same Eppendorf tube along with a small amount of liquid nitrogen and returned to the container with liquid nitrogen. After the collection and grinding of all the samples, 6–8 samples were prepared at one time for the Luc assay. Ground tissue was added to 0.5 ml extraction buffer containing 100 mM potassium phosphate pH 7.0, 1 mM dithiothreitol, 1 mg ml^{-1} bovine albumin (Sigma), mixed thoroughly, then centrifuged for 5 min in a microcentrifuge at 4 °C. Supernatants were transferred to clean tubes. Then, 120 µl of extract was mixed with 120 µl of 60 mM ATP at pH 7.0 and 1080 µl of assay buffer containing 14 mM glycylglycine buffer (Gly-Gly, Sigma) at pH 7.8, 14 mM magnesium chloride, 1 mg ml^{-1} bovine albumin and 6 mM ATP. Aliquots of 400 µl were dispensed into luminometer tubes, 100 µl of 1 µM luciferin was injected into the same tube and the relative light unit (RLU) was measured. Since luciferase activity diminishes rapidly, usually only 2 samples were prepared at a time. By doing the collection and grinding of tissue in liquid nitrogen, it was possible to prepare 6–8 samples at a time without any significant decrease in luciferase activity. Luciferase assays were performed in triplicate for each sample to minimise any instrument variation. Protein concentration was determined by the method of Bradford (1976), and the RLU of each sample was calculated per milligram of protein extract.

Southern blot analysis

Genomic DNA was extracted from fully developed leaves following the procedure of Ellis et al. (1994). The genomic DNA was digested with the restriction endonuclease *Hin*dIII which also linearises the plasmid. Digested DNA was size-fractionated by electrophoresis on 0.8% agarose gels and then transferred under alkaline conditions to a positively charged nylon membrane, following the protocol of Reed and Mann (1985). Hybridisation was performed with α-[^{32}P]-dCTP-labelled probes using a standard protocol (Feinberg and Vogelstein 1983). The probes used contained either the 1.1-kb fragment of the luciferase coding region and or the 974-bp fragment of the hygromycin-coding region, both synthesised by the polymerase chain reaction (PCR). The hybridised filters were exposured to Kodak XAR-5 X-ray film.

Segregation analysis

Forty-four T_1 seeds were divided into two groups, imbibed for 24 h in water and imaged using the luminograph to determine expression of the luciferase gene in caryopsis, coleoptiles and roots. The goodness-of-fit of the segregation ratios (3:1 model) of those found to be Luc positive/Luc negative was tested using Chi-square tests. Expression of the *hmr* gene, was checked by germinating all the seeds of the first group, irrespective of *luc* expression, and all the seeds of the second group with *luc* expression in hygromycin at a concentration

50 mg l^{-1}, while the seeds of the second group without *luc* expression were germinated in water. Later, the presence of the *luc* and *hmr* genes was confirmed in these progenies by Southern blotting. A quantitative Luc assay was performed on tissues of the fourth leaf of each of the T_1 progenies to check if there was any difference in Luc activity between homozygotes and hemizygotes.

Seeds were collected from T_1 progeny plants that were positive for the *luc* and *hmr* transgenes, and 20 seeds of each plant were germinated in 50 mg l^{-1} hygromycin to analyse the inheritance of the transgenes as well as to assess which of the T_1 progeny plants were homozygous and which were hemizygous for the transgenes. The luciferase expression of these T_2 progenies was checked using the luminograph.

