Introduction of a chimeric gene encoding an oryzacystatin-β-glucuronidase fusion protein into rice protoplasts and regeneration of transformed plants

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ABSTRACT

In order to construct transgenic rice plant with an introduced oryzacystatin (OC)-β-glucuronidase (GUS) fusion gene, we first introduced it into rice protoplasts by electroporation, together with a marker gene conferring hygromycin-resistance (pUC-HPH). In a transient assay using the transfected protoplasts, both OC and GUS activities were detected. The GUS activity was higher when the OC-GUS fusion protein was expressed than when only a single GUS protein was expressed. Next, to isolate stable transformants, hygromycin-resistant calli were selected. Forty one out of 116 hygromycin-resistant calli expressed a 2.2 kb mRNA transcribed from the chimeric gene and their extracts exhibited the activities of both OC and GUS. Finally, the transgenic calli were regenerated into rice plants whose tissues (leaves, roots and seeds) exhibited GUS activity probably derived from the fusion protein.

INTRODUCTION

Studies on establishment of rice transformation have been carried out by many groups of the world (Zhang and Wu 1988, Shimamoto et al. 1989, Terada and Shimamoto 1990, Tada et al. 1991). Recently, reports relevant to transgenic rice plants expressing useful genes have also been studied (Hayakawa et al. 1992, Toki et al. 1992, Shimamoto et al. 1993).

Oryzacystatin has a biochemical activity of inhibiting cysteine proteinases and may play a phytophysiologically important biodefense role in the seed of rice (Abe et al. 1987a, Abe et al. 1987b, Abe et al. 1988, Kondo et al. 1989). The enhancement of this protein is thus significant in an agroindustrial point of view.

In order to establish transgenic systems for development of crops with novel functions, we attempted to construct a chimeric gene encoding a OC-GUS fusion protein with two activities. GUS reporter enzyme, which has been well-defined (Jefferson et al. 1987), is easily detected for its activity and, therefore, its expression can be used as a selection marker. The use of a chimeric gene is of merit in that, when, for example, a chimeric gene A-B made of genes A and B is introduced and expressed, the resulting products, protein A and protein B, will be formed as a fusion protein A-B which is in principle a covalently fused molecule. It follows that, theoretically, both proteins should always be stoichiometrically formed at any particular sites of plant tissues. Therefore, it is possible to quantify one protein (A or B) by determining the amount of molecule of the other protein (B or A). In addition, if the activity of the one (protein B) is determined with difficulty, it is possible to estimate its activity by measuring the activity of the other (protein A). Our strategy is thus to propose a new method for getting otherwise unobtainable fruits with such stoichiometric accuracy.

The measurement of the GUS activity to be elicited after expression of the introduced OC-GUS chimeric gene would stoichiometrically reflect the possible formation of OC in various tissues of regenerated transgenic rice plants. It is also of merit to introduce the OC-GUS chimeric gene, because the use of exogeneous OC gene in such a form would facilitate to distinguish it from the endogeneous OC gene. We describe here that such an attempt is successfully made in our particular case and will be generally applicable as a new method in plant cell biology researches.

MATERIALS AND METHODS

Construction of Expression Plasmids

OC cDNA fragment encoded a 94 amino acid residues was excised from the plasmid pOC26-3' (Abe et al. 1988) by digestion with SphI and EcoRI. The fragment was then treated with T4 DNA polymerase and Klenow fragment to be blunt-ended. A plasmid to express an OC-GUS fusion protein, designated pOG-1, was constructed by insertion of this fragment into the filled-in XbaI site of pBI221 (Jefferson et al. 1987) as shown in Fig. 1.

Protoplast Isolation, Electroporation, Culture and Selection of Transformed Calli

Seeds of Oryza sativa L., cultivar Nipponbare, was used as the source of protoplasts for regeneration. Oc cells (Baba et al. 1980) from root explants were used for transient assay. Scutella of mature rice seeds were cultured in vitro in Murashige and Skoog medium (Murashige et al. 1962) containing 2 mg/l 2,4-D (2,4-dichlorophenoxyacetic acid) and calli were obtained. The callus...
the unit of inhibition of the Z-Phe-Arg-MCA hydrolyzing activity and at 370 nm (excitation). Inhibitory activity was represented as resultant extracts were preincubated at 37°C for 20 min in the buffer (pH4.3). Fluorescence was measured at 460 nm (emission) stopped by addition of 0.1 M sodium monochloroacetate in acetate and incubation was conducted for 20 rain. The incubation was

Institute, Inc., Japan) was added at a final concentration of 1 uM pH 6.0, containing 3 mM dithiothreitol and 2 mM EDTA, and the resistant calli were sonicated in 100 mM sodium phosphate buffer, containing Oc cells as the nurse (Kyozuka et al. 1987). After incubation of the protoplasts at 25°C in R2 medium, extracts they were transferred to a plastic container containing 70 ml of

were cultured in N6 liquid medium at 26.5°C by suspension culture (Chu et al.1975, Murashige et al.1962) containing 2 mg/l 2,4-D and 3% sucrose.

