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EVALUATION OF TRANSGENIC INDICA RICE (*Oryza sativa* L.) THROUGH REPORTER AND DESIRED GENE

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ABSTRACT

Rice is one of the most important food crops in the world. It is greatly affected by various abiotic stresses. Among them, to improve the complex stress salinity we introduced a desired gene *kat*E, a catalase gene of *Escherichia coli*, and reporter gene GUS into the indica rice cultivar Kasalath. Plant survival under different salt water concentration was checked. Transformation was carried out using *Agrobacterium tumefaciens* strain EHA101 harboring a binary vector pIES6/Hm/*kat*E and pIG121/ Hm/ GUS which contains genes for catalase *kat*E, GUS gene, hygromycine resistance gene HPT and kanamycine resistance gene NPTII in the T-DNA region. Transformation was confirmed by PCR with *kat*E and GUS primer. Transgenic plants at very young stage (3 days) were able to grow up to 15 days in 100 mM NaCl solution and 7 days in 250 mM NaCl solution where as non transgenic plants could not survive even up top 5 days in 100 mM condition and 7 days in 250 mM NaCl concentration. Twenty eight days matured plants could survive and were able to form iflorescence. Here a single gene introduction significantly improved the salt tolerance of this crop rice.

Key words: Rice (*Oryza sativa* L), *Agrobacterium tumefaciens*, transformation, reporter gene, acetosyringon, binary vector.

INTRODUCTION

Rice productivity is greatly affected by a complex trait salt stress. This is regulated by various mechanisms. Organisms that thrive in hyper saline environments possess different mechanisms to adjust internal osmotic status. One of the such mechanisms is the ability to accumulate low molecular organic compatible solutes such as sugars, some amino acids and quaternary ammonium compounds, which are believed to be essential for adaptability of plant cells to high salinity (Bohnert *et al*., 1995).

The mechanisms of adaptation to salt stress are the exclusion of Na cations from the sodium sensitive sites which has been proposed as a function a Na^+/H^+ antiporter and Na^+ ATPase (Serrano, 1996). Salt loading also induces oxidative stress. Upon salt stress, the CO_2 fixation rate may be decreased causing the over reduction of the photosynthetic electron transport chain and the production of active oxygen species such as singlet

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oxygen ($^1O_2$*), super oxide anion ($^{\cdot}O_2$-), hydrogen peroxide (H_2O_2) and hyhdroxyl radical ($^{\cdot}OH$). H_2O_2 is the most stable active oxygen species, but produces the most reactive oxygen species, $^{\cdot}OH$, if H_2O_2 is present together with O_2- and Fe^{2+}.

The best documented source of H_2O_2 in photosynthetic organisms is O_2- generated by Mehler reaction. Therefore, the decomposition of H_2O_2 is most pivotal in the protection of the cells from oxidative damage. Early report of the effect of overexpression of the catalase gene on the oxidative stress as well as on the salt stress in cyanobacteria indicated that, the quenching of H_2O_2 was an important factor for salt tolerance (Kaku *et al*., 2000). Catalase is an antioxidant enzyme which has the more stable and difficult to denature character than the other antioxidants like ascorbate peroxidase. Normally catalase is expressed in microbody, mitochondria and chloroplast but not in the cytosol. Catalase breaks down hydrogen peroxide and produces H_2O and usually O_2 which helps for the survival of the plants.

The present research was conducted, to transform recalcitrant indica variety (Kasalath) with a gene encoding catalase, *kat*E, derived from *E. coli* which decomposes H_2O_2 (Kaku *et al*., 2000) and reporter gene GUS. Salt tolerance of transgenic plants in different growth stages between transgenic and nontransgenic plants were investigated.

Material and Methods

Plant material and bacterial strain

Indica rice (*Oryza sativa* L) Kasalath was used for the present study and mature seed was used as explant. Transformation was conducted by using *Agrobacterium tumefaciens* strain EHA101 (Hood *et al*. 1986) harboring the vector pIES6/Hm/*kat*E and pIG121/Hm/GUS. Binary vector contains genes for catalase *kat*E, GUS gene, hygromycine resistance gene HPT and kanamycine resistance gene NPTII in the T-DNA region. This *Agrobacterium* strain was provided from Dr. Elizabeth E. Hood of Washington University and *kat*E gene was provided by Dr. Tetsuko Takabe of Nagoya University, Japan. The vector posses an enhancer region from CaMV promoter and connected to the 35S promoter of cauliflower mosaic virus, there by helping for expression (Fig. 1 a & b).