Results and discussion

Recovery of transformed tissue and regeneration

Transient expression of the luciferase gene was observed using the luminograph LB 980 (low-light imaging system) in almost all of the scutella of bombarded immature embryos (Fig. 1a) . Ten days after transfer onto selection medium, most parts of the scutellum, from 90% of the bombarded embryos, had turned brown, but some tissue proliferation was observed from the remaining parts. Tissue proliferation was totally inhibited in 10% of the bombarded scutella. At this stage callusing scutella were subjected to imaging for a second time. This time, 3 out of 45 calli showed luciferase activity. Despite this result, all 45 calli were split into three to five pieces, plated on the same selection medium and imaged for a third time, 26 days after selection. The number of calli showing luciferase activity was now reduced to 2 out of the 45 original callus lines. One of these lines was a fast-growing, vigorous callus, while the other was a chimeric callus. During further proliferation, when calli were split and plated onto medium under similar conditions, the fast-growing callus proliferated much more vigorously than the other lines. All the calli derived from this line were positive for luciferase expression (Fig. 1b) when checked with the luminograph LB 980. Up to this stage in the experiment, hygromycin did not totally inhibit the proliferation of non-transformed callus.

After 12 days, the fast-growing callus line and most of the slow-growing callus lines containing embryogenic calli were transferred to regeneration medium, still with 50 mg l^{-1} hygromycin, and incubated under 16 h of light at 28 °C. Within 10 days, growth of most of the callus lines ceased, except for the vigorously growing line and a few slow-growing lines that produced shoots. Within the next 15 days, further development of the slow-growing callus lines was totally inhibited and they underwent rapid necrosis. The fast-growing, luciferase-positive callus line produced healthy shoots and roots when transferred to the rooting medium. A total of 19 plants were regenerated from this single clone and raised under glasshouse conditions.

During the tissue culture phase of development of transformed plants the luciferase gene has an advantage over the more widely used reporter gene β-glucuronidase in that the transformed tissue/callus containing the *luc* gene can be monitored for the expression of the *luc* gene without destroying the tissues. Proliferation of embryogenic calli is not affected by treatment with the substrate luciferin. Transformed plants can be generated conveniently using luciferase as a reporter gene.

DNA analysis of transformants

Southern blot analysis of the 19 T_0 plants derived from the single callus line showed similar banding patterns, indicating homogeneity within the clone (Fig. 2). *Hin*dIII-digested genomic DNA gave a single band of more than 12 kb, i.e. larger than the size of the linearised plasmid, when hybridised with a probe complementary to either the *luc* or *hmr* coding region.

The results of Southern blot hybridisation with the DNA of 27 T_1 plants that were expressing luciferase and also resistant to hygromycin showed a banding pattern similar to the T_0 parental DNA. This confirmed the inheritance of the transgenes in the progeny plants. A PCR analysis with the DNA of 9 T_1 progeny that did not express Luc and were germinated in water without hygromycin confirmed the absence of the *luc* transgene.

Image analysis of luciferase

Activity of the luciferase gene was visualised in transgenic calli and regenerating transformed plantlets (Fig. 1b, c). In seeds of the segregating generation, Luc activity was observed in the caryopsis (Fig. 1fI, fII), and later in the leaves and roots (Fig. 1d, e) of germinating seedlings. The phenotypic ratio for *luc* expression (14 Luc-positive: 8 Luc-negative in group 1; 13 Luc-positive and 9 Luc-negative in group 2) fitted a 3:1 ratio, confirming Mendelian inheritance of a single gene locus for the transgene. In terms of intensity, no observable variation in Luc activity was observed between different parts of the homozygotes and hemizygotes of segregating progenies when imaged with the luminograph. The T_2 generation progenies expressed Luc activity in the leaves and roots in a way similar to their parental generation.

Germination test for hygromycin resistance

Twenty-two seeds, in two replications, from a T_0 plant were imbibed in water for 1 day, then checked for *luc* expression. Nine seeds from one replication that were negative for *luc* expression were germinated in water, while the remaining seeds were germinated in the presence of 50 mg l^{-1} hygromycin. Seeds in both replications, that were positive for *luc* expression were also resistant to hygromycin, while 8 seeds that did not show any *luc* expression failed to germinate in the presence of hygromycin (Fig. 1gI, gII). The phenotypic ratio for hygromycin resistance fitted with a 3:1 ratio, confirming Mendelian inheritance of a single

