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Full Length Article

Expression of Synthetic *hsr1* Gene in Transgenic Tobacco (*Nicotiana tabacum*) for Enhanced Tolerance to Drought and Salt Stresses

Tayyaba Huma[1], Tayyaba Shaheen[1*], Azeem Asghar[1], Farwa Nurjis[2], Ahmad Sattar Khan[3] and Zahid Mukhtar[2]
[1]*Department of Bioinformatics and Biotechnology, Government College University, Faisalabad, Pakistan*
[2]*Agricultural Biotechnology Division, National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, Pakistan*
[3]*Institute of Horticultural Sciences, University of Agriculture, Faisalabad, Pakistan*
[*]For correspondence: tayaba_pgmb@yahoo.com

Abstract

Drought and salinity are causing significant loss in crop yield worldwide. Genetically engineered salt and drought tolerant crop varieties can be a potential remedy for the recovery of lost uncultivated saline and low rain fall areas. In eukaryotes, transcription factors are involved in gene expression which activate or repress the transcription under specific conditions. Hence, the present study has been designed to characterize a fully modified synthetic *hsr1* gene. This gene encodes for a transcription factor of heat shock family and has been found to be involved in drought and salinity tolerance in yeast. The synthetic *hsr1* gene was cloned under double 35S promoter and CaMV terminator for high level of expression in the transgenic plants. Initially the gene was cloned into pJIT163 and then into expression vector pGreen0029. *Agrobacterium tumefaciens* strain GV was used as a vector for transformation into tobacco. In three different experiments about 34.5% transformation efficiency was observed. Putative transgenic plants were confirmed by PCR analysis using *hsr1* gene specific primers. Further screening of these putative transgenic plants against different salinity levels and drought stress showed that transgenic plants were tolerant to drought and salinity conditions. Transformation in the model plant paves the way to genetically engineer vital crops for the development of drought and salinity resistant genotypes. © 2015 Friends Science Publishers

Keywords: *Agrobacterium tumefaciens; hsr1*; Heat shock proteins (HSP); *Nicotiana tabaccum*; Salt and drought resistance

Introduction

Among abiotic stresses, salinity and droughts are the major limiting factors in crop production. Drought has severe effects on plant growth and development causing crop losses worldwide (Farooq *et al*., 2009, 2013; Tsonev *et al.*, 2014). The latest approach to combat these stresses is to analyze cell adaptation at genetic level to salt and drought stress by introducing the desired genes in model plant (Yu *et al.,* 2013; Ahmad *et al.*, 2014). Being sessile, plants adapt some morphological and physiological changes to cope with these stresses. In all living organisms under stress conditions stress-induced proteins or heat-shock proteins (HSPs) are induced. There are different families of HSPs but most diverse group is low molecular weight small HSPs (sHSPs) (Bechtold *et al.,* 2013). HSPs are involved in a variety of cellular processes which include membrane transport and stability, protein folding, and cell signaling (Glatz *et al.,* 1999; Bechtold *et al.,* 2013). The regulatory proteins called heat stress transcription factors (HSFs) control the transcription of HSPs encoding genes (Al-Whaibi, 2011). HSFs are vital for survival of all organisms under severe stress conditions being inducible transcriptional regulators of genes (Anckar and Sistonen, 2011). Numerous plant species including crops have been genetically engineered using heat shock transcription factors for various traits (Al-Whaibi, 2011). Heat shock transcription factors are well studied regarding the heat stress-related pathway; however, their role in other stress responses is yet to be explored (Miller and Mittler, 2006; Swindell *et al.,* 2007; Hwang *et al*., 2014). In a recent study the role of *Arabidopsis thaliana* transcription factor AtHsfA6a in drought and salinity stress responses through ABA-dependent signaling pathway has been reported (Hwang *et al.*, 2014).

Hsr 1 mutants were first reported in yeast and were found to be involved in heat shock tolerance (Iida and Yahara, 1984). The mutant gene was called *hsr1* because of its homology related to heat shock family of transcription factors, and the gene sequence is present in single copy in *Candida tropicals* genome (Ali *et al.,* 2001)**.** It induces up-regulation of *ENA1*, in *S. cerevisae,* a main determinant of salt tolerance encoding cation-extrusion pump. Its open reading frame encodes a predicted size of 728 amino acids of the heat shock family with homology to transcription factors (Ali *et al.,* 2001).

