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Improved salt tolerance of jute plants expressing the *katE* gene from *Escherichia coli*

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Abstract: There is an urgent need for developing crops with greater tolerance to environmental stresses. This is even more important for fiber crops, which are being pushed to the marginal low-productive lands in order to make more room for food crops. Jute is the source of a highly versatile and environmentally friendly natural fiber, and is second only to cotton in terms of production and variety of uses. In this study, we used a tissue culture independent method for introducing a CMV 35S promoter driven *katE* gene from *Escherichia coli* K12 into a popular jute cultivar (*Corchorus olitorius* var O-72) in Bangladesh. Molecular analysis of the transgenic plants using PCR, reverse transcription PCR, and Southern blot confirmed the insertion of the katE gene into the jute genome and its successful expression. Salt stress regimens (150 mM NaCl) showed that transgenic plants were more tolerant as compared to wild plants.

Key words: Jute, salt tolerance, *katE*, fiber crop, transgenic

1. Introduction

High salinity causes ion imbalance and hyperosmotic stresses, which alter plant metabolism by affecting the content and activities of many enzymes (1). As a secondary effect of persistent salinity, oxidative damage may occur (1). To survive, plants have to synthesize carbohydrates through highly energetic light-dependent reactions of photosynthesis. Excess light, especially in combination with other environmental stresses, interferes with this oxygenic photosynthesis and can sometimes be a major threat to plant survival (2,3). High light intensity disrupts the flow of electrons through photosystem II (PSII) by damaging its D1 subunit (4,5). Plants, however, repair the photo-damaged D1 component by rapidly replacing it with a newly synthesized subunit (6–8). Like other stresses, salinity can complicate this scenario by interfering with the repair process of PSII (9). The influx of sodium ions inside the plant cell initiates a chain of events, including inhibition of CO_2 fixation, over-reduction of the electron transport chain, and oxidative stress leading to reactive oxygen species (ROS)-induced inhibition of PSII repair (10). High sodium concentration can also affect seed germination, slow down plant development, cause leaf senescence, and eventually decrease crop yield and quality (11,12).

Among abiotic and biotic stresses, the increase in soil salinity is the most alarming. Of the 230 million ha of global irrigated land, 20% (45 million ha) is affected by salinity (13), and this situation is aggravated by the global rise in sea water levels.

Jute is considered to be the second most important natural fiber after cotton in terms of cultivation and usage. It is mainly grown in eastern India, Bangladesh, China, and Burma. Until recently the fiber was used mostly as a packaging material. With the increasing global awareness of a sustainable environment and with the expanding diversified application of jute fibers, the demand for jute is on the rise. However, jute, being a self-pollinating dicot with only 2 commonly cultivated species (*Corchorus olitorius* and *C. capsularis* (14)), has limited genetic diversity (15) and is severely susceptible to most biotic and abiotic stresses (16–18). As arable lands decrease gradually due to high population pressure, jute is being pushed constantly to marginal and sub-marginal lands. Development of salt-tolerant jute varieties will allow cultivation of this useful fiber crop in these inhospitable

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terrains. In order to develop such a jute variety, we have augmented an ROS scavenging pathway commonly used by plants and many other organisms.

Tolerance to salinity varies from plant to plant, and this variation is greater in dicotyledonous species (19). Plants have developed various mechanisms to cope with ROS-induced damages, such as changing the leaf morphology and pigment composition, and increasing the expression of ROS-detoxifying enzymes (20,21). The introduction of an ROS-scavenging enzyme like the bacterial *katE* gene into tobacco and rice was found to reduce the production of ROS under salt stress and ensure salinity tolerance. Here we report the enhancement of salinity tolerance in a transgenic *C. olitorius* (var O-72) plant that expresses the *E. coli* (K-12) *katE* gene downstream of a constitutive CaMV 35S promoter.

2. Materials and methods

2.1. Construction of entry and destination vectors

The *katE* gene was amplified from *E. coli* (K-12) genomic DNA in a GeneAmpR PCR System 9700 (Applied Biosystems) using gene-specific primers (Table) after the following thermal cycling profile: initial denaturation at 95 °C for 4 min, 35 cycles of denaturation at 95 °C for 50 s, annealing at 60 °C for 1 min, extension at 72 °C for 1 min 30 s, and a final elongation step at 72 °C for 10 min. The amplified fragment was inserted in a pCR/GW/TOPO TA® cloning vector (Invitrogen) following the supplier's instructions and subsequently introduced into chemocompetent *E. coli* (DH5α) cells. The presence of the insert in the transformed *E. coli* was confirmed by nested PCR with NestF and NestR primers (35 cycles, 95 °C for 50 s, 52 °C for 30 s, and 72 °C for 1 min). To check the orientation of the inserted gene, the plasmid was amplified with 2 primer pairs, NesF and NesR, and sequenced (Table). This entry vector was recombined with the pH7WGF2 binary vector using LR clonase (Invitrogen) according to the instructions of the supplier (22).

