Original Article

Evaluation of *iceA1* Gene Expression of *Helicobacter pylori* Risk Factor of Gastric Cancer in Transgenic Brinjal

Mohammad Javad Mehran[1], Basaralu Yadurappa Sathish Kumar[1,2,*], Nanjundappa Haraprasad[3], Rambod Barzigar[1], Bashasab Fakrudin[4], Sayan Paul[5,6]

[1]JSS Research Foundation, SJCE Technical Campus, Mysore, Karnataka, INDIA.
[2]Department of Biotechnology, JSS College, Ooty Road, Mysore, Karnataka, INDIA.
[3]JSS Science and Technology University, SJCE, Technical Campus, Mysore, Karnataka, INDIA.
[4]Department of Biotechnology and Crop Improvement, College of Horticulture, University of Horticulture Sciences Campus, GKVK Post, Bengaluru, Karnataka, INDIA.
[5]Department of Biotechnology, Manonmaniam Sundaranar University, Tirunelveli, Tamil Nadu, INDIA.
[6]Centre for Cardiovascular Biology and Disease, Institute for Stem Cell Science and Regenerative Medicine (inStem), Bangalore, Karnataka, INDIA.

ABSTRACT

Background: The advancement of plant biotechnology improved crop production by revolutionizing plant science. Humans were commonly infected by *Helicobacter pylori*, and it was closely linked to stomach ulcers and cancer. In addition to traditional vaccines for *H. pylori*, transgenic plants have also been produced to produce its antigens as well as edible and non-edible parts that can produce an immune response after consumption. The protein present in *H. pylori* associated with virulence (*iceA1*) is believed to predispose to stomach cancer. **Objectives:** The current study was aimed to evaluate the *iceA1* gene expression within brinjal plant to produce novel transgenic lines LC420461-B7, -B10, -B15, -B22 and -B27. **Materials and Methods:** In the present study, amplified *iceA1* gene from *H. pylori* strain 26695 was transformed into callus of brinjal (from leaf explants) through *Agrobacterium tumefacians* (EHA105). pBI121 vector was used in constructing the plant expression vector, and the transgenics generated were further evaluated by quantitative Real-Time PCR and western blot analysis. **Results:** Out of the 46 plants obtained five of them were found to be positive for the *iceA1* expression. Both real time and western blot confirmed of the presence of expressed gene *iceA1* within the plant sample leaves. By studying transgenic brinjal, the study may result in an *H. pylori* vaccine candidate. As well, the data can be used by researchers to get valid scientific information.

Key words: *Helicobacter pylori*, Transgenic brinjal, *iceA1*, *Agrobacterium*, Callus induction, Real-time PCR.

INTRODUCTION

Agricultural biotechnology has always worked towards improved crop production and increased sustainability since its inception.[1] A vaccine, in contrast, generates an immune response through the inactivation of bacteria or viruses forming a revolutionary phenomenon in the 19th century. These are simply proteins found in an inactivated form that resemble pathogens and stimulate the immune system to recognize the foreign antigen later on.[2] A number of *H. pylori* biomarkers/traits have been associated with the onset of gastric cancer. Some of these factors, including cytotoxin-associated gene A (*cagA*), vacuolar cytotoxin (*vacA*), and outer inflammatory protein A (*oipA*) are responsible for the development of gastric cancer. Due to these factors, *H. pylori* is considered a potent carcinogen. Since *H. pylori* infection is associated with a reduced risk of gastric cancer, eliminating *H. pylori* infection has been beneficial, but

Submission Date: 29-08-2021;
Revision Date: 05-10-2021;
Accepted Date: 08-11-2021.

DOI: 10.5530/ijper.55.4.219

Correspondence:
Dr. Basaralu Yadurappa Sathish Kumar,
[1]JSS Research Foundation, SJCE Technical Campus, Mysore-570006, Karnataka, INDIA.
[2]Postgraduate, Department of Biotechnology, JSS College, Mysore-570025, Karnataka, INDIA.
Phone: +91 9242208001,
Email – bysathish@gmail.com

www.ijper.org

an antibiotic-resistant strain of *H. pylori* makes any eradication of the infection difficult. In light of the antibiotic resistance in different parts of the world, it is imperative that there are new and effective treatments to treat *H. pylori*.[3]

Helicobacter pylori virulence factors play a crucial role in pathogenesis of the infection. During attachment of the bacterium to the gastric mucosa, the *iceA* gene of *Helicobacter pylori* is induced by an epithelial contact which is subdivided into 2 variants *iceA1* and *iceA2*. Although some data suggest a link between *H. pylori iceA1* and peptic ulcers, this concept still remains controversial.[4] In addition to cytotoxin-associated gene A (*cagA*) and epithelium antigen-induced virulence factor (*iceA*),it has several other virulence factors.[5] Urease enzyme, flagella, adhesins, cytotoxin-associated gene *A (cagA)*, vacuolating cytotoxin A (*vacA*), and the induced by contact with epithelium (*iceA*) gene are all considered as virulence factors.[6] *iceA* genes were found in *H. pylori* isolated from gastritis and Peptic ulcer disease (PUD) patients.[7] When human epithelial cells were in contact with Hpylori, *iceA1* expression increased. An increased expression of mucosal IL-8 and acute antral inflammation were associated with the *iceA1* genotype. Additionally, it was demonstrated that adhesion to gastric epithelium *in vitro* stimulated *iceA1* transcription.[8] The *iceA1* variant is associated with PUD according to several studies.[9] As for *iceA2*, its functions remain unknown despite the fact that this allele is associated with asymptomatic gastritis and nonulcer dyspepsia.[9]

Genes of pathogens (bacteria) are inserted into genetically modified plants, without altering the immunogenic capacity, to manufacture mass-produced vaccines. Vaccines called edible vaccines seem to trigger both mucosal and systemic immunity against disease-causing pathogens.[2] Cheng *et al*., 2007, developed plant expression vectors carrying *Helicobacter proteins* like *cagA* and *ureB* that expressed fusion proteins against *Helicobacter* strains.[10] Although traditional vaccine production is urgently needed, it is stalled due to the high cost of production and the complicated process of purification.[11] The robustness and sensitivity of real-time PCR and blotting have made them dominant in recent years for studying gene expression.[12,13] Furthermore, and with respect to expression levels, these techniques are applied over microarray assays.[14,15] In fact, leafy greens, fruits, and vegetables can easily produce edible vaccines using plant expression systems. By using oral delivery systems, traditional vaccines will be able to be administered without the side effects of sterile administration. Additionally, the purification procedure and old storage are not necessary for conventional vaccines.[16]