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This study was aimed to characterize the potential of *hsr1* gene for development of drought and salinity stress tolerant transgenic plants to cope with the extreme environmental conditions. Hence, a synthetic modified *hsr1* gene deduced from *Candida tropicales* was introduced into model plant (*Nicotiana tabacum* L.) by transformation. Different salinity and drought treatments were used to analyze the drought and salinity tolerance of transgenic tobacco.

Materials and Methods

Cloning of *hsr1* Gene into pJIT163, pGreen0029 and then into *Agrobacterium tumefaciens*

A synthetic *hsr1* gene was used in this study. The gene sequence originally was deduced from *Candida tropicales*. Codon optimization of the gene *hsr1* was done so that only the gene sequence was changed without changing the final proteins. The gene sequence after codon optimization was synthesized by Chinese company (Generay Biotech Co, Ltd.). Restriction sites for *Hind*III and *Eco*RI were generated at the both ends of the gene for cloning purpose. The gene was first ligated into pJIT163 which is an intermediate vector. The synthetic gene and pJIT163 vector were restricted with *Eco*RI and *Hin*dIII (Fermentas) restriction enzymes at 37°C for 1 h. The digestion was confirmed by visualizing the products on 1% agarose gel. Digested fragments were precipitated with phenol-chloroform and ligated at 16°C overnight (total volume of ligation mixture was 25 µL including 2.5 µL ligase buffer, 0.5 µL vector (pJIT163), 15 µL of insert DNA (HSR1) and 2.5 µL T4 DNA ligase). The ligated product was then transformed into *E. coli* 10b by electroporation. The transformation mixture was incubated for one h at 37°C and then spread on 100 mg/mL LB ampicillin petri plates. Plates were incubated overnight at 37°C and the next day colonies were cultured in LB media containing ampicillin and placed overnight in a shaking water bath at 37°C. Plasmids were isolated from cultures using miniprep kit (Fermentas) and recombinant clone was confirmed by digestion with *Eco*RI and *Hin*dIII. The resultant vector was named as pJHSR1. The gene was further transferred from this vector into binary vector pGreen0029. Both pJHSR1and pGreen0029 were digested with *Xho*I and *Xba*I endonucleases, ethanol precipitated and ligated at 16°C for 18 h. This ligated mixture was used for further transformation into *E. coli* and colonies were confirmed by restriction analysis. The resultant vector was named as pGHSR1. Transformation of both pGreen0029 and pGHSR1 was done into *Agrobacterium tumefaciens* strain (GV) by electroporation. The transformation mixture after 1 h incubation at 28°C was then spread on LB medium plates, which contained 100 µg/mL tetracycline, 50 µg/mL of kanamycin and 25 µg/mL of rifampicin antibiotic. Plates were incubated at 28°C till the appearance of colonies. Then the colonies were grown in LB medium containing 50 µg/mL of kanamycin, 25 µg/mL of rifampicin and 100 µg/mL tetracycline and placed at 28°C for 48 h. To confirm the cloning of *hsr1* expression cassette into pGreen0029, ten different colonies were picked and plasmid was isolated using plasmid miniprep kit (Fermentas) and digested with *Kpn*1 and *Xho*1. Upon digestion of plasmid DNA with *Kpn*1 and *Xho*1, fragments of expected sizes *i.e.,* 3.6 kb representing expression cassette (bearing 2x35S promoter, *hsr1* gene CaMV terminator) and 4.6 kb representing pGreen0029 vector backbone were recovered. Transformant were also confirmed by PCR analysis with specific primers of *hsr1* gene F 5′ TGTCGATTTGGAGCCTCTTT 3′ and R 5′ AGCATGAATTGGGCTTATGG 3′.

Transformation of pGHSR1 into Tobacco

About 210 explants (leaf discs) of *N. tabaccum* cvs. Spade-28 and Samsun were transformed with *A. tumefaciens* (Hiei *et al.,* 1994) having pG*hsr1* in three different experiments (Table 1).

