The Possible Final Localization of GUS (β-glucuronidase) Reporter Gene Product in Transgenic Plants is Vacuole

Taka Murakami, Dajjiro Hosokawa* and Yuko Ohashi

(National Institute of Agrobiological Resources, Kanonnodai, Tsukuba, Ibaraki 305, Japan; *Faculty of Agriculture, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183, Japan)

Received January 31, 1992

Abstract: Localization of the product of a bacterium-terminating reporter gene (β-glucuronidase: GUS) was studied using histochemical and electron-immunocytochemical methods in transgenic tobacco plants introduced by GUS reporter gene with no targeting signals. In leaf and stem cells the insoluble blue dye associated with GUS gene product was found without exception on the subcellular organelles and in the vacuole. However, in the cells with higher GUS activity, uniform blue color was often observed all over the cell as if blue ink had dissolved. When such cells, which have high GUS activity, were treated with 1 M K2SO4, solution, the closed area with blue uniform color began to shrink and could be distinguished from cytoplasm and the cell wall as a result of plasmolysis. The blue round compartment was very similar to a vacuole which could also be detected as a red closed area after neutral red-staining and plasmolysis. GUS localization was further studied electron microscopically by the method of protein A-immunogold. Many gold particles were found in the electron-dense small bodies in vacuoles, however they were sparsely found in the other areas. These results indicated that GUS was produced in the cytoplasm, and was finally secreted into the vacuole in these cells.

Key words: Gene targeting, β-glucuronidase, Neutral red, Nicotiana tabacum, Plasmolysis, Reporter gene, Vacuole.

Transgenic plants have been used for the analysis of regulated expression of the introduced genes. Cis-acting elements can be studied by the fusion of the promoter with a suitable reporter gene and introducing them into plants. Quantitative determination of the product of the reporter gene in transgenic plants provides much information on the regulation of gene expression under induced or non-induced condition and at different developmental stages in different organs and tissues. Such experiments are also important for basic studies in order to determine the practical use of transgenic plants in the pro-
duction of useful proteins or antisence RNA. The reporter gene, β-glucuronidase (GUS) has been most frequently used due to its following advantages: 1) The reporter enzyme can be very easily and sensitively assayed in vitro by a fluorometric method. 2) It can be detected in situ by histochemical methods. 3) Endogenous GUS or GUS-like activity is very low in plants and further the low intrinsic GUS-like activity can be almost completely suppressed by an improved GUS assay method. 4) GUS-fusion proteins can be used for protein-targeting into cell organelles.

The information on localization and the half-life of the product of the reporter gene are very important for the analysis of gene regulation and its practical usage. On the half-life of GUS, Jefferson et al. have described that the GUS enzyme is very stable in extracts and in living cells with a half-life 50 hr. However, no evidence on the localization of the product of the introduced GUS gene with no targeting signals has been reported. In this paper, we describe the evidence of GUS localization in the cells of GUS gene fusion-introduced transgenic plants using the GUS vital staining method and electron immunochemical method.

Materials and Methods

1. Plant materials

Three kinds of GUS gene fusion-introduced transgenic plant types were used. The first type was tobacco plant (Nicotiana tabacum cv. Samsun NN), containing a chimeric gene which consisted of cauliflower mosaic virus 35S promoter and GUS reporter gene (35S-GUS). In this plant, introduced GUS chimeric gene is expressed constitutively. The second type was transgenic tobacco plant containing PR-GUS chimeric gene consisted of 2.4 Kb of 5’ flanking region of tobacco pathogenesis-related (PR)1 protein gene fused to GUS reporter gene (PR-GUS). In the plant, GUS activity was induced to a high level by treatment with salicylic acid, which is an effective inducer of PR 1 protein in tobacco plant. Two days after the salicylic acid treatment, GUS activity increased to 20-fold from that at 0 time. Self pollinated progenies of the original GUS gene fusion introduced plants were used in this study for both the above type of chimeric genes. The third type was originated from an interspecific hybrid of Nicotiana glutinosa × Nicotiana debneyi, which produces PR1 protein constitutively at a high level. We had introduced the PR-GUS chimeric gene into the hybrid, and used in this experiments as transgenic plants which had high GUS activity constitutively at healthy state as if the parents’ plants were treated with salicylic acid. All plants were grown in a temperature-controlled green house.