Indians consider brinjal, commonly known as eggplant, as a common vegetable.[17] Due to its popularity in usage within the country, it has been crowned a common man's vegetable.[18] The vast range of pharmaceutical products, medicines, and dietary supplements it produces makes it the 'King of Vegetables'.[19] It is a common dish in every Indian household, regardless of income level and food preference. In addition to its nutritional benefits and water content, it is low calorific. In addition, it is a good source of fiber and minerals such as calcium, phosphorus, and folate.[20] In current Indian culture, the brinjal is deeply entrenched in the Indian culture with numerous folk songs being performed and tuned for the humble vegetable.[21]

In order to trace the significant application of plants in the production of *H. pylori*, the current was done to express the clone of the *iceA1* gene from *H. pylori* strain 26695 in the brinjal plant, *Solanum melongena* L. *Cultivar* Arka Keshav. By expressing *iceA1* in brinjal plants, we demonstrated that *H.pylori* can be countered by the immune system in plants. These results may lead to the development of possible vaccine candidates against *H. pylori* from the brinjal plant.

MATERIALS AND METHODS

Plant material and Experimentation

We collected eggplants (*Solanum melongena* L. *cultivar* Arka Keshav) from the Division of Vegetable Crops, IIHR, Bangalore. Experimental protocols were approved by the JSS College Institutional Biosafety Committee (IBSC), Mysore at Postgraduate Department of Biotechnology, JSS College, Mysuru, Genei India Pvt. Ltd., Bangalore and Department of Biotechnology and Crop Improvement, College of Horticulture, University of Horticulture Sciences, GKVK Post, Bangalore before work began. The College of Horticulture, University of Horticulture Sciences, GKVK Post, Bangalore has granted permission for the collection of plant or seed specimens. In accordance with procedures of international guidelines and legislation, all plants were discarded immediately after growing and before flowering.

Chemicals

Genomic DNA from *Helicobacter pylori* 26695 was procured from (ATCC® 700392DQ™) ATCC and the chemicals and reagents used in the study were bought from Sigma Chemical Co and HiMedia and were of molecular grade and primers were ordered from Sigma

Ltd, Bangalore. Wile DNA ladders, *SacI* and *BamHI* and enzyme T4 DNA ligase was bought from Genei Laboratories, Bangalore and *Helicobacter pylori* polyclonal antibody, Biotin (HSN/SAC: 38220090) was procured from Thermo Fisher Scientific. *E. coli* (DH5α) (F', *end A1 hsdR17* (rk^-mk^+) *glnV44 thi*-1 *recA1gyrA* (Nalr) *relA1*(*lacZY, AargF*) *U169deo* RΦ 80d*lac* (*lacZ*) M15 and pBI121 with T7 promoter was bought from Genei Laboratories Pvt Ltd and Invitrogen Life Technologies, USA respectively. *A. tumefaciens* strain (EHA105) was provided by the Department of Biotechnology of the IIHR, Bangalore. During our study, a callus was transformed with *Agrobacterium tumefaciens* (EHA105) and T-DNA carrying the *iceA1* gene was inserted into the pBI121 vector.

PCR amplification of *iceA1* gene: An amplification of the gene,[1] was performed with primers designed from *H. pylori iceA1*, which are specific for forward and reverse amplification of the gene (Table 1) corresponding to 519bp. The PCR mixture contained of 50ng of total gDNA as template, Hotstart Taq DNA polymerase (1U), dNTPs (0.2mM each), 1× PCR buffer, $MgCl_2$ (3mM) and primers (10pmol each) in a total reaction volume of 25µl. Initial denaturation at 94°C for 2 min was followed by 35 cycles (94°C, 40 sec, 56°C, 45 sec, 72°C, 1 min), followed by a final extension at 72°C for 15 min. In the next step, the PCR products were resolved on 1.0% agarose gels and visualized with a UV transilluminator. The PCR products were purified from the gel using the Genei clean purification system (Gel Extraction Kit, Genei). Using Miniprep Kits (Genei), we isolated high-yielding plasmid DNA (30µl) according to the manual.

Cloning of *iceA1* gene into pBI121: We cloned *iceA1* fragment of 519bp into bacterial strains which was then purified after amplification with *BamHI* and *SacI* (37°C for 2hr). we intend to create transgenics in this manner so as to induce an immune response among the patients of *H. pylori*. In brief, the purified PCR product was ligated into the pBI121 vector (Fermentas, USA) and transformed into the competent *E. coli* (DH5α) (F', end *A1 hsdR17* (rk^-mk^+) *glnV44 thi*-1 *recA1gyrA* (Nalr) *relA1*(*lacZY, AargF*) *U169deo* RΦ *80dlac* (*lacZ*) M15. About 30µl of ligation reaction was used with 3:1 ratio of insert and vector DNA. 5µl of PCR product (0.55pmol) was then ligated with T4 DNA ligase (5 weiss units). Final vector concentration of 50ng/µl was used in the study. The mixture was then incubated at room temperature (25°C) for about one hour and then used for further transformation experiments. The clones were then screened for digests to confirm the positive transformation of the gene.

Preparation of competent cells and Transformation: The competent DH5α *E. coli* cells prepared prior were transformed with pBI121 vector containing *iceA1* gene and incubated on ice for 30 min and heat shock was given at 37°C for 5 min and the cells were then plated onto LB agar plates and screened further for the *iceA1* inserts. Single isolated colonies were inoculated into 5ml of LB broth (with Kanamycin 20µg/ml) and incubated at 37°C for 12 hr on a shaker incubator (120rpm). Plasmids were purified using alkaline lysis method, and checked for purity on 0.8% agarose gel electrophoresis and the clones which are positive for the insert were confirmed by restriction digestion and then DNA sequencing.