Protoplasts were prepared from the two-month suspension cultures, essentially as described previously (Akagi et al. 1989, Ohtsuki et al. 1988). The calli formed were incubated at 30 °C for 3 h in a solution (pH 5.6) containing 0.05% pectrime y-23 (Seisin Pharmaceutical Inc., Japan), 2% cellulase Onozuka R2 (Yakult Inc., Japan), 0.01% CaCl2, 0.1% dextran sulfate potassium salt and 9% mannitol (Takebe et al.1968). Protoplasts formed were collected by centrifugation (100 x g) after sieving through a nylon mesh (30μm) and washed twice with 0.5 M mannitol containing 0.1 mM Mg2SO4. The protoplasts were finally suspended at a population density of 2 x 106/ml in the same mannitol solution and mixed with 40 μg/ml of plasmid pOG-1, 20 μg/ml of pUC19-HPH (hygromycin B resistance) (Gritz and Davis 1983) and 20 μg/ml carrier DNA (calf thymus, Sigma). Electroporation of the suspension was done using CET-100 (Nihonbunko Inc., Japan) under the following conditions: voltage, 1000V/cm; pulse width, 0.5 msec; and pulse time, 6. The electroporated protoplasts were suspended at a density of 106/ml in R2 medium (Ohira et al. 1987) containing Oc cells as the nurse (Kyozuka et al. 1987). After they were cultured for two weeks, hygromycin B was added at a concentration of 40 μg/ml. The each resistant colony cultured in fresh N6 medium containing hygromycin B (40 μg/ml) then was transferred to hygromycin B-deprived N6 medium containing 0.01 mg/l of naphthalene acetic acid and 0.1 mg/l of 6-benzyladenine (Fujimura et al. 1985). When shoots become 4-5 cm in length, they were transferred to a plastic container containing 70 ml of hormone-free N6 regeneration medium with 0.8 % agarose.

For transient assay, Oc cells were maintained in MS medium and protoplasts were isolated from its four-day-old calli and electroporated by the same method described above. After incubation of the protoplasts at 25°C in R2 medium, extracts were prepared to measure their cystatin and GUS activities. Incidentally, the endogenous level of OC in Oc cell was 135 ± 5(units).

Measurement of Cystatin Activity

Cystatin activity was worked out by measuring the papain inhibitory activity by the method of Barrett and Kirschke (1981). Protoplasts on 1 or 2 days after electroporation and hygromycin-resistant calli were sonicated in 100 mM sodium phosphate buffer, pH 6.0, containing 3 mM dithiothreitol and 2 mM EDTA, and the resultant extracts were preincubated at 37°C for 20 min in the above buffer. The fluorescent substrate Z-Phe-Arg-MCA (Peptide Institute, Inc., Japan) was added at a final concentration of 1 μM and incubation was conducted for 20 min. The incubation was stopped by addition of 0.1 M sodium monochloroacetate in acetate buffer (pH4.5). Fluorescence was measured at 460 nm (emission) and at 370 nm (excitation). Inhibitory activity was represented as the unit of inhibition of the Z-Phe-Arg-MCA hydrolyzing activity of papain in the assay mixture. One unit of papain activity was defined as the release of 1 μmol of the MCA per 1 min. One unit of cystatin activity was shown by the inhibition of 1 unit of the papain activity.

Measurement of GUS Activity

GUS activity was measured using the substrate 4-methylumbelliferonyl-β-D-glucuronide (4-MUG, Sigma) as described by Jefferson et al. (1987). Protoplasts collected by centrifugation (120 x g for 10 min) after electroporation with expression vectors pOG-1 and pBI221 (Ohtsuki et al. 1990) were each resuspended in a lysis buffer (50mM sodium phosphate, pH7.0, 10mM EDTA, pH7.0, 0.1% Triton X-100, 0.1% Sarkosyl, 2mM DTT). GUS activities of the extracts of the protoplasts were assayed in the lysis buffer containing 1mM 4-MUG at 37°C for 30 min and the fluorescence was measured at 448 nm. The protein concentration of extracts was determined by using BCA protein assay reagent (Pierce).

Histochemical Analysis of GUS Activity

Calli, leaves, roots and seeds cut by hand were incubated at 37°C for up to 16 h in a solution containing 1mM 5-bromo-4-chloro-3-indoly-glucononide (X-gluc) (Clontech) and 50 mM sodium phosphate, pH 7.0.

Northern Blot Analysis

Total RNA was extracted from calli by a standard method (Brawerman et al. 1972). The RNA (10 μg) was denatured and subjected to electrophoresis on a formaldehyde agarose gel (Maniotis et al. 1982). The RNA was transferred onto a nylon membrane (Hybond N; Amersham) and hybridized with 32P-labeled OC or GUS DNA probe.

RESULTS

Introduction and Expression of an OC-GUS Chimeric Gene in Rice Protoplasts

Plasmids pBI221 and pOG-1, encoding GUS and OC-GUS fusion protein, respectively, were each introduced into the protoplasts, and the GUS activities of the protoplast extracts were measured. As Fig. 2 shows, GUS activity was distinctly detected after transfection and reached a plateau 24 and 48 hr after introduction of pOG-1 and pBI221, respectively. The GUS activity was constantly higher in protoplasts transfected with pOG-1 than in those transfected with pBI221. On the other hand, OC activities represented as cystatin activities were also distinctly enhanced in pOG-1-introduced protoplast

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**Fig. 2.** Temporal change of GUS activity after the introduction of expression plasmids. GUS activities were measured after introduction of pOG-1 and pBI221. Each plot is due to an average ± standard errors.