2. Histochemical assay for GUS activity

GUS active staining in situ was performed by the improved method described by Kosugi et al. which is essentially similar to the one described by Jefferson et al. In this modified method, chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc) was used as the substrate and 20% (v/v) methanol was added to the reaction mixture. The endogenous GUS-like activity in the plant was suppressed to a negligible level by methanol. After the reaction, ethanol was added to denature GUS and to remove chlorophyll for easy observation of blue colored GUS reaction product in the tissue. The sections were transferred to water and then mounted in 70% glycerol for photography using light microscopy.

3. Identification of vacuole

Vacuole can be identified by staining with neutral red (NR). It changes to orange-red color by selective absorption of the dye in a viable cell. However, if the cells are damaged or dead, the cells are stained non specifically all over. Plasmolysis by 1 M KNO₃ solution was used to confirm the presence of vacuoles in the cells. After vital staining of the cells by diluted aqueous neutral red, the mounted solution was gradually changed to 1 M KNO₃ solution on a glass slide on the microscopic stage. Red stained vacuole covered with unstained cytoplasm gradually separated from the cell wall by plasmolysis, and the red vacuole became more distinguishable from the pale yellow cytoplasm and the cell wall.

4. Immunoelectron microscopy

The sections from transgenic tobacco leaves treated with salicylic acid at 20°C for 3 days were stained with anti-GUS anti serum followed by protein A-gold. Fixation and immunocytochemistry were performed as described in Hosokawa and Ohashi.
Results

1. Histochemical analysis of GUS localization

Fresh sections were prepared from various tissues of transformed plants with various GUS gene fusions. We could easily locate where the gene was expressed by observing the blue indigogenic dye which is the end product of GUS reaction, using light microscopy (Fig. 1). Young leaf blades from fully expanded upper leaves (Fig. 1 A–1 G), and young stem near the shoot apex (Fig. 1 H and 1 I) were the materials in this study.

In the 35 S-GUS introduced cells with low or moderate GUS activity, cellular organelles such as chloroplast, mitochondria and nucleus and the moiety around them were stained blue, but its cytoplasmic matrix stained little at all (Fig. 1 F). However, in cells where GUS activity was high, the homogeneous blue staining was often observed all over the cells of several tissues, such as palisade mesophyll tissue, spongy mesophyll tissue, xylem parenchyma, phloem, epidermis, and/or trichome. The tone of the blue color looked like blue ink being dissolved inside the cell, with cell walls being clearly marked (Fig. 1 A and 1 G). Fig. 1 G shows heavy GUS staining in the leaf tissue where introduced PR-GUS chimeric gene expressed following induction by the treatment of salicylic acid. Homogeneous blue color was observed in the immature trichome and palisade cells under similar conditions. In mature cells, vacuole covers almost all the volume of the cytoplasm, with the vacuole outline being similar to that of the cell wall.