Callus induction

Seeds of the variety Arka Keshav (pre-treated with 250ppm of GA3; gibberellic acid) were surface sterilized with sodium hypochlorite (6%) and water in a 1:1 ratio and rinsed with sterile distilled water four times. 20-25 seeds are inoculated onto Petri plates containing N6 callus induction medium (with 10,000mg/l Myoinositol, 200mg/l glycine, 50mg/l Thiamine HCl, 50mg/l Pyridoxine HCl, and 50mg/l of Nicotinic acid) of pH 5.75. 10µM of BAP and 1µM NAA were added to the autoclaved medium. Following inoculation, the plates were then incubated at 25±2°C in dark and the callus obtained from the germinating seeds was then sub-cultured on a fresh callus induction medium. The current study used transversely cut hypocotyl segments and median portions of cotyledonary leaves as explants.[22]

Callus transformation with *Agrobacterium*

As per the Patel *et al*., 2013 protocol, the calli were transformed with *Agrobacterium* (EHA105),[23] but with slight modifications. Electroporation was used to introduce vectors into *Agrobacterium*. About 5µl of plasmid DNA was added to 50µl of cells, and mixed thouroughly. The contents were then transferred into a prechilled cuvette for electroporation. The *Agrobacterium tumefaciens* mobilized with vector inserts, were then inoculated into 5ml of YEP (yeast-extract-mannitol medium; pH 7.0) medium (with 20mg/l Rifampicin and 50mg/l Kanamycin) and incubated at 28°C in a shaking incubator (200rpm) overnight. Overnight culture was then transferred into 45ml of infection media (MS basal media with Thiamine HCl [1mg/l], Myoinositol [250mg/l], Casein hydrolysate [1.0g/l], Proline [690mg/l], Glucose [30g/l], 2,4-D [5.0mg/l] and Acetosyringone [200µM]) of pH 5.2 and incubated at 28°C in a shaker incubator (200rpm). The incubation was done for 48hr until the OD_{600} reaches 0.5–0.6.

To confirm the *iceA1* insert, we performed a colony PCR of *Agrobacterium*.

After being heat shocked at 42°C for 3 min, the embryogenic calli obtained were then incubated at room temperature for 12 min before being infected with *Agrobacterium*. After blotting the calli on to Whatman papers to remove excess bacterial suspension, it was co-cultivated on co-cultivation media (media (MS basal media with Myoinositol [690mg/l], Casein hydrolysate [1.0g/l], Proline [1gm/l], maltose [0.5mg/l], 2,4-D [0.1mg/l], BAP [0.1mg/l] and Acetosyringone [200µM], Phytagel [3gm/l) of pH 5.8 for 2 days in dark at 25°C.

Explants were maintained on the cocultivating medium for two days before using the selection medium (media with 100mg/l of kanamycin). The selection medium was subcultured every six days for about 20 days, then transferred to fresh cocultivation medium.

Three to four weeks after selection, the calli growing on kanamycin alone were transferred to regeneration media (MS media with 2.0mg/l BAP, 30g/l Maltose, 3.0g/l Phytagel, 200mg/l Timentin and 50mg/l hygromycin) and then incubated in light at 25°C for about 3 weeks. Incubation media with kanamycin (25mg/1) and cefotaxime (250mg/l) were used for the newly regenerated shoots. The shoots obtained were then transferred onto rooting medium (half-strength MS medium with 250mg/l cefotaxim along with IBA 5µM) and selection medium without kanamycin. The rooting was then performed in new bottles with rooting medium, and the plantlets were left to grow for an additional 15 days. A number of primary transgenics about 8-10cm in size were carefully removed from the soil and replanted into 6inch pots containing peat-lite and sand 50:50 in the greenhouse (26/22°C, 16hr light/8hr dark and high humidity). Upon acclimatization, the plants were placed in growth chambers (28/24°C, 16hr light, 8hr dark) and given care and nutrients according to standard management practices.

Primer design: Using mRNA sequences deposited with GenBank, all primers (Table 1) were designed using Primer 3 software (version 4.13).[24] A standard nucleotide-nucleotide BLAST (blastn; provided by NCBI online) was used to check the specificities of the primers against the original GenBank sequences.[25] The amplification of the cDNA was verified by gel electrophoresis, followed by Real-time PCR.

RNA extraction

The Brinjal plant leaves were flash frozen with liquid nitrogen and ground into powder using liquid nitrogen using a mortar and pestle after the expression of the Brinjal plant had been stabilized. The manufacturer's instructions were followed to extract RNA from 100mg of ground tissue using the RNeasy Plant Mini Kit (Genei Laboratories Pvt. Ltd). The purified RNA (2µl) was then quantified using a Nanodrop® system and 2µl of RNase free water. Genei's M-MuLV RT-PCR Kit (Genei) with Oligo(dT) was used to revert 1g of total RNA into 20l of cDNA, as described in the manual (Genei).

cDNA synthesis: cDNA was synthesized as described by Palikša *et al*., with M-MuLV RT-PCR Kit (Genei).[26] About 2µg of the RNA obtained in the previous section was used as a starting material. The RNA concentration obtained was 40µg/ml, so we used approximately 1.02µl of total RNA was used, along with random primers and 1µL of RT enzyme. The contents were then incubated at 25°C for 10min and then at 70°C for 45 min following the first incubation. The cDNA (20µl) obtained was then used to amplify the whole *iceA1* gene and for the qRT-PCR expression studies.

PCR amplification of *iceA1* from cDNA of transgenic plants. *IceA1* gene was amplified to estimate the expression level of *iceA1* mRNA within the tissues. Amplification was done as mentioned in the previous chapter and cDNA was used as the template. The products were then run on 1.2% agarose gel to confirm the *iceA1* gene insert.

Real-time PCR: RT-PCR was conducted with cDNA templates,[27] using Realplex 4 (Eppendorf) and SYBR

Table 1: The list of primers used in the study is shown in the following table. RT: Real time primers; FP: Forward primer, RP: Reverse primer. Tm: melting temperature. Actin was used as housekeeping gene.