Screening for Salt and Drought Tolerance

NaCl stress treatment: Salt stress treatment was imposed by NaCl stress treatment. For salt tolerance four lines of *N. tabacum* were developed. Lines 01, 02 and 03 were transgenic plants each line consisting 10 plants while line number 04 was control (non-transgenic plants) consisting of 10 plants. Plants were grown and maintained in a growth room at 25°C±1 in a 16/8 h light/dark cycle. Transgenic and control plants were germinated in small glass jars containing MS medium (Murashige and Skoog, 1962). Five weeks old plants were subjected to saline stress by providing nutrient solution (1/8 strength Murashige and Skoog salt mixture) supplemented with NaCl, incrementally increasing with each successive salt solution from 0 m*M*, 100 m*M*, to a final concentration of 200 m*M* NaCl. Plants were watered after 5 days interval. Effect of different salt concentrations on plant growth, wilting and recovery was recorded and final data was recorded after 15 days.

Drought stress treatment: For drought stress treatment, water deficit was imposed to transgenic plants. For drought tolerance six lines of *N. tabacum* were developed. Lines L1, L2 and L3 were transgenic plants each containing 10 plants while line number C1, C2 and C3 was control (non-transgenic plants) each containing 10 plants. Effect of drought (water deficit) on plant growth, wilting and recovery was recorded after 24 h of stress treatment. All plants at real-leaf stage were exposed to 20% PEG6000 stress for 24 h as was done by Zhang *et al*. (2007). After 24 h of water deficit, plants were grown in another MS recovery medium (Murashige and Skoog, 1962) under a fully watered regime at 25°C for one week to observe recovery.

Molecular Analysis of Putative Transgenic Plants

For molecular analysis the DNA of transgenic and control plants was extracted using the method of Iqbal *et al*. (1997). The PCR was performed on genomic DNA of transgenic plants with *hsr1* specific internal forward and reverse primers. F-5′ TGTCGATTTGGAGCCTCTTT 3′ and R-5′ AGCATGAATTGGGCTTATGG 3′. PCR was performed in a total volume of 20 µL using 2.5 µL (15 ng μL^{-1}) of genomic DNA; 10X PCR buffer without $MgCl_2$ (10 mM Tris-HCl, 50 m*M* KCl, pH 8.3); 3 m*M* $MgCl_2$; 0.1 m*M* each of dATP, dGTP, dCTP, and dTTP; 0.5 units of Taq DNA polymerase; and 0.15 m*M* of each primer. Taq DNA polymerase, 10X PCR buffer, $MgCl_2$, and dNTPs were purchased from MBI Fermentas. Following 1 min at 94°C, PCR consisted of 35 cycles of 94°C for 30 s, 56°C for 30 s, a 72°C extension for 1 min, and a final extension at 72°C for 10 min. The amplification was visualized on 1% agarose gel.

Statistical Analysis

All experiments were conducted in three biological replicates and statistical analysis was performed using excel 2010.

Results

Development of Transgenic Plants

A synthetic gene *hsr1* was used to develop transgenic tobacco plants; the gene was cloned first into pJIT163. The fragments representing the pJIT163 (3.720 kb) and *hsr1* gene (2.4 kb) were recovered on restriction analysis which confirmed the cloning of gene into the vector. Further the construct was cloned into pGreen009 vector and this new construct was named as pG*hsr1*. Upon digestion of plasmid DNA with *Kpn*1 and *Xho*1, fragments of expected sizes *i.e.* 3.6 kb representing expression cassette (bearing 2x35S promoter, *hsr1* gene and CaMV terminator) and 4.6 kb representing pGreen0029 vector backbone were recovered (Fig. 1). The recombinant plant expression vector (pG*hsr1*) was then transformed into *Agrobacterium tumefaciens* strain GV. PCR analysis confirmed the cloning of right sized gene. Further the transformation of the construct into *N. tabaccum* cvs. Spade-28 and Samsun was done with *A. tumefaciens* having pG*hsr1*. Out of 210 explants transformed with recombinant *A. tumefaciens*, only 69 explants could survive on selection media (Fig. 2) and regenerated plants with an average transformation frequency of 34.50% (Table 1). The rest of the explants bleached out indicating that these were non-transformed. The first signs of regeneration of plantlets were evident after 28 days of transformation (Fig. 2).