Table. List of primers used in this study.

Name of primer	Sequence (5′–3′)
catalase For	GTTCAATGTCGCAACATAACGAAAAGA
catalase Rev	CTATGTAAATCATTTGAGGCGGCGCAAT
NesF	ACGTTTCCACTGGAAACCACTGGC
NesR	GCACCAGTTCTTCCGGGATAAGTT
Hyg For	GATGTTGGCGACCTCGTATT
Hyg Rev	GCGAAGAATCTCGTGCTTTC

2.2. *Agrobacterium*-mediated transformation of jute plant

A. tumefaciens (LBA4404) carrying the pH7WGF2 vector (Figure 1) harboring the *katE* gene and spectinomycin-resistance gene was grown overnight on yeast mannitol broth (YMB) medium containing spectinomycin as the selective agent at 28 °C. Bacterial concentration was determined by a spectrophotometer at a wavelength of 600 nm. Jute (*C. olitorius* var O-72) transformation was performed following a protocol established in our lab (23). Seeds from the mature plants (T_1 jute seeds) were grown on MS medium containing hygromycin (25 mg/L) as the selective agent. For comparison, seeds from non-transgenic control plants from the same species were grown on the same medium. These died within a week after germination.

2.3. PCR analysis

Genomic DNA was isolated from control (non-infected) and T_2 transgenic plants by the CTAB method (24). The presence of the newly introduced sequences in the genomic DNA was confirmed by PCR with Hyg For and Hyg Rev primer pairs (Table) using a thermal cycling profile of 35 cycles of 95 °C for 40 s (denaturation), 60 °C for 50 s (annealing), and 72 °C for 50 s (extension).

2.4. Reverse transcription PCR (RT-PCR) analysis

Total RNA was extracted from the T_2 transgenic plants and the untransformed control plants by the acid guanidinium thiocyanate-phenol-chloroform extraction method (25), and first-strand cDNA was synthesized using reverse transcriptase (Superscript II; GIBCO BRL) and oligo-dT primers. Expression of the transgenes was checked by RT-PCR using primers for catalase (Catalase For and NesR) and hygromycin (Hyg For and Hyg Rev) (Table).

2.5. Southern blot analysis

In order to verify the presence of the *katE* gene and to determine its copy number in the T_2 plant genomes, 15 µg of gDNA was digested with *Eco*RI and detected by

Figure 1. Schematic representation of the constructed destination vector pH7WGF2 (LB left border; RB right border; p35S CMV promoter; *katE* catalase gene; Egfp enhanced green fluorescent protein; Hyg and SpR hygromycin and spectinomycin resistance genes).

Southern blot analysis using an immunologic detection protocol (DIG Nucleic Acid Detection Kit, Roche). A 602 bps product of the hygromycin (*hyg*) resistance gene was amplified, labeled with digoxigenin-dUTP using a DIG DNA labeling Kit (Roche), and used as a probe.

2.6. Leaf disk assay

Leaf disk assay was carried out to evaluate the sensitivity of the transformed and untransformed jute plants to sodium chloride (NaCl) stress as described by Fan et al. (26). Fully developed healthy leaves of wild-type and transgenic T_2 plants (of similar age, about 50 days old) were washed with distilled deionized water. Leaf disks (~1 cm diameter) were excised and floated on 7 mL of 250 mM NaCl solution and sterile distilled water (used as an experimental control) for 10 days. The same were kept under continuous white light at 25 °C. The effect of salt treatment on leaf disks was observed by monitoring phenotypic changes.

2.7. Salinity stress and transgenic plants

Seeds from previously selected T_1 transgenic plants (selection was carried out in hygromycin selection medium) were transferred to earthen pots and grown in a greenhouse. Two weeks after transfer to soil, plants were watered with a 150 mM NaCl solution once every 3 days. The relative growth of the seedlings in the presence of continuous salt stress was monitored until the control plants died.

3. Results

In this study, we transformed a high yielding jute variety (*C. olitorius* var O-72) with the *E. coli* (K-12) *katE* gene using a previously developed tissue culture independent transformation method apposite for recalcitrant plants like jute. The gene was cloned in the pH7WGF2 binary vector under the control of a constitutive promoter (35S promoter of cauliflower mosaic virus). Young plants (*C. olitorius* var O-72, 5 weeks old) were infected with *A. tumifaciens* LBA4404 harboring the construct. Seeds from these plants were transferred to a hygromycin selection media and later evaluated for *katE* expression, function, and salt tolerance.