Gene		Sequence	Length	Tm	GC%	Product length
iceA1	FP	TATCTGGATCCATGGAGTTTGATAAAGGGCAAACTC	36	76	41.7	519bp
	RP	ATATCGAGCTCCTATAAAGTAGTTTTTTGATGGT	40	67.4	32.4	
iceA1 (RT)	FP	AAAGGGCAAACTCTAGGGAA	20	61.5	45	178bp
	RP	CGGCCGTCTTTATGATCCAC	20	66.4	55	
Actin (RT)	FP	TCAGGTGTCCAGAGGTGTTGTA	22	64.5	50	150bp
	RP	ATGGTTGTGCCTCCTGAAAGTA	22	64.5	45.4	

Green PCR master mix (BioRad) according to the manual instructions (BioRad) with the designed primers listed in Table 1. In order to minimize the suppression of PCR by RT solutions, the cDNA samples were then diluted tenfold with sterile DEPC water. Genei's iQTM SYBR Green Supermix (2.5µl of 10× PCR buffer with SyBr green, 2.5µl of 10mM dNTPs, 10pmoles/µl of each primer, 10.4µl of PCR water, 0.1µl of 5U Taq polymerase (Genei), and 2µl of cDNA),[28] was used to amplify the *iceA1* gene along with the housekeeping gene (Actin).

cDNA was initially denaturated at 94°C for 10 min, followed by 35 cycles of amplification at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec. We finished the run with a 10 min extension at 72°C. A negative control was run on samples that did not contain cDNA. In our study, all samples were run in duplicate. We calculated the relative expression of the target genes in the samples using the following formula: ΔCt = Ct (target gene) - Ct (housekeeping gene) where Ct (target gene) indicates the value of the threshold cycle for the gene of interest, and Ct (housekeeping gene) indicates the value of the threshold cycle for the housekeeping gene used as normalizer. The relative expressions of all samples (both transgenic and control) were calculated as follows: RE = $2^{-\Delta\Delta Ct}$ [where ΔΔCt = ΔCt (sample) – ΔCt (control)] where RE indicates the relative expression, ΔCt (sample) indicates the difference between Ct values of the target gene and the housekeeping gene(s) calculated for the test sample, ΔCt (control) indicates the difference between the Ct values of the target gene and the Ct values of the housekeeping gene(s) obtained from the corresponding with control.[29,30]

Protein extraction

Liquid N2 was used to grind leaf samples (transgenic and control) in a mortar and pestle. In 2ml cold acetone, 0.2gm of tissue powder is resuspended for 30 sec and vortexed thoroughly.[31] A pellet was obtained and centrifuged at 10000g for 3 min at 4°C after washing with acetone. The powder was transferred into a mortar and ground further with quartz sand, and then transferred into 2ml microfuge tubes, washed with ice-cold 10% TCA 3 to 4 times, followed by addition of cold 80% acetone. After centrifugation, the pellet was used for extraction at room temperature. A slightly modified phenol extraction method was used in the study.[31] 0.1 g of powder was resuspended in 0.8 ml of phenol (Tris buffered, pH 8.0) and 0.8 ml of SDS buffer (sucrose 30%, SDS 2%, 0.1M Tris-HCl, pH 8.0, 5% 2-mercaptoethanol) containing 0.8% distilled water. Immediately after mixing thoroughly for 30 sec, the contents are centrifuged at 10,000g for 3 min. Proteins were precipitated out of the upper phenol phase using 5 volumes of cold methanol and ammonium acetate (0.1M). After precipitation, the proteins were centrifuged for 5 min at 10000 g before being washed twice with ice cold 80% acetone and cold methanolic ammonium acetate. Pellets thus obtained were dried, rehydrated in 2-DE rehydration solution (8M urea, 4% CHAPS, 2% IPG buffer, 20mM dithiothreitol) and quantified (Genei, Bangalore) using bovine serum albumin as standard.

Western blotting

Transgenic plants expressing *iceA1* were evaluated using western blotting following the protocol described by C. Yang *et al*., 2011,[32] with slight modifications. Separation of soluble proteins was accomplished with SDS–polyacrylamide gel electrophoresis (4.75 stacking density and 12% resolving density with 12% glycerol) and electrotransfer to nylon membrane (Genei, Bangalore). Resolving buffer concentration (Tris-HCl, pH 8.8) was modified to 0.75M rather than 0.375M. Bio-Rad mini-SDS PAGE gels were used for denatured protein samples (not shown in this manuscript) under constant 200V and resolution. TBST buffer (20 mM Tris-HCl, pH 7.6, 0.8% NaCl, 0.1% Tween 20) was used as a blocking buffer and antibodies against *Helicobacter pylori* polyclonal antibody was used for hybridization. (Thermo Fisher, Biotin dilution 1:500) at room temperature for about 1hr. A western blot detection kit (Genei, Bangalore) was used to detect the secondary antibody streptavidin (dilution 1:1000, Thermo Fisher). Beta-actin of ~42KDa was used as a housekeeping gene.

RESULTS

A total of 46 independent brinjal plants were regenerated by *Agrobacterium* transformation [Figure 1]. Colony PCR of *Agrobacterium* confirmed the positive expression of *iceA1*. Bands at the size of 519bp (GenBank® accession no. LC420461.1) could be seen in the agarose gel and also from the cDNA of the transgenic plants which suggests the positive expression of the *iceA1* gene [Figure 2] were further screened for the *iceA1* gene expression by Real-Time PCR and western blot assay.

Expression of the *iceA1* gene by Real-time PCR: The PCR products from the real-time PCR were run on 2% agarose gel to trace the quality of the expression among the samples [Figure 3]. HKG was seen to be expressed in all the samples at about 150bp. The *iceA1* was seen to be expressed in all five samples of interest. Though the expression was positive across the samples and seen at about 178bp, the samples B15, B22, B27

Figure 1: Transgenic brinjals expressing *iceA1* protein.

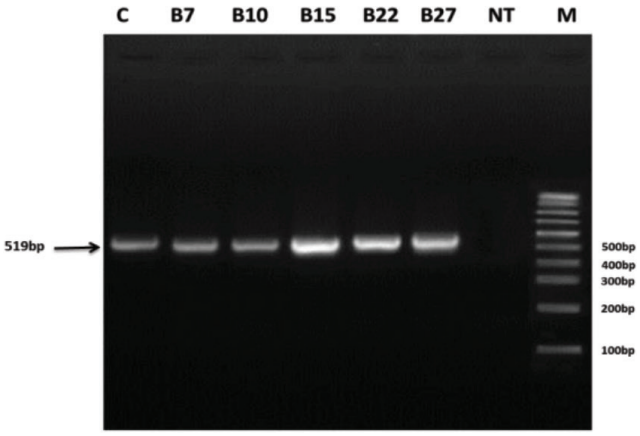


Figure 2: RT-PCR products amplified from the total RNA of transgenic plant. Primers designed for PCR amplification of the *iceA1* gene which specifically amplified DNA fragments of 519bp. Lane description: molecular-weight ladder (M), RT-PCR product of cDNA from a non-transformed plant (NT), RT-PCR products of cDNA from transformed plant lane No. LC420461-B7, LC420461-B10, LC420461-B15, LC420461-B22 and LC420461- B27. pBI121-*iceA1* plasmid was used as positive control (C).

