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Improved Salt Tolerance and Morphological Variation in Indica Rice (*Oryza sativa* L.) Transformed with a Catalase Gene from *E. coli*

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Key words: Rice, Oxidative stress, Enhanced salt tolerance, Transformation, Morphological variation

Abstract

In an attempt to improve the salt tolerance of rice, we introduced *kat*E, a catalase gene of *Escherichia coli*, into the indica rice cultivar Kasalath. Transformation was carried out using *Agrobacterium tumefaciens* strain EHA101 harboring a binary vector pIES6/Hm/*kat*E which contains genes for catalase *kat*E, hygromycine resistance gene HPT and kanamycin resistance gene NPTII in the T-DNA region. With the inclusion of acetosyringone, higher amount of transgenic cells and regenerated plants were obtained. Transformation was confirmed by PCR with *kat*E and HPT primer. Transgenic plants at a very young stage (three - four days) were able to grow up to 15 days in 100 mM NaCl solution and seven days in 250 mM NaCl solution whereas control plants died within five days in 100 mM and seven days in 50 mM NaCl. Plants stressed for four weeks could survive for a long time and were able to flower. Different morphological characters varied in transgenic compared to control plants. Introduction of *kat*E gene significantly improved the salt tolerance of the transgenic indica lines which could mature and set seed under stress.

Introduction

Salinity is one of the major abiotic stresses that cause severe loss of rice production. Salt tolerance is a complex trait because it is regulated by various mechanisms. Organisms that thrive in hyper saline environments possess different mechanisms to adjust the internal osmotic status under stress. One mechanism 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).

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Another mechanism is the the ability of some plants to sequester. Na cations away from sodium sensitive sites, by proteins such as the Na^+/H^+ antiporter and Na^+ ATPase (Serrano 1996). Salt accumulation also induces oxidative stress. Enzymes that can remove the reactive oxygen species (ROS) produced by the salt stress play an important role in tolerance. Thus quenching of H_2O_2 was an important factor for salt tolerance as observed in cyanobacteria (Kaku et al. 2000). Nagamiya et al. in 2007 were able to produce salt tolerant japonica rice (at 100 mM salt concentration) by over expressing the catalase gene *kat*E. Catalase is one of the major and stable antioxidant enzymes which break down hydrogen peroxide producing H_2O and O_2, helping plants to survive. Normally catalase is expressed in microbodies, mitochondria and chloroplasts but not in the cytosol. This research was conducted to transform the recalcitrant indica rice Kasalath, with the *E. coli* derived *kat*E gene (Kaku et al. 2000). Salt tolerance of the transgenic plants at different growth stages and their morphological variation has been reported and compared with control plants.

Materials and Methods

Plant material and bacterial strain: Oryza sativa L. indica cultivar Kasalath was used in this research and mature seeds were used as explant. The vector was constructed by Nagamiya et al. 2007 and the vector pIES6/*kat*E/Hm was transformed into *Agrobacterium tumefaciens* strain EHA101 by electroporation. Transformation was carried out by using the *Agrobacterium tumefaciens* strain EHA101 harboring the vector pIES6/Hm/*kat*E (Hood et al. 1986). This is a binary vector that contains genes for catalase *kat*E, hygromycin resistance gene HPT and kanamycin resistance gene NPTII in the T-DNA region.

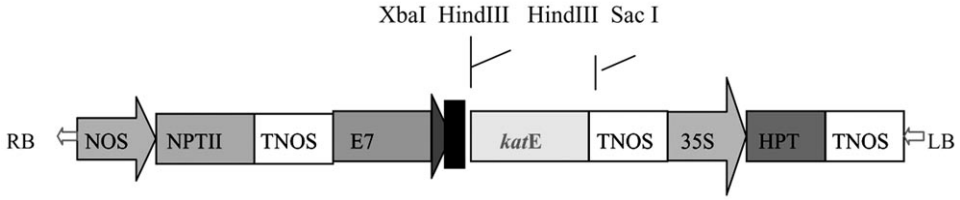


Fig. 1. Schematic diagram of a part of the T-DNA region of plant expressing vector pIES6*kat*E/Hm. RB, right border; NOS, nopaline synthatase promoter; NPTII, neomycin phosphotransferase II; TNOS, terminator of nopaline synthatase; 35S, Cauliflower mosaic virus promoter; E7, seven enhancer region (– 290 to – 90) from CaMV promoter; *kat*E, catalase *kat*E gene; HPT, gene for hygromycin phosphstransferase; LB, left border.