Table 1: Transformation efficiency in tobacco plants in three different experiments

Exp. No.	Total explants used	No. of kanamycin resistant explants	Transformation efficiency
01	75	23	30.67%
02	80	19	23.75%
03	55	27	49.09%
Total	210	69	34.50%

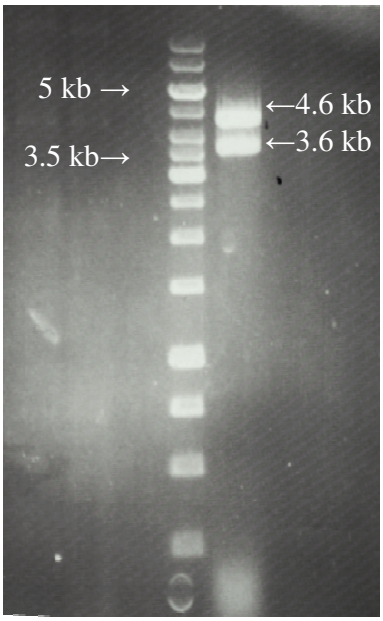

Fig. 1: Confirmation of *hsr1* gene under double 35S promoter and CaMV terminator in pGreen0029. Resultant vector was named as pGHSR1. Double digestion with *Kpn*I and *Xho*I enzymes generated two fragments 3.6 kb showing *hsr1* with 2X35S promoter and CaMV terminator 4.6 kb representing vector backbone (pGreen0029)

Molecular Analysis of Transgenic Plants

In total 60 putative transgenic green plants having pGHSR1 were regenerated. Total eight plants representing all experiments, from individual transformation events were randomly selected and analyzed by PCR. The DNA isolated from eight putative transgenic plants and four non-transformed controls were subjected to PCR analysis (Fig. 3) using *hsr1* specific internal forward and reverse primers. A fragment of 650 bp from the internal region of the *hsr1* gene was amplified as expected from all transgenics. No amplification from the non-transformed control authenticated the results.

Physiological Effects of *hsr1* on Salt (NaCl) Medium

Three lines of transgenic plants and one line of non-transgenic control plants were used for this study. Data were recorded after 15 days of exposure to different salt

concentrations. There was no change in transgenic and control tobacco plants those were placed on 0 m*M* NaCl medium. Non-transgenic plants that were placed on 200 m*M* NaCl medium had severe wilting and death. Leaf margins were blackish, leaf colour yellowish and no more growth was observed. On the other hand, 30–75% transgenic plants showed no effect like wilting or cell death treated with 200 m*M* NaCl (Fig. 4).

Effect of Drought Stress on Transgenic *hsr1* Plants

Three lines of each of control and transgenic plants were used in this experiment. Medium having 20% PEG6000 was used. All transgenic and control plants were grown on PEG6000 liquid medium. After 24 h the control plants showed more signs of wilting compared to the transgenic tobacco plants. Both transgenic and the control plants were placed on MS recovery medium. After 1 week 40–80% transgenic plants recovered from wilting but none of the control plants were unable to recover and showed permanent wilting (Fig. 5).

Discussion

The present study is an endeavor to express synthetic *hsr1* gene under a strong promoter (CaMV35S) for high-level expression in model plant tobacco. The *hsr1* having homology with heat shock transcription factor play a role by up-regulating the expression of the *ena1* cation extrusion pump in *S. carevisiae* thus proving to be a major determinant of salt and drought tolerance. In earlier reports a number of genes from yeast (*S. cerevisiae*) have been examined, over expression of such genes in yeast show a positive result on salt tolerance by maintaining a decreasing intracellular Na^+ and high K^+ concentration during salt stress (Mascuda *et al.,* 2000). In a recent study the functional characterization of *Arabidopsis thaliana* heat shock transcription factor *AtHsfA6a* has revealed its up-regulation under drought and salinity stress conditions (Hwang *et al.,* 2014).