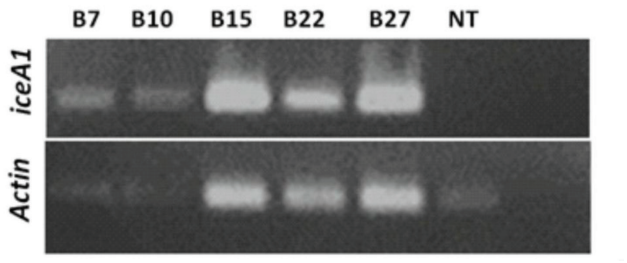


Figure 3: Image showing the real-time PCR amplification of *iceA1* gene from cDNA extracted from both control and transgenic plants. PCR products were run on 2% agarose gel. 100bp ladder was used as a molecular marker (not shown). Bottom: HKG of B7, B10, B15, B22 and B27 and NT respectively. Top: *iceA1* amplification of B7, B10, B15, B22 and B27 and NT respectively.

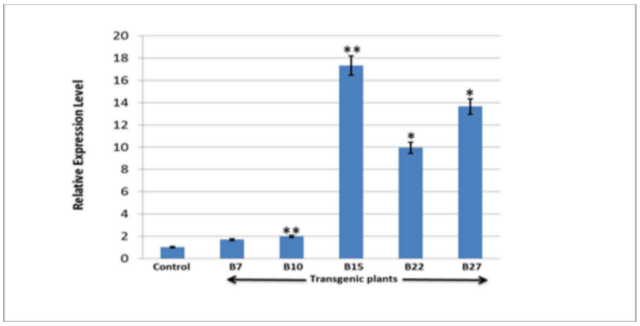


Figure 4: Graph showing the relative expression levels of *iceA1* gene of the transgenic and control plants. Control expression was considered to be 1 and 100%. All the values are average of Duplicates. Values are expressed as value ± SD. ** Significant at 5% (0.05) and * Significant at 10% (0.10) level.

were found to be overexpressed than B7 and B10. *iceA1* gene was reported to be overexpressed in samples B15 and B27 with 17.329 ± 0.42466 and 13.646 ± 0.416804 respectively followed by B22 (9.954 ± 0.284). B7 and B10 although showed response, but the expressions were very little when compared to B15, B27 and B22 [Figure 4]. These values are in accordance to the band expression on the gel. The result shows that F- value (0.002**) is significant. Therefore, equal variance is not assumed. As the p-value (0.064*) is significant at 10% level, we rejected the null hypothesis and concluded that the relative expression levels of brinjal transgenic *IceA1* plants were significantly greater than the relative expression levels of the control plant. There was a marginal difference in the average relative expression of Brinjal transgenic *iceA1* plants and the relative expression level of the control plant and the difference was statistically significant. The result showed that the t- value was significant for four pairs, B10 *iceA1* - control (0.013**) and B15 *iceA1* - control (0.007**) at 5% level and B22 *iceA1* - control (0.101*) and B27 *iceA1* - control (0.059*) at 10% level whereas B7 *iceA1* - control (0.232) was found to be insignificant [Figure 5]. As the p-value is significant at 5% and 10% level we rejected the null hypotheses and conclude that there is a statistically significant difference between B10, B15, B22, B27 *iceA1* plants and the control plant.

Expression of *iceA1* protein in transgenic brinjal plants

The main objective of our study was to produce *iceA1* proteins within the brinjal plant, *Solanum melongena*

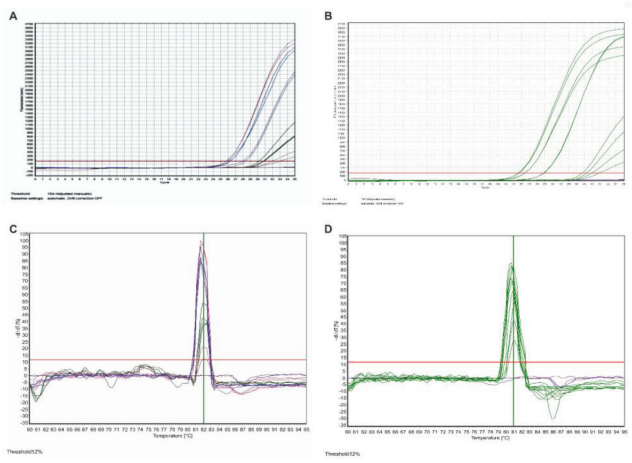


Figure 5: Images showing the Ct and melt curves obtained from quantitative real-time PCR. A, B: Ct curves of Actin and *iceA1* gene respectively; C, D: Melt curves of Actin and *iceA1* gene respectively. All the values are average of duplicates.

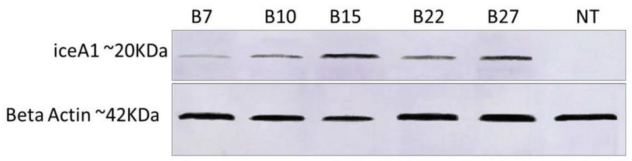


Figure 6: Image showing the western-blot analysis of *iceA1* from independent transgenic plants and control. NT: Non-transgenic plant; B7, B10, B15, B22 and B27 are the transgenic plants. Bands seen at ~20KDa corresponds to *iceA1*. Beta Actin bands (HKG) were seen at ~42KDa.

L. *Cultivar* Arka Keshav. The western-blotting study revealed significant amounts of *iceA1* protein produced in transgenic brinjal. The blot of protein obtained from the non-transgenic or control showed no protein band at the respective molecular size confirming negative expression. On the other hand, transgenic lines B7, B10, B15, B22 and B27 suggested that is a strong positive correlation between the transcripts and protein level of *iceA1* among the transgenic brinjal. The B15 and B27 samples showed higher expression of the *iceA1* gene compared to the other samples [Figure 6]. The expression of *iceA1* protein obtained from the western-blot analysis was found to be in concordance with the qRT-PCR data.