To confirm the localization of GUS in the vacuole, the technique of plasmolysis was used. Epidermal cell is usually very flat and convenient for direct observation of vacuole. In sections cut from young leaf tissues containing a whole epidermal cells which had higher GUS activity. GUS staining seemed to be in the vacuoles of the cells which had just began to plasmolysis (Fig. 1 A and 1 A’). Using similar sections as that of Fig. 1 A, vital staining was carried out by using neutral red (Fig. 1 B and 1 B’). When the mounting medium was changed to 1 M KNO₃ solution, convex plasmolysis occurred (Fig. 1 C and 1 C’). Homogeneous red staining of vacuoles was seen at first (Fig. 1 B and 1 B’), followed by shrinkage of vacuoles which were covered with the pale yellow cytoplasm, and lastly red-stained vacuoles gradually contracted to a smaller size as plasmolysis progressed (Fig. 1 C and 1 C’). The shape and action of the blue compartment in Fig. 1 A was quite similar to the red closed area observed in Fig. 1 B and 1 C. In this case, methanol was omitted from substrate mixture and ethanol was also omitted after GUS–staining because of their inhibitory effect on plasmolysis. In the basal cells of the trichomes in the same tissue and same treatment as Fig. 1 A–1 C, neutral red-positive vacuole occupied less than half of the cell (Fig. 1 E). On the other hand, GUS activity in these cells was found in a closed compartment in the basal portion of the cell with homogeneous blue staining being distinguishable from that of the cytoplasm where GUS activity was scarcely found (Fig. 1 D). The pattern of the blue closed area in fig. 1 D was also very similar to the vacuole in Fig. 1 E.

In the longitudinal section of young stem near the shoot apex, immature vacuoles were recognized without plasmolysis by heavy red color in the xylem parenchyma with the background of weak red color using neutral red staining (Fig. 1 I). By the GUS staining of a similar section, only similar convex closed area as those which were stained by heavy red color in Fig. 1 I was GUS positive and showed heavy blue color in weak blue background (Fig. 1 H), suggesting that the heavy blue compartments were vacuoles.

2. Electron microscopic observation of GUS localization

Immuno-gold labels were found spread over the electron-dense bodies in the central vacuole. The shape of these bodies were spherical or oval with a diameter of 3–6 μm. They were entirely filled with the electron-dense proteinaceous materials (Fig. 2 A and 2 B) or had translucent vacuole-like areas in them (Fig. 2 C). One to three electron-dense bodies were usually observed in a central vacuole on the sections. In many cases, small odd-shaped electron-dense structures labeled with gold particles and with stretched strings were observed in central vacuoles near the tonoplast, as if they have been just translocated from the cytoplasm (Fig. 2 D).

The other areas of the cells were sparsely labeled with gold particles in organelles and in
the cytoplasm. Few labels could be observed on sections of water-treated PR–GUS transgenic tobacco. In 35S–GUS transgenic tobacco in which GUS activity was low or not very high, treatment with preimmune serum also showed no specific labels in any part of similar cells (data not shown).

Discussion

Light microscopic observation showed that GUS activity was generally found in organelles and in the moiety around organelles in transgenic cells where introduced GUS gene fusion was expressed. From electron microscopic observation, it was also obvious that GUS was immunologically found in organelles or in the cytoplasm by protein A-immuno gold method, through the gold label was found only sparsely. On the other hand, in the cells which had high GUS activity, uniform blue colour was often detected all over the cells as if blue ink was dissolved within the cell. Such stained cells correspond to vacuoles of plant cells. In the mature leaf cells, vacuoles occupy a large volume of the cell, and can be identified by the uniform red color after staining with neutral red. To confirm the presence of vacuoles, the technique of plasmolysis has been often used. When living cells are treated with neutral red and then with 1 M KNO₃ solution, vacuole shrinks to a smaller volume showing concentrated red color with its shrunken volume. Such a shrunken vacuole can be easily distinguished from the pale yellowish cytoplasm covered on vacuole. When the cells with uniform blue color after GUS reaction were treated with 1 M KNO₃ solution, the blue compartments shrank to a smaller volume showing concentrated blue color in the same manner as that of vacuole. Protein A-immunogold method showed that many gold labels accumulated specifically in the electron dense bodies in the vacuole, whereas they were only scarcely found in other areas of the vacuole where the electron dense bodies were not present nor in other parts of the cell. We have two contradictory observations on the localization of GUS in vacuoles using two different methods of GUS detection. The first is that homogeneous GUS staining was clearly found in the vacuole microscopically, but not by electron immunochemistry. The second is that many gold labels were electron microscopically detected in the electron dense bodies in vacuole, however under a light microscope such bodies did not contain the blue color after GUS reaction. We rule out the first contradiction by the possibility that soluble GUS content in the vacuole is not sufficient to be detected electron microscopically but the enzyme activity is sufficient to be detected under light microscope following GUS staining. The second contradiction could be illustrated by the possibility that electron dense bodies in vacuoles may contain sufficient GUS protein to react with the antibody, but there is a loss of β-glucuronidase activity due to possible attack by endogenous protease in vacuoles.