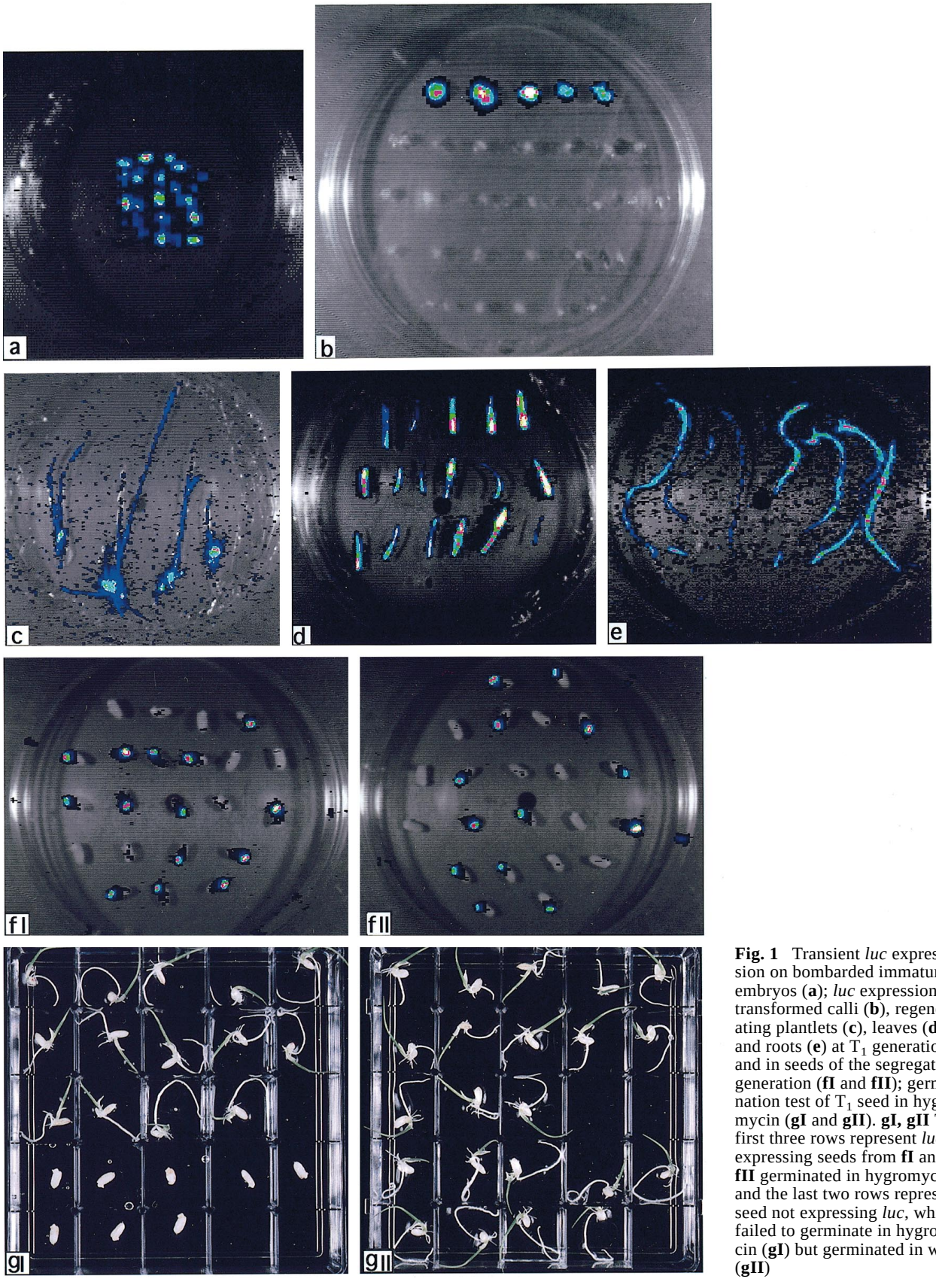

Fig. 1 Transient *luc* expression on bombarded immature embryos (**a**); *luc* expression in transformed calli (**b**), regenerating plantlets (**c**), leaves (**d**) and roots (**e**) at T_1 generation, and in seeds of the segregating generation (**fI** and **fII**); germination test of T_1 seed in hygromycin (**gI** and **gII**). **gI, gII** The first three rows represent *luc*-expressing seeds from **fI** and **fII** germinated in hygromycin, and the last two rows represent seed not expressing *luc*, which failed to germinate in hygromycin (**gI**) but germinated in water (**gII**)