DISCUSSION

A wide variety of expression systems have been used previously to produce therapeutic proteins in plants, with mostly partial success.[33] Recombinant therapeutics like vaccines, which are cheaper than conventional vaccines, are created from leaves, vegetables, and fruits of plants.[34] In humans, *Helicobacter pylori* is one of the most prevalent bacterial strains causing peptic ulcers and gastric cancer in more than 40 to 50% of cases.[35] *H. pylori*, is known to colonize the stomach lining with gram-negative strains and cause chronic gastritis and ulcers, both of which may lead to adenocarcinoma and lymphoma.[36,37] To elicit an immune response against deadly diseases, vaccines are inactivated protein suspensions from bacteria or viruses.[2] Through the immune system, they help the body detect foreign antigens. Recent decades have seen an increase in the production of plant-based vaccines, not only to reduce harmful effects of traditional vaccines, but also to minimize the costs.[38,39] Plants have been genetically altered to produce biotechnology vaccines that can be injected into tissues without affecting immune function.[40] *iceA1* protein was manufactured by the brinjal plants in this study.

As far as to our knowledge this is the first report of expressing *iceA1* proteins in brinjal plants. Although we still must investigate the immune response capacity of brinjal plants by immunization experiments with mice, the results suggest that the *iceA1* protein can be expressed in brinjal plants. *Agrobacterium* has been shown to transform rice plants with the *ureB* gene, and this has been confirmed by gene amplification and blotting.[41] As described above, we used brinjal plants to express the *iceA1* gene and further evaluated this using RT-PCR and western blotting. *iceA1* gene amplification and SDS-PAGE both established the role of the *iceA1* gene in the plant leaves confirming the transgenic lines. Additionally, the immune blot assay revealed the presence of *iceA1* protein within the leaf tissues. Despite the availability of many antibiotics to treat Helicobacter infections, many of these strains have become resistant and have forced scientists to develop a new vaccine candidate.[32] Gastric cancers are predominantly caused by *H. pylori* in both developing and developed nations.[42] Transgenic plants produce therapeutic proteins cheaply, which reduces contamination from animal viruses as well as production costs.[43] Genes encoding *iceA1* antigen was cloned into binary vector pBI121 and transformed into brinjal callus using a kanamycin resistance marker gene.

From the 46 plants, 5 had been identified as transgenic, and this was further confirmed in the expression studies. RT-PCR and Immunoblot analysis confirmed the presence of the *iceA1* gene in the five plants. According to the results of our experiments, the *iceA1* transgenic brinjal can be further studied as a vaccine candidate against *H. pylori*. *iceA1* gene was found to strongly expressed in B15 and B27 samples with 17.329 ± 0.42466 and

13.646 ± 0.416804 respectively, which was significantly higher than similar studies.

Numerous studies are being conducted currently to generate edible vaccines from ready-to-eat fruits and vegetables to follow the oral route of administration.[2] Transgenes are generally used to make edible vaccines by introducing them into plants. Furthermore, these vaccines are more affordable and safer than conventional vaccines on the market.[44] Our work conclusively shows that the transgene is fully integrated into the brinjal plant's nuclear genome without any limitations. Based on our studies, it appears that this methodology could be used to express a number of valuable proteins.

CONCLUSION

In the current investigation, we transformed brinjal plants with the *iceA1* gene of *Helicobcater pylori* and evaluated it level of expression among the leaves of the plants. Out of the 46 plants, B7, B10, B15, B22 and B27 were found to be transgenic with *iceA1* gene expressed successfully, and further we evaluated the expression levels using real time PCR and western blotting. RNA extracted and cDNA synthesized were qualitatively screened and used for the evaluation studies. Analyses by real time PCR clearly indicated that the *iceA1* gene had been successfully transformed in transgenic brinjal plants among the five transgenic lines. B15 and B27 samples. Analyses by western blot indicated that the integrated *iceA1* gene was expressed in all the five lines of transgenics but more expressive were B15 and B27 samples.

The present study only demonstrated that the *iceA1* gene was transformed and expressed within the transgenic brinjal plants.Further studies need to be done on their stable expression for generations so as to be applied as edible vaccines.

ACKNOWLEDGEMENT

The authors acknowledge the support from JSS Research Foundation, JSS Technical Institutions Campus, Mysore; Genei Laboratories Pvt Ltd, Bangalore; Department of Biotechnology and Crop Improvement, Postgraduate Centre, College of Horticulture, University of Horticulture Sciences Campus, GKVK, Bangalore and Postgraduate Department of Biotechnology, JSS College, Mysore.

CONFLICT OF INTEREST

The authors declare no potential conflicts of interest.

ABBREVIATIONS

WHO: World Health Organization; **MALT:** Mucosa Associated Lymphoid Tissue; **PCR:** Polymerase chain reaction; **TE buffer:** Tris EDTA buffer;**HCl:** Hydrochloric acid;**BAP:** 6-Benzyl Amino Purine;**NAA:** Naphthalene acetic acid; **PUD:** Peptic ulcer disease.

Author contributions

MJM, BYSK and RB conceived and designed the study, MJM and RB performed the experiments, MJM, BYSK, NH, RB, BF and SP analyzed the data, MJM and SP drafted the manuscript and all the authors reviewed the manuscript.