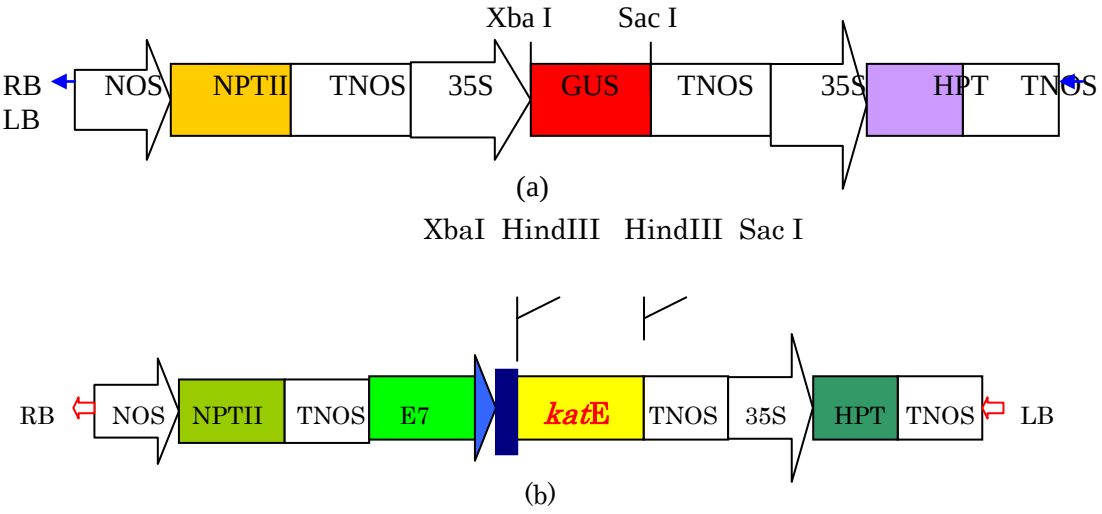

Fig.1. Schematic diagram of the constructed vector

Ttransformation and culture condition

Rice transformation was conducted using the established methods of Hiei et al (1994) and Toki (1997). Callus was induced from indica rice (*Oryza sativa* L) seeds using N6 medium supplemented with 2mg/l 2,4-D. Two weeks later induced calli, were infected with *Agrobacterium tumefaciens* EHA101 carrying pIG121/Hm/*kat*E and pIG121/ Hm/GUS.

After three days of co culture, callus was sterilized with 500 mg/l carbenicillin and transferred to N6 selection medium containing 50 mg/l hygromycin and 500 mg/l carbenicillin for two weeks. Selected, calli were transferred to MS hormone free medium for regeneration. Regenerated plantlets were acclimatized in pots (15 cm diameter) with soil and submerged with water in a growth incubator at 30°C (light phase) and 25°C (dark phase) with 12 hours light at 71 m mole m^{-2} s^{-1} photon flux density and 62 % relative humidity. Progenies were obtained from those transgenic plants by selfing under the same condition as mentioned above.

PCR analysis

Genomic DNA was extracted from young leaf tissues of T_0 transgenic and non transgenic control plants. PCR was performed in a reaction mixture containing about 25 ng plant genomic DNA, 100 µ mole dNTP, 0.2 µ mole of each primer and 1 U of *Taq* polymerase (Takara, Japan). PCR analysis was carried out under standard condition with 30 second denaturation, 40 second annealing, 50 second extension at 94°C, 50°C and 72°C respectively, for 30 cycles. The sequence of the PCR primers for *kat*E gene were as follows: 1165U (CCACCAAgTTCTATACCgAAgAgg) and 1165 L (gTgATATTCAgCTggTCgTCAgTC) and for GUS gene was GUS-4 (AGGCTGTAGCCGACGATG) and GUS-F (ATCACCGAATACGGCGTGGA).