3.1. Molecular characterization of transgenic jute lines overexpressing the *katE* gene

The catalase *katE* gene (GenBank accession number NC_000913) was previously characterized in rice for salt tolerance. The aim of this study was to investigate the overexpression effect of this gene in jute. For this purpose, full length ORF of *Kat E* was cloned in the binary vector pBI121 under the control of a constitutive promoter (p35S CAMV) (Figure 1). After *Agrobacterium*-mediated transformation of jute plants and selection with hygromycin, several transformants were identified. These transgenic lines were grown up to the T_2 generation, from which plants were isolated for further analysis.

Integration of the *katE* gene into the jute genome was confirmed by PCR analysis. DNA was isolated from both non-transgenic and transgenic plants and PCR was performed using *hyg* specific primers. The desired amplification was observed in all transgenic plants, whereas no amplification was observed in the untransformed jute plants (Figure 2).

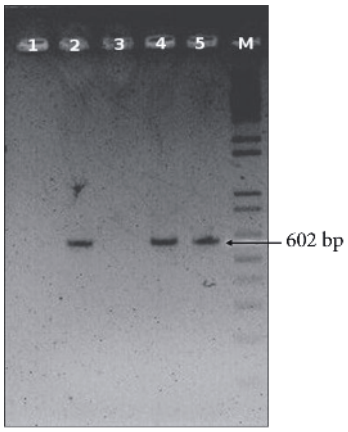

Figure 2. PCR result confirming the presence of transgene in T_2 Jute plant genome. Lanes 2, 4 and 5 for genomic DNA from different T_2 plants. Lanes 1 and 3 for genomic DNA isolated from non-transformed jute plants (taken as negative controls). Lane M for reference 1 kb plus ladder.

To determine the inheritance of the *katE* gene from T_0 to the subsequent progenies and to estimate the copy number of the transgene integrated into the jute genome, Southern blot analysis using the hygromycin gene sequence as a probe was carried out. The copy number of the integrated *katE* gene in different transgenic plant genomes ranged from 1 to 2 among the tested T_2 plants (Figure 3). The observed hybridization signal pattern was

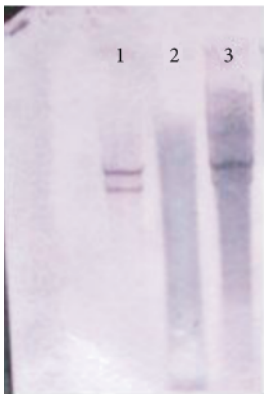

Figure 3. Southern blot analysis of transgenic jute plants (T_2) probed for the *hyg* gene; 15 µg DNA was digested with Eco R1 and probed with a 602 bp *hyg* gene fragment. Lanes 1 and 3 contain digested DNA from T_2 generation plant; Lane 2 contains digested DNA from non-transgenic plants.

different for the 3 plants in the blot, indicating independent integration events at different locations of the genome for each plant. However, no hybridization signal was detected in untransformed control plants.

Expression of the transgene in engineered plants was confirmed by RT-PCR with mRNA isolated from leaves of the transgenic and the control plants. A 1101bps amplification product of the *katE* gene was detected only in the transgenic lines (Figure 4a). A 602 bps product in RT-PCR with *hyg*-specific primers also indicated that both the genes (*katE* and *hyg*) were stably expressed in T_2 plants.

3.2. Increased salt resistance of jute plants expressing the *Escherichia coli katE* gene

In order to compare the relative salt tolerance, both the transgenic and non-transgenic jute plants were transferred to soil pots. After 2 weeks of growth under optimum conditions, plants were watered with 150 mM NaCl. Salt

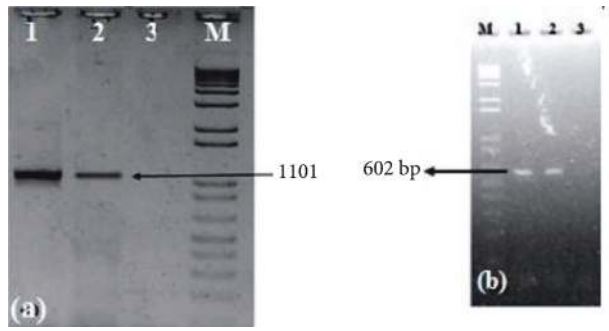

Figure 4. (a) RT-PCR analysis for expression of transgene in the T_2 generation using NesR and Catalase For primers. Lane 1 is positive control (recombinant vector); Lane 2 contains RT-PCR product of T_2 generation plants; Lane 3 contains RT-PCR product of non-transgenic jute plant. (b) RT-PCR analysis for expression of transgene in the T_2 generation using Hyg For and Hyg Rev primers. Lanes 1 and 2 contain T_1 generation plants; Lane 3 contains non-transgenic jute plant.