From the results described here, we speculate that the process of GUS localization into electron dense bodies in the vacuole are as follows; 1) Firstly, GUS is synthesized and accumulated on/in organelles or in the moiety around them in the cytoplasm; 2) Secondly, GUS is secreted vigorously into the vacuoles in the cells where GUS activity is high; 3) Thirdly, GUS protein in the vacuole may have had β-glucuronidase activity at first. However, as it accumulated in electron dense bodies in the vacuole, it may have lost the enzyme activity by subsequent inactivation in the vacuole such as protease attack. The degree of GUS degradation may not be so severe since GUS protein as an antigen can react with its antibody.

There are other reports on the formation of electron-dense bodies in the vacuole and accumulation of enzymes into such electron dense bodies. Halperin reported the presence of acid phosphatase-positive vesicles in the vacuole of Daucus carota. The electron micrographs of these vesicles are very similar to odd-shaped electron dense bodies in our study. However in this case, electron dense bodies still had the enzyme activity. This phenomenon is reasonable because acid phosphatase is well known protein which is contained in vacuoles and protease resistant. Another report on basic β-1, 3-gluconase and chitinase in tobacco leaves showed that these enzymes were secreted into vacuoles and further accumulated into electron dense bodies, which was similar to that observed in our study. However, in this case these proteins were synthesized with transit peptides for the targeting signal to vacuoles. There are other reports on GUS protein target-
Fig. 1. Histochemical localization of GUS activity in the vegetative shoot of GUS gene fusion introduced-transgenic tobacco plants.

(A-E) Epidermal system in fresh sections of young lamina from PR-GUS introduced interspecific hybrid of N. glutinosa × N. debneyi was used. In those transgenic plants, constitutive high GUS activity was observed in the leaf[10]. A', B' and C' are schematic diagrams of the respective micrographs of left side.

(A) Epidermal cells stained with X-gluc for 4 h without methanol, and then plasmolysis agent (1 M KNO₃) was treated. GUS staining shown in vacuole-like compartment (arrowhead), and plasmolysis was just beginning (arrow).
Fig. 2. Ultrathin sections from transgenic tobacco leaves stained with anti-GUS anti-serum followed by protein A-gold.

Fig. 1 continued
(B) Homogeneous red staining were shown all over the vacuole after staining the section with neutral red (arrowhead). Before the plasmolysis.
(C) In the same sections to (B), 40 minutes after treatment of plasmolysis agent, the vacuoles were shrunken to convex-plasmolysis (arrowhead).
(D) After GUS reaction with X-gluc for 4 h, plasmolysis agent was treated. Homogeneous staining was shown in the vacuole-like compartment (arrowhead) on the basal part of trichome. In this cell, no blue color was found in the cytoplasm. Plasma membrane (arrow) and tonoplast (small arrowhead) were shown.
(E) Almost the same view as (D) was observed by the vital staining with diluted neutral red and subsequent plasmolysis. Only vacuole was positive in homogeneous red staining (arrowhead). Plasma membrane (arrow) and tonoplast (small arrowhead) were able to be distinguished.
(F, G) Transverse section of young lamina from 35S-GUS (F) and PR-GUS (G) introduced tobacco plants. GUS reaction was carried out for 1 h.
(F) GUS staining were shown in organella and moiety arround them in cytoplasm.
(G) Homogeneous heavy GUS staining in salicylic acid-treated tobacco leaf. In such leaf, very high GUS activity was induced. The immature trichome (t) and palisade tissue were stained heavy homogenous blue in the cells in addition to the concentrated blue around organella.
(H, I) Longitudinal sections of young stem near the shoot apex of transgenic interspecific hybrid. Immature vacuoles were recognized in the xylem parenchyma without the treatment of plasmolysis. GUS-activity were observed in the cytoplasm (arrow) and vacuole (arrowhead). GUS-reaction was carried out for 3 h.
(I) NR-staining were also observed in the vacuoles (arrowhead).
Size bars indicate 30 μm.
g: guard cell, p: palisade tissue, t: trichome, v: vessel.
ing to other cell organelles. Using fusions to
the transit peptide of a chlorophyll a/b pro-
tein, GUS reporter protein was used effectively
to target chloroplasts. Only N-terminal–GUS
fusion involving constructs containing the
DNA binding domain of the dASIC-zipper
region of TGA-1A or TGA-1B conferred
nuclear import. However, in the above case,
specific peptide for targeting was fused to the
N-terminal of GUS protein. Therefore, our
report on the secretion of GUS into vacuole is
the first evidence on the localization of GUS
protein without targeting signal in transgenic
plants.