Transformation and culture condition : Transformation of rice was conducted using the established methods of Hiei et al. (1994) and Toki (1997). Callus was induced from mature rice seeds on N6 medium supplemented with 2 mg/l 2,4-D. After two weeks induced calli were infected with *Agrobacterium tumefaciens* EHA101 carrying pIES6*kat*E/Hm. After three days of co-culture with the inclusion of acetosyringone, callus was sterilized with 500 mg/l carbenicillin and grown in N6 selection medium containing 50 mg/l hygromycin and 500 mg/l carbenicillin for two weeks. Resistant calli were transferred to hormone-free MS for regeneration. Regenerated plantlets were acclimated 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.

PCR analysis: Genomic DNA was extracted from young leaf tissues of T_0 transgenic and 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, 50 and 72°C, respectively for 30 cycles. The sequence of the PCR primers were as follows: 1165U (CCACCAAGT TCTATACCGAAGAGG) and 1165 L (GTGATATTCAGCTGGTCGTCAGTC).

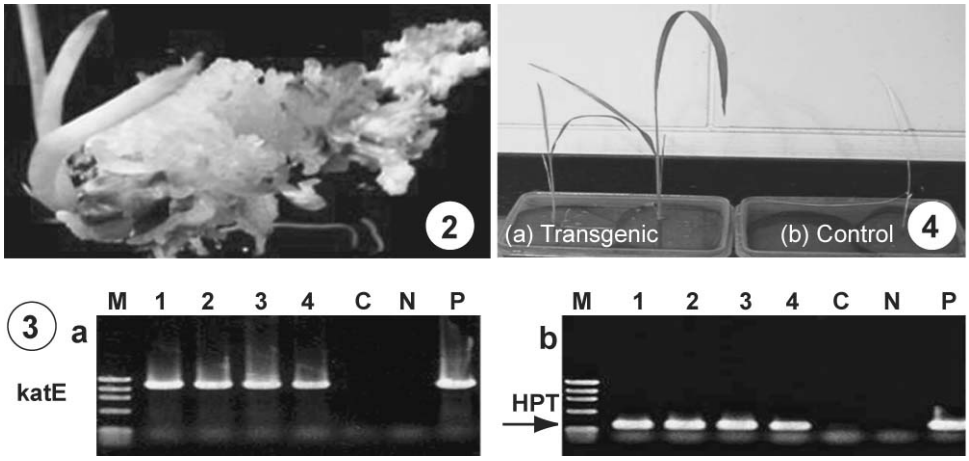
Morphological variation and salt stress study: Mature salt water treated transgenic plants were checked for various morphological characters comparing with the non-transgenic plants such as plant height, first leaf length, root length, number of panicles per hill, panicle length, number of spikelets per panicle, filled grain percentage etc. together with control plants. Relative plant height was also measured.

Transgenic and non-transgenic plants at two different stages were used to evaluate salt tolerance. In first stage after acclimatization of T_0 transgenic and wild plants were grown for three days in pot soil and then in 0, 50, 100, 150, 200, 250 and 300 mM (half of the sea water level) of sodium chloride solutions were added. In case of second growth stage acclimatized T_0 transgenic and control plants were grown for four weeks in pot soil and then they were treated with the above mentioned solutions. For each concentration, at least two plants were used. Water was added untill the mark on vessels every two days to maintain the same concentration of NaCl in the vessels. T_1 plants were also used for salt water test.

Results and Discussions

Transformation and regeneration with PCR analysis: We transformed the recalcitrant Indica rice cultivar Kasalath with the *kat*E gene derived from *E. coli*. The gene was expressed under the control of the 35S RNA promoter of the cauliflower mosaic virus with E7 enhancer. We infected the calli with a binary vector *pIES6kat*E which was derived from the 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 more effective for transformation of rice. Hiei et al. (1994) described that recalcitrant cultivars can be more easily transformed by using this super binary vector. However, Hashizume et al**.** (1999) obtained high transformation frequencies by using a conventional binary vector. In this study a conventional binary vector was used to obtain transformants from recalcitrant indica rice cultivars.

Transformation and regeneration frequencies were 80.0 ± 2.9 and 51.4 ± 7.9%, respectively. This was obtained using the 100 µM acetosyringone in comparison with 50 µM concentration. We have detected the insertion of *kat*E and HPT genes by PCR from randomly selected T_0 plants (Fig. 3 a, b). The results showed the presence of transgene in all transformants examined and transgenic plants carrying *kat*E gene were obtained with high efficiency. At 100 µM acetosyringone concentration, transformation and regeneration values were as high as some japonica cultivars (Hiei et al. 1994) and the indica cultivar Basmati 370 (Rashid et al. 1996). According to James et al. 1993, acetosyringone treatment has been reported to be highly effective for increased transformation. In this experiment also addition of higher concentration of acetosyringone (100 µM) showed better results both transformation and regeneration.