Observation of salt tolerance

Transgenic and non transgenic plants at different stages were used to evaluate the salt tolerance. One was at very young stage (3 days after root formation) another was 4 weeks after root formation. After acclimatization, T_0 transgenic plants and wild plants were soaked in 0 mM, 50mM, 100mM, 150 mM, 200 mM, 250 mM and 300 mM (half of the sea water level) concentrations of sodium chloride solution. For each sodium chloride concentration, at least two plants were used.

RESULTS AND DISCUSSION

We could transform recalcitrant Indica rice cultivar Kasalath with the *kat*E gene that was derived from *E. coli* and designed to be expressed under the control of the 35S RNA promoter of the cauliflower mosaic virus with E7 enhancer and with GUS gene.

In Kasalath transformation with the vector *pIES6kat*E and pIG121/ Hm/GUS were successful where T_0 transformants and their progeny were investigated. We infected the calli with binary vector which were derived from a most common binary vector, pBI121 (Ohta *et al*., 1990). Rashid *et al.* (1996) used the same vector to obtain transgenic Basmati rice. In some reports, a super binary vector, in which a DNA fragment from the virulence region was introduced in to a binary vector, was more effective and easy for transformation of rice and they also described that recalcitrant cultivars can more easily be transformed by using the super binary vector (Hiei *et al.,* 1994). Hashizume *et al.* 1999,

also obtained high transformation frequencies even using conventional binary vector. In this study also conventional binary vector was used to obtain higher transformed recalcitrant Indica rice cultivars.

Transformation efficiency

Transformation frequency was 82% with the vector pIG121/ Hm/ Gus and 80% with the vector pIES6/*kat*E/Hm at 100 μM acetosyringone concentration. This report shows that, transgenic plants carrying *kat*E gene were obtained with high efficiency. These values are as high as some Japonica cultivars (Hiei *et al*. 1994) and Indica cultivar Basmati 370 (Rashid *et al*. 1996). One of the most commonly used techniques in transformation of monocotyledonous plant is the addition of phenolic compound acetosyringon (Van Wordragen *et al*. 1992). According to James et al. 1993, acetosyringon treatment has been reported to be highly effective for increased transformation. Vijayachandra *et al*. 1995 reported that, rice tissues could induce expression of *vir* genes but that induction was greatly enhanced by acetosyringon. In this experiment also addition of higher concentration of acetosyringon (100 μ M) showed better result both in transformation and regeneration than 50 μM concentration. This result shows similarity with Hiei *et al*. 1994, where they demonstrated that, acetosyringon at 100 μM is a key factor for successful transformation of rice.

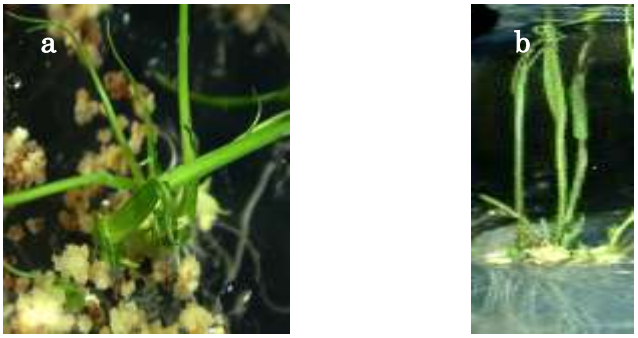

Fig. 2. (a) Regeneration (b) root formation of transgenic plants

(a) (b) PCR with short *kat*E primer (1165 bp)

Fig.3. PCR amplification from the genomic DNA of transgenic Kasalath plants showing expected (a)GUS (between 310 and 610 bp), (b) *kat*E fragment at 1165 bp. M, marker *Hae* III; C control; P positive control; N negative control.

PCR analysis

We have detected the insertion of *kat*E and GUS gene by PCR from randomly selected regenerated T_0 plants (Fig. 3 a, b). This result shows that, transgene was proven to be integrated in all transformants examined.