Plants deal with unfavorable conditions using various mechanisms at different levels, including physiological, molecular and biochemical processes (Todaka *et al.,* 2012). All cellular organisms have essential mechanism to control the intracellular ion homeostasis. In cytosol mostly cell maintain low Na^+ and high K^+ concentration and it is compulsory requirement for survival of glycophytic plants under stress which includes the majority crop species. Plants and yeast have achieved this through coordinated regulation of transporters for H^+, Na^+ and K^+. This regulation is achieved through a variety of transcription factors, phosphates and protein kinases and *hsr1* is the transcription factor having homology with the heat shock family of transcription factors (Ali *et al.,* 2001) and has potential to be utilized for development of salinity and drought resistant plants.

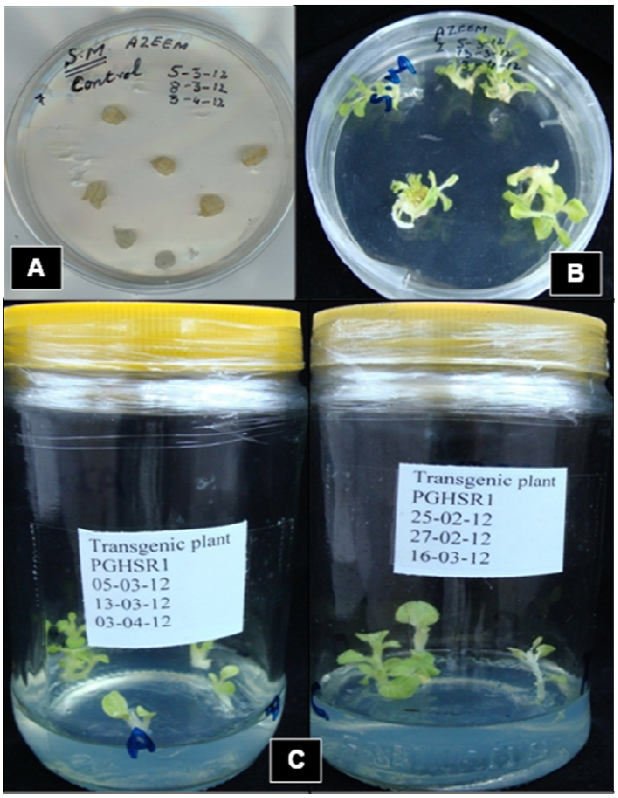

Fig. 2: Different stages of growth of the transgenic plants: Leaf discs on selection medium without agrobacterium inoculation showing necrosis and cell death (A), *Agrobacterium* inoculated leaf discs showing regeneration of plants on selection medium (B) and Shoot formation of transgenic plants on selection medium (C)

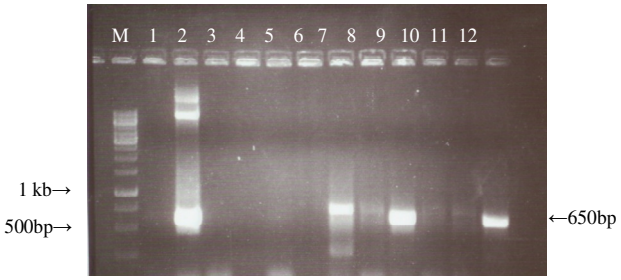

Fig. 3: Agarose gel electrophoresis of PCR amplified product from genomic DNA isolated from different putative transgenic tobacco plants showing amplification of correct sized (650 bp) fragment of the internal region of *hsr1*gene. M is marker lane 1, 3, 4, 5 are control plants while rest of the lanes represent putative transgenic plants

A synthetic hsr1 gene was used to develop transgenic tobacco plants using agrobacterium transformation to enhance drought and salinity tolerance. Transgenic technology has a great potential and applications in the crop improvement (Zhong, 2001; Yu *et al.,* 2013).

Fig. 4: Comparison of non-transgenic with transgenic *N. tabacum* grown on same concentration of NaCl medium. Non transgenic *N. tabacum* grown on 200 mM NaCl (A), and Transgenic *N. tabacum* on 200 mM NaCl (B)