Figs. 2-4: 2. Regeneration of transgenic calli on MS regeneration medium. 3(a) PCR analysis of T_0 Kasalath plants for presence of *kat*E gene (1165 bp) and (b) HPT gene (350 bp). M, for marker λHind III and Hae III; Lane 1-4, transgenic plants, C, control; N, negative control and P for

positive control. 4(a) three-day-old T_0 plants and (b) control plant in 100 mM NaCl solution five days after salt water treatment.

Phenotypic characterization and salt tolerance: Various morphological traits of transgenic plants were observed at the time of maturity in comparison with non-transformants. As shown in Table 1, transformants had shorter plant height, leaf , root and panicle length, smaller panicle and spikelet number and seed fertility compared to the non-transformants. Some transgenic lines without salt water treatment showed seed fertility from 62 to 97% and control plants showed seed fertility from 92 to 98% (data not shown).

Table 1. Phenotypic variation of transgenic and non-transgenic Kasalath plants.

Character evaluated	Transgenic plants	Non-transgenic plants
Plant height (cm)	103.0 ± 3.5	111.7 ± 3.2
First leaf length (cm)	8.9 ± 0.6	11.1 ± 1.7
Root length (cm)	13.8 ±1.3	13.06 ±1.4
No. of panicle/hill	3 ± 0	3 ± 0
Panicle length (cm)	22.6 ± 0.6	24.1 ± 0.9
Filled grain %	89.2 ± 3.4	95 ± 5.1
Awn	Some are awned	Mostly awned

*Four to six plants were tested for the experiment.

We evaluated T_0 transgenic and non-transgenic plants for salt tolerance. At 100 mM sodium chloride solution, plants at young stage (three days grown on pot soil) survived until 15 days and control plants for four days (Fig. 4).

Same aged transgenic plants at 250 mM concentration survived up to eight days whereas control plants died on the second day. Four weeks grown transgenic plants were treated with salt water where T_0 plants could form inflorescence at 100 mM and could grow 25 days in 250 mM sodium chloride (Table 2). On the contrary wild rice plants could not survive even in the presence of 50 mM sodium chloride for more than seven days and no seed was formed.

Table 2. Salt water treatment of transgenic (after four weeks grown on pot soil) and wild rice plants.

In 100 mM conc.	Days after treatment									
	3	6	9	10	13	15	18	20	25	35
Wild plants	+++	+++	++	++	+	-	-	-	-	
T_o	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
In 250 mM conc.	Days after treatment									
	3	6	9	10	13	15	18	22	25	35
Wild plants	++	-	-	-	-	-	-	-	-	-

T_o	+++	+++	+++	+++	+++	++	++	++	+	-

+++ = All leaves are green at good condition, ++ = Some portion of leaves are bleached,
+ = Maximum portion of leaves is bleached, - = Almost dead.

At the time of salt water treatment relative plant height was measured. Transgenic plant height at 200 mM increased and survived up to 20 days but the height of the control plants was lower and on the ninth day their growth stopped and died at the 12th day (Fig. 5).

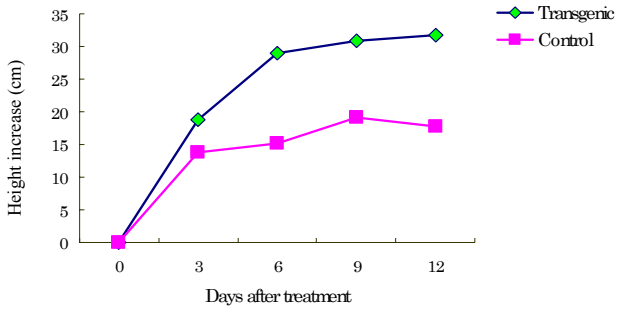


Fig. 5. Increase of height after treatment of T_0 plants under 200 mM NaCl conc..

A good deal of efforts have been made to develop 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) etc. Transformed rice plants with GS2 gene could survive for two 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) that could produce a compatible solute regulating internal osmotic balance was introduced into rice genome and rice plants could survive one week at 150 mM sodium chloride (Sakamoto et al. 1998). Another gene encoding CDPK was introduced to rice plants where transformed rice plants could survive for three days at 200 mM sodium chloride (Saijo et al. 2000). In cyanobacteria, the introduction of a catalase gene *kat*E from *E. coli* was found to reduce ROS production under salt stress and to confer salt tolerance (Kaku et al. 2000). However, salt tolerant transgenic rice plants, which have been reported so far, were able to form seeds in japonica transformants at 100 mM NaCl at the maximum (Nagamiya et al. 2007). Transgenic indica rice plants with *kat*E gene derived from *E. coli* as reported here, were able to form inflorescence in 100 mM or lower concentration of NaCl at different growth stages. They could survive for one month in 150 mM and for 20 days in 200 and 250 mM NaCl. Here, we report the expression of *kat*E gene and its salt resistance effect in T_0 indica rice plants with their phenotypic variation.

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