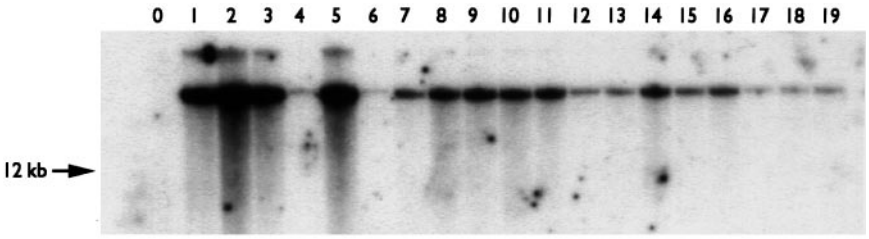

Fig. 2 Southern blot analysis of DNA from primary transformants. *Lane 0* DNA of a non-transformed plant, i.e. the negative control, *lanes 1–19* DNA from 19 different plants derived from a single clone

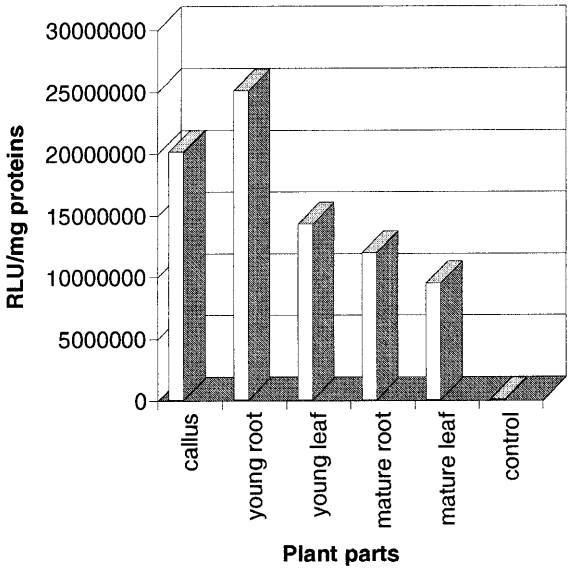

Fig. 3 Results of Luc assay in putative transformed callus and different plant parts of primary transformants, along with a non-transformed negative control

gene locus of this transgene. A co-expression frequency of 100% for the *luc* and *hmr* gene was observed in the segregating progenies.

Twenty seeds from each of the T_1 plants were tested in a similar way to examine expression of the *hmr* gene. Seeds of 9 plants that were negative for *luc* expression did not germinate in hygromycin. However, seeds of 21 Luc-positive plants germinated in hygromycin and gave a 3:1 ratio of resistant:sensitive seeds, confirming the hemizygous state of the transgene, while seeds of the remaining 6 plants gave 100% germination in hygromycin, confirming that they were homozygous for the transgene.

Quantitative luciferase activity

Luciferase activity was quantified using a luminometer (Lumat LB 9501) to measure the light emission in terms of relative light units (RLU) per milligram of protein in tissue extracts of different parts of the T_0 plants (Fig. 3). A large range existed for the number of RLU per milligram protein within the same clone in mature leaf samples at different stages (5) (591) (539-13) (670) (843 RLU) and root tissues (4) (520) (543-27) (029) (447 RLU) of different plants. This range was still high in regenerating plantlets, (6) (879) (427-25) (028) (640 RLU) in leaves and (15) (477) (242-29) (629) (342 RLU) in roots. In general, however, the values decreased as the tissues matured.