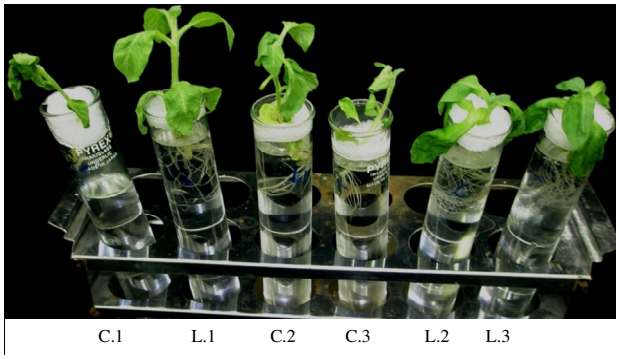

Fig. 5: Transgenic and control *N. tabacum* plants placed at 20% PEG6000. Control plants show severe wilting compared to the transgenic plants. C. 1, 2 and 3 are non-transgenic *N. tabacum* while L.1, 2 and 3 are transgenic *N. tabacum*

Abiotic stresses like salt and drought stresses causes the adverse effects on the growth of plants and the productivity of crops (Farooq *et al.,* 2013) The major constraints in crop production in the world are drought and salinity, which have significant factors affecting the food sanctuary and thus harmfully impact the socio-economic fabric of various developing countries (Yu *et al.,* 2013).

In present study, the overall transformation efficiency was observed 34.50% (Table 1) in three batches. In first, second and third batch, it was 30.67, 23.75 and 49.09%, respectively. It was low in the second batch due to the physiological condition of the ex-plants used. The transformation efficiency of *Agrobacterium* depends on large number of factors such as physiological state of the ex-plant, *Agrobacterium* strain nature used in transformation and lastly more or less the *vir* genes of Ti plasmid (Park, 2006). Due to these factors transformation efficiency varies from experiment to experiment. Sohail *et al.* (2013) observed 27.14% efficiency during transformation in tobacco.

The over-expression of the *hsr1* gene in transgenic *N. tabaccum* plants resulted in enhanced salt and drought tolerance. When transgenic plants were exposed to periodic drought stress and salinity, they showed better efficiency as compared to control plants, which directly co-relates with the over-expression of vacuolar H^+-pyrophosphatase (Gaxiola *et al.,* 2001). In earlier studies transformation of HAC1 genes from yeast in plants such as tomato (Gisbert *et al.,* 2000) and melon (Bordas *et al.,* 1997) resulted in high level of salt tolerance in transgenic plants (Gisbert *et al.,* 2000).

Results have shown that 20% PEG6000 treatment exhibited wilting after 24 h and 30–75% of the transgenic plants were fully recovered after one week of drought treatment. In earlier study Zhang *et al.* (2007) revealed that with 17% PEG6000 treatment, the rate with which seedlings revived corresponded with the results in drought-shed after 12 h. The method with osmotic adjustment of PEG is undemanding, rapid and easily operated, can be used to evaluate the drought tolerance. Liu *et al.* (2011) observed that 36.7% of the transgenic tobacco plants revived after drought stress using 20% PEG treatment and no watering for 35 days as compared to control plants. They developed transgenic tobacco plants with wheat MYB transcription factor to enhance resistance to *Ralstonia solanacearum* and increased drought and salinity tolerance, the HSR1 gene has effectively enhanced the drought tolerance of tobacco plants.

In the present study, transgenic plants against different salinity levels (0–200 m*M* NaCl) showed that 0 and 100 m*M* are not effective for screening. At 200 m*M* NaCl concentration 40–80% transgenic plants were tolerant whereas the control plants showed wilting within 15 days of salt treatment. In the study of characterization of MYB transcription factor in tobacco the survival rate of the transgenic tobacco was 20–60% as compared to control plants after treatment with 250 m*M* NaCl for 40 days (Liu *et al.,* 2011). Ibrahim *et al.* (2009) stated that screening of putative transgenic plants against different salinity levels (50–250 m*M* NaCl) showed that transgenic plants were tolerant to 250 m*M* NaCl whereas the control plants showed wilting within 36–48 h of salt treatment. It is evident that *Hsr1* is effective in enhancing salinity tolerance of tobacco plants.

Conclusion

Using this strategy which allows plants to modulate gene expression programs, which are necessary for adaptation to stressful environments, genes encoding for salt, drought and cold tolerance like *hsr1* is an important aspects for improving the crops like wheat, sugarcane, potato and maize

against abiotic stresses. Once the potential of this transcription factor for improving salt/drought tolerance is proved in a model plant system, this gene can further be exploited to generate other important crop plants with enhanced salt/drought tolerance characteristics.

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