Figure 5. Comparison of salt tolerance between the control (non-transgenic) and T_1 transgenic jute plants at different growth stages. After 2 weeks of growth under normal growth conditions, plants were watered with 150 mM NaCl. Photographs were taken after (a) 4 weeks of salt treatment; (b) 7 weeks of salt treatment; and (c) 9 weeks of salt treatment. All non-transgenic plants died whereas transgenic plants survived (d). Transgenic plants displayed normal growth, flower formation, and seed setting.

stress provoked phenotypic changes at the whole plant level between the control (non-transgenic) and transgenic plants are shown in Figure 5. While the transgenic plants showed significant tolerance to salt stress and exhibited normal growth characteristics even after 4 weeks of 150 mM NaCl treatment, the control plants showed typical yellowing of leaves as well as stunted growth (Figure 5a). A total of 9 weeks of 150 mM NaCl treatment ultimately killed the control plants, while the transgenic plants were still growing normally without showing any noticeable change (Figure 5c). After completing their growth phase, these transgenic plants could finally move to their reproductive phase and set fruit (Figure 5d).

Leaf disk senescence assay of transformed and non-transformed plants was performed as a bioassay for the estimation of salt tolerance potential. Leaf disks from both non-transgenic and transgenic plants were floated on 250 mM NaCl salt solutions for 10 days to investigate the effect of *katE* over expression on improving the tolerance to the toxic effects of NaCl. Figure 6 reveals that the leaf disks from non-transgenic plants show extensive bleaching, which is a symptom of chlorosis (Figure 6a), while the transgenic lines appear to have considerably less damage (Figure 6b).

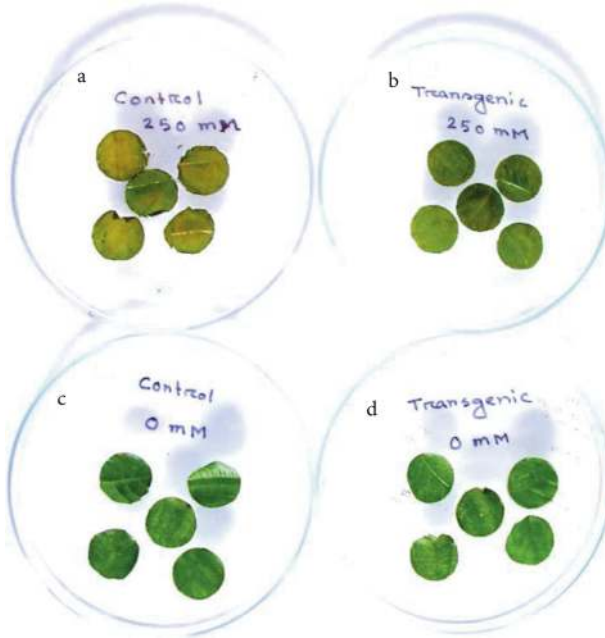

Figure 6. Leaf disk senescence assay. (a) Leaf disks from non-transgenic plant on 250 mM NaCl for 10 days and (b) leaf disks from transgenic plant in 250 mM NaCl for 10 days; (c) leaf disks from non-transgenic plant in water for 10 days served as the experimental control and (d) leaf disks from transgenic plant in water for 10 days served as the experimental control.

4. Discussion

Our present work shows that insertion and overexpression of the *E. coli katE* gene in jute plants improve tolerance to salt stress. Wild-type plants display progressive chlorosis, reduced leaf area, and general growth inhibition when treated with high salt concentrations. Although salinity tolerance is a multigenic trait, considerable progress has been achieved in developing salt-tolerant plants by introducing transgenic approaches using a single gene (27,28). A considerable number of the reports on the transgenic improvement of plant salt tolerance involved detoxifying genes, namely, catalase (29), glutamine synthase (30), superoxide dismutase (31,32), and Na^+/H^+ antiporter genes (33–36). In cyanobacteria, the introduction of the *E. coli* catalase gene ensured salt tolerance and reduced ROS production (37). Transgenic japonica rice plants were able to form seeds at 100 mM NaCl stress while the transgenic indica rice cultivar BR5 was able to tolerate up to 200 mM NaCl stress for 2 weeks. A recent study also found similar results for the transgenic indica rice cultivar Kasalath. In line with the previous findings, we have found that transgenic jute plants expressing the *E. coli katE* gene can tolerate 150 mM NaCl stress for 8 weeks under natural growth conditions. This is the first transgenic approach for developing jute plants tolerant to an abiotic stress, and hence could be considered a significant achievement in the field of jute biotechnology.

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