RLU values for Luc activity were measured in the leaf tissue of T_1 progenies using the fourth leaf of every plants. The 9 plants that were negative for *luc* expression, as visualised under the luminograph, also gave RLU values that were similar to that of the negative non-transformed control. However, high RLU values were observed for the 27 plants that showed luciferase activity and were resistant to hygromycin. A significant difference was observed

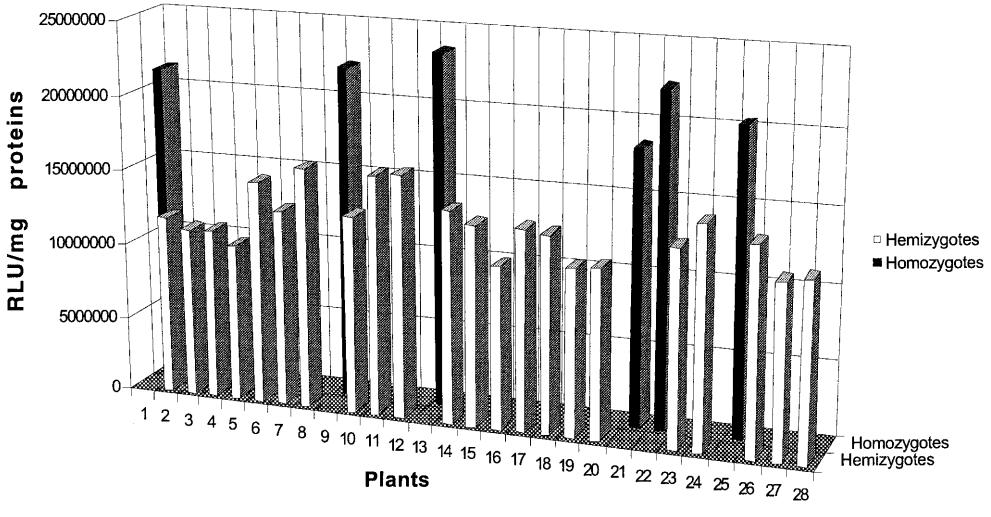

Fig. 4 Results of Luc assay in leaf tissue of T_1 plants (*plants 1–27*) and a T_0 plant (*plant 28*) as control

between the different T_1 plants. Out of 27 plants, 21 could be grouped under one category and these had RLU values which were close to that of the parent (T_0) which was hemizygous for the transgene, while the remaining 6 plants fell into a different category with significantly higher RLU values per milligram of protein, indicating that these plants were probably homozygous (Fig. 4). This was confirmed by analysing the T_2 seeds for expression of *luc* and *hmr* transgenes. The 21 T_1 plants in the first category produced seed that segregated in a 3:1 ratio for *hmr* and *luc* expression, whereas seed from the second category T_1 plants were all positive for *hmr* and *luc* expression. Thus, the quantitative Luc assay gives early information about the homozygous and hemizygous state of the *luc* transgene and possibly other genes carried on the plasmid without analysing the progenies. This could save time and the expense of maintaining a large number of transformed plants in segregating and successive generations, since hemizygotes could be identified and discarded at an early stage of the T_1 generation. Zygosity of transgenic plants in segregating generations was also determined by comparing the protein level of transgene potato proteinase inhibitor II in japonica rice (Duan et al. 1996).

Conclusion

In this report the stable expression of the firefly luciferase gene, which is under the control of the maize *ubi1* promoter, in transgenic rice and its inheritance in segregating generations is demonstrated. The firefly luciferase gene has not been used extensively as a reporter in the production of transformed plants although the expression of the *luc* gene, which is under the control of a number of promoters including CaMV 35S, in transgenic tobacco (Ow et al. 1986), *Dendrobium* (Chia et al. 1994), rice protoplasts (Sadasivam and Gallie 1994), and the maize *ubi1* promoter in rice callus (Cornejo et al. 1993) has been examined. The results of these experiments indicate that the luciferase gene, linked with other agronomically useful genes, could be used conveniently for the production of transgenic plants and later as a marker for genetic crosses.

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