When bacterial GUS gene is expressed in
transgenic tobacco, it should be recognized as a
foreign protein by tobacco cells. It is well
known that many useful foreign proteins are
produced in bacteria by gene engineering. In
such cases, the content of newly produced
protein sometimes reached to 10–40% of
total bacterial protein, and the protein ag-
ggregated into vesicles or inclusion bodies
which is a proteinous non-crystallized aggreg-
gate without a membrane structure. In
plants, vacuole is known as the location of
degradation and accumulation of many
metabolites. In such a sense, secretion into
vacuole and accumulation into electron dense
bodies of bacterial GUS protein as a foreign
protein in transgenic tobacco may be reason-
able even if the GUS gene does not have any
signals for targeting.

Recently, many foreign proteins from virus,
bacteria, yeasts, insects and animals have been
over produced in many plants by gene-
manipulation techniques for both funda-
mental studies and for practical applica-
tions. In such systems, the localization or the
fate of the gene product in the cells would be
very important in studies on expression and
regulation of introduced gene and on the
effective protein production in practical usage
of such transgenic plants. The results in this
report on the localization of GUS gene product
into vacuole will provide some direction to
these studies.

References

1. Ahl, P. and S. Gianinazzi 1982. b-Proteins as a
constitutive component in highly (TMV) resis-
tant interspecific hybrids of N. glutinosa × N. deb-
2. Garhan, P.B. 1984. Plant Histochemistry and
Cytochemistry. An Introduction. Academic Press,
London. 124—130.
acid phosphatase in cultered cells of Daucus carota.
Planta (Berl.). 88 : 91—102.
chemical localization of pathogenesis-related pro-
teins secreted into the intercellular spaces of
salicylate-treated tobacco leaves. Plant Cell
Physiol. 29 : 1035—1040.
1987. GUS fusions: Ap-glucuronidase as a sensi-
tive and versatile gene fusion marker in higher
plants. EMBO J. 6 : 3901—3907.
1988. Targeting a foreign protein to chloroplasts
using fusions to the transit peptide of a chloro-

Fig. 2 continued
(A) Gold labels were very frequently found all over the electron-dense bodies in the central vacuoles.
The shape of the first type of these bodies was spherical or oval. They were entirely filled with the
electron-dense proteinaceous materials.
(B) High magnification micrograph of the electron-dense spherical body in (A).
(C) The second type of electron-dense bodies observed in central vacuoles. Many gold particles were
specifically found on the electron dense parts in the bodies.
(D) The third type of electron-dense structures in a central vacuole. They were small odd shape and
labeled with many gold particles. In the cytoplasm, there were only small number of gold signal on
chloroplast. The electron-dense amorphous bodies were often observed near tonoplast in the
vacuoles, as if they has just been translocated from the cytoplasm. These three types of electron
dense bodies shown in Fig. 2 A→2 D were found in a vacuole with every one to three.
Size bars indicate 1 µm.
c : chloroplast, v : vacuole, w : cell wall.