Salt tolerance of transgenic plants

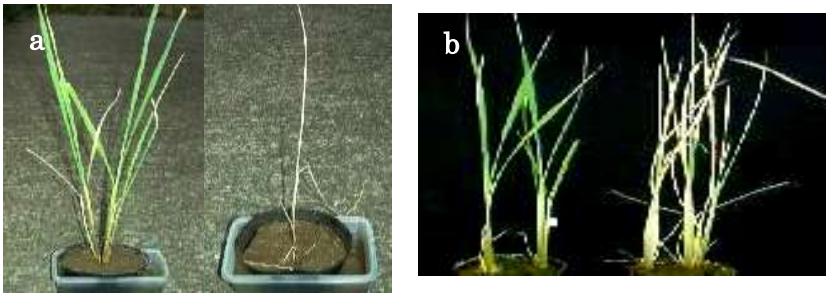

Fig. 4. (a) T_0 plants under 150 mM NaCl concentration and photograph was taken 15 days after treatment (b) T_1-5 plants, 10 days after salt water treatment at 250 mM NaCl solution

Table 1. Salt tolerance test of T_1 plants with *kat*E gene and T_o plants with GUS gene.

In 50 mM conc.	Days after treatment									
	3	6	9	10	13	15	18	21	25	30
Non tr. Kasalath	+++	+++	+++	+++	+++	+++	+++	++	++	+
T_1 - L-5	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
T_1 - L-11	+++	+++	+++	+++	+++	+++	+++	++	++	++
Kasalath by GUS*	+++	+++	+++	+++	+++	+++	+++	++	+	+
In 100 mM conc.	Days after treatment									
	3	6	9	10	13	15	18	21	25	30
Non tr. Kasalath	+++	+++	++	++	+	-	-	-	-	
T_1 - L-5	+++	+++	+++	+++	++	++	++	+	+	-
T_1 - L-11	+++	+++	+++	+++	++	++	++	+	+	-
Kasalath by GUS*	+++	+++	+++	++	+	+	+	-	-	-
In 250 mM conc.	Days after treatment									
	3	6	9	10	13	15	18	21	25	30
Non tr. Kasalath	++	++	+	-	-	-	-	-	-	-
T_1 - L-5	+++	+++	++	++	+	+	+	-	-	-
T_1 - L-11	+++	+++	++	++	+	+	+	-	-	-
Kasalath by GUS*	++	++	+	+	-	-	-	-	-	-

+++, All leaves are green good condition; ++, some portion of leaves are bleached; +, maximum portion of leaves are bleached; - almost dead and * transformed Kasalath with GUS gene but not with *kat*E gene

We evaluated T_0 transgenic and non transgenic plants for salt tolerance. At 100 mM sodium chloride solution, 3 days aged plants survived until 15 days and control plants survived until 4 days. Same aged transgenic plants at 250 mM concentration until 8 days where as control plants died at 2^{nd} days. T_1 plants could grow for more than 14 days in the presence of 100mM sodium chloride solution and and in the presence of 250 mM concentration plnts could survive up to 18 days (Fig. 4). On the contrary nontransgenic rice plants could not survive even in the presence of 50 mM sodium chloride for more than 7 days and no seed were formed.

Many efforts have been made to produce salt tolerant rice plants by introduction of genes encoding proteins involved in protecting plants from environmental stress such as

chloroplastic glutamine synthase (GS2), betain aldehyde dehydrogenase, calcium dependant protein kinase (CDPK) and others. Transformed rice plants with GS2 gene could survive for 2 weeks at 150 mM sodium chloride (Hoshida *et al*. 2000). A gene encoding betaine aldehyde dehydrogenase, an enzyme involved in the synthesis of glycine betaine (CodA) produce a compatible solute regulating internal osmotic balance was introduced into rice genome and transgenic rice plants could survive 1 week at 150 mM sodium chloride (Sakamoto *et al*. 1998) A gene encoding CDPK was introduced to the rice plant and the transformed rice plants could survive for 3 days at 200 mM sodium chloride (Saijo *et al*. 2000). However, salt tolerance transgenic rice plants, which have been reported so far, have been able to grow in 150 mM sodium chloride solution for 2 weeks at maximum. Transgenic rice plant with *kat*E gene derived from *E. coli* as reported here, were able to form inflorescence in 100 mM or lower concentration of NaCl. They could survive for one month in 150 mM and for 20 days in 200 and 250 mM NaCl concentration.

Salt tolerance of T_1 plants from two lines seemed to be slightly weaker than that of T_0 plants. They survived for 25 days in 100 mM and for 18 days in 250 mM concentration of NaCl. So far reported here, this is one of the strongest salt tolerant Indica rice plants that could form inflorescence.

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