Ethics approval

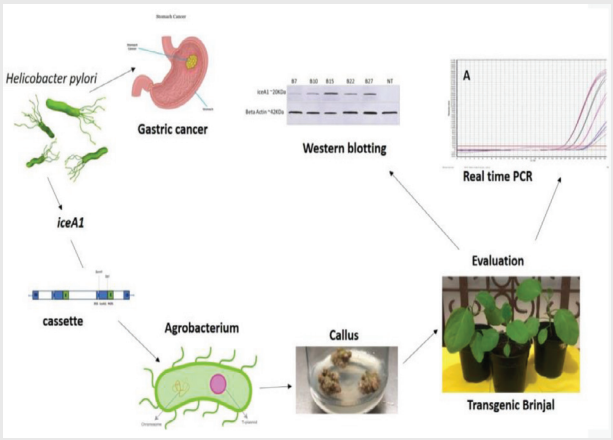
IBSC Registration number: JSSC111220191080

REFERENCES

1. Mehran MJ, Zendehbad SH, Malla S. Cloning and expression of a partial UreA antigen for the production of vaccine against *Helicobacter pylori*, the risk factor for gastric cancer. Asian J Pharm Clin Res. 2014;7:111-7.
2. Kurup VM, Thomas J. Edible vaccines: promises and challenges. Mol Biotechnol. 2020;62(2):79-90. doi: 10.1007/s12033-019-00222-1, PMID 31758488.
3. Muzaheed. *Helicobacter pylori* Oncogenicity: Mechanism, Prevention, and Risk Factors. ScientificWorldJournal. 2020;2020:3018326. doi: 10.1155/2020/3018326. PMID 32765194.
4. Ciftci IH, Uslan I, Dilek FH, Aşık G, Ozgür MA, Dilek ON. Investigation of *Helicobacter pylori* iceA1 and iceA2 genes in patients with chronic gastritis and gastric cancer. Mikrobiyol Bul. 2011;45(2):228-33. PMID 21644065.
5. Abu-Taleb AMF, Abdelattef RS, Abdel-Hady AA, Omran FH, El-Korashi LA, Abdel-Aziz El-Hady H, El-Gebaly AM. Prevalence of *Helicobacter pylori* cagA and iceA genes and their association with gastrointestinal diseases. Int J Microbiol. 2018;2018:4809093. doi: 10.1155/2018/4809093, PMID 29849647.
6. Yamaoka Y. Mechanisms of disease: *Helicobacter pylori* virulence factors. Nat Rev Gastroenterol Hepatol. 2010;7(11):629-41. doi: 10.1038/nrgastro.2010.154, PMID 20938460.
7. Yakoob J, Abbas Z, Khan R, Salim SA, Abrar A, Awan S, Ahmad Z. *Helicobacter pylori*: correlation of the virulence marker iceA allele with clinical outcome in a high prevalence area. Br J Biomed Sci. 2015;72(2):67-73. doi: 10.1080/09674845.2015.11666799, PMID 26126322.
8. Shiota S, Suzuki R, Yamaoka Y. The significance of virulence factors in H elicobacter pylori. J Dig Dis. 2013;14(7):341-9. doi: 10.1111/1751-2980.12054, PMID 23452293.
9. Amjad N, Osman HA, Razak NA, Kassian J, Din J, bin Abdullah N. Clinical significance of *Helicobacter pylori* cagA and iceA genotype status. World J Gastroenterol. 2010;16(35):4443-7. doi: 10.3748/wjg.v16.i35.4443, PMID 20845512.
10. Cheng C, Chen Z, Zhu C. Construction of plant expression vectors with fusion gene of *Helicobacter pylori* cagA, ureB and ctb and its genetic transformation in tobacco. Wei sheng wu xue bao. 2007;47(1):29-33. PMID 17436619.
11. Sabalza M, Christou P, Capell T. Recombinant plant-derived pharmaceutical proteins: current technical and economic bottlenecks. Biotechnol Lett. 2014;36(12):2367-79. doi: 10.1007/s10529-014-1621-3, PMID 25048244.
12. Gachon C, Mingam A, Charrier B. Real-time PCR: what relevance to plant studies? J Exp Bot. 2004;55(402):1445-54. doi: 10.1093/jxb/erh181, PMID 15208338.
13. Bustin SA, Nolan T. Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction. J Biomol Tech. 2004;15(3):155-66. PMID 15331581.

14. Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible WR. Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. Plant Physiol. 2005;139(1):5-17. doi: 10.1104/pp.105.063743, PMID 16166256.
15. Chuaqui RF, Bonner RF, Best CJ, Gillespie JW, Flaig MJ, Hewitt SM, Phillips JL, Krizman DB, Tangrea MA, Ahram M, Linehan WM, Knezevic V, Emmert-Buck MR. Post-analysis follow-up and validation of microarray experiments. Nat Genet. 2002;32;Suppl:509-14. doi: 10.1038/ng1034, PMID 12454646.
16. Streatfield SJ, Howard JA. Plant production systems for vaccines. Expert Rev Vaccines. 2003;2(6):763-75. doi: 10.1586/14760584.2.6.763, PMID 14711360.
17. Wei Q, Du L, Wang W, Hu T, Hu H, Wang J, David K, Bao C. Comparative transcriptome analysis in eggplant reveals selection trends during eggplant domestication. Int J Genomics. 2019;2019:7924383. doi: 10.1155/2019/7924383, PMID 31211132.
18. Kalloo G 1993. Eggplant: Solanum melongena L. genetic improvement of vegetable crops, 587-604.
19. Meyer RS, Karol KG, Little DP, Nee MH, Litt A. Phylogeographic relationships among Asian eggplants and new perspectives on eggplant domestication. Mol Phylogenet Evol. 2012;63(3):685-701. doi: 10.1016/j.ympev.2012.02.006, PMID 22387533.
20. Hirakawa H, Shirasawa K, Miyatake K, Nunome T, Negoro S, Ohyama A, Yamaguchi H, Sato S, Isobe S, Tabata S, Fukuoka H. Draft genome sequence of eggplant (Solanum melongena L.): the representative solanum species indigenous to the old world. DNA Res. 2014;21(6):649-60. doi: 10.1093/dnares/dsu027, PMID 25233906.
21. Mishiba KI, Nishida K, Inoue N, Fujiwara T, Teranishi S, Iwata Y, Takeda S, Koizumi N. Genetic engineering of eggplant accumulating β-carotene in fruit. Plant Cell Rep. 2020;39(8):1029-39. doi: 10.1007/s00299-020-02546-8, PMID 32382812.
22. Prakash D, Deepali B, Asokan R, Ramachandra Y, Shetti D, Anand L, Hanur VS. Effect of growth regulators on in vitro complete plant regeneration in brinjal. Indian J Hortic. 2008;65:371-6.
23. Patel M, Dewey RE, Qu R. Enhancing Agrobacterium tumefaciens-mediated transformation efficiency of perennial ryegrass and rice using heat and high maltose treatments during bacterial infection. Plant Cell Tissue Organ Cult (PCTOC). 2013;114(1):19-29. doi: 10.1007/s11240-013-0301-7.
24. Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG. Primer3—new capabilities and interfaces. Nucleic Acids Res. 2012;40(15):e115-. doi: 10.1093/nar/gks596, PMID 22730293.
25. Smith CT, Elfstrom CM, Seeb LW, Seeb JE. Use of sequence data from rainbow trout and Atlantic salmon for SNP detection in Pacific salmon. Mol Ecol. 2005;14(13):4193-203. doi: 10.1111/j.1365-294X.2005.02731.x, PMID 16262869.
26. Palikša S, Alzbutas G, Skirgaila R. Decreased K m to dNTPs is an essential M-MuLV reverse transcriptase adoption required to perform efficient cDNA synthesis in one-Step RT-PCR assay. Protein Eng Des Sel. 2018;31(3):79-89. doi: 10.1093/protein/gzy003, PMID 29608777.
27. Schmidt GW, Delaney SK. Stable internal reference genes for normalization of real-time RT-PCR in tobacco (Nicotiana tabacum) during development and abiotic stress. Mol Genet Genomics. 2010;283(3):233-41. doi: 10.1007/s00438-010-0511-1, PMID 20098998.
28. Expósito-Rodríguez M, Borges AA, Borges-Pérez A, Pérez JA. Selection of internal control genes for quantitative real-time RT-PCR studies during tomato development process. BMC Plant Biol. 2008;8:131. doi: 10.1186/1471-2229-8-131, PMID 19102748.
29. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc. 2008;3(6):1101-8. doi: 10.1038/nprot.2008.73, PMID 18546601.
30. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25(4):402-8. doi: 10.1006/meth.2001.1262, PMID 11846609.
31. Wang W, Scali M, Vignani R, Spadafora A, Sensi E, Mazzuca S, Cresti M. Protein extraction for two-dimensional electrophoresis from olive leaf, a plant tissue containing high levels of interfering compounds. Electrophoresis. 2003;24(14):2369-75. doi: 10.1002/elps.200305500, PMID 12874872.
32. Yang CY, Chen SY, Duan GC. Transgenic peanut (Arachis hypogaea L.) expressing the urease subunit B gene of *Helicobacter pylori*. Curr Microbiol. 2011;63(4):387-91. doi: 10.1007/s00284-011-9991-4, PMID 21833666.
33. Furuta Y, Namba-Fukuyo H, Shibata TF, Nishiyama T, Shigenobu S, Suzuki Y, Sugano S, Hasebe M, Kobayashi I. Methylome diversification through changes in DNA methyltransferase sequence specificity. PLOS Genet. 2014;10(4):e1004272. doi: 10.1371/journal.pgen.1004272, PMID 24722038.
34. Gauntlett JC, Nilsson HO, Fulurija A, Marshall BJ, Benghezal M. Phase-variable restriction/modification systems are required for *Helicobacter pylori* colonization. Gut Pathog. 2014;6:35. doi: 10.1186/s13099-014-0035-z, PMID 25349630.
35. Nešić D, Buti L, Lu X, Stebbins CE. Structure of the *Helicobacter pylori* CagA oncoprotein bound to the human tumor suppressor ASPP2. Proc Natl Acad Sci U S A. 2014;111(4):1562-7. doi: 10.1073/pnas.1320631111, PMID 24474782.
36. Lyon F. s on the evaluation of carcinogenic risks to humans. IARC monograph. Some Ind Chem. 1994;60:389-433.
37. Furuta Y, Kawai M, Uchiyama I, Kobayashi I. Domain movement within a gene: a novel evolutionary mechanism for protein diversification. PLOS ONE. 2011;6(4):e18819. doi: 10.1371/journal.pone.0018819, PMID 21533192.
38. Ma Y, Lee CJ, Park JS. Strategies for optimizing the production of proteins and peptides with multiple disulfide bonds. Antibiotics (Basel). 2020;9(9):541. doi: 10.3390/antibiotics9090541, PMID 32858882.
39. BARZIGAR R, HARAPRASAD N, Kumar BYS, Mehran MJ. Challenges and recent developments associated with vaccine antigens production against *Helicobacter pylori*. Int J App Pharm. 2020;12:45-50. doi: 10.22159/ijap.2020v12i4.37722.
40. Stern AM, Markel H. The history of vaccines and immunization: familiar patterns, new challenges. Health Aff (Millwood). 2005;24(3):611-21. doi: 10.1377/hlthaff.24.3.611, PMID 15886151.
41. Gu Q, Han N, Liu J, Zhu M. Expression of *Helicobacter pylori* urease subunit B gene in transgenic rice. Biotechnol Lett. 2006;28(20):1661-6. doi: 10.1007/s10529-006-9141-4, PMID 16912927.
42. Schillberg S, Fischer R, Emans N. Molecular farming of recombinant antibodies in plants. Cell Mol Life Sci. 2003;60(3):433-45. doi: 10.1007/s000180300037, PMID 12737305.
43. Yao J, Weng Y, Dickey A, Wang KY. Plants as factories for human pharmaceuticals: applications and challenges. Int J Mol Sci. 2015;16(12):28549-65. doi: 10.3390/ijms161226122, PMID 26633378.
44. Kalbina I, Engstrand L, Andersson S, Strid A. Expression of *Helicobacter pylori* TonB protein in transgenic Arabidopsis thaliana: toward production of vaccine antigens in plants. Helicobacter. 2010;15(5):430-7. doi: 10.1111/j.1523-5378.2010.00786.x, PMID 21083749.

PICTORIAL ABSTRACT



SUMMARY

This study highlights the role of *iceA1* gene of *Helicobacter pylori* in causing gastric cancer along with pathogenesis among the global population. *iceA1* gene could be cloned and expressed into brinjal plants as to create transgenic lines and potentially use them later as edible vaccines. It was found that *iceA1* gene was strongly expressed within the 5 transgenic lines out of 46 plants which we confirmed it by using qulantitative real time PCR and protein blotting methods. The expression levels were screened and confirmed within the five transgenic lines and further need to be screened for their stable expression for generations. This could surely aid the scientific community to effectively design an edible vaccine candidate for this gene *iceA1*.

About Authors

Mohammad Javad Mehran, is a PhD Research Scholar at JSS Research Foundation, University of Mysore, He worked on transgenics and vaccine, Area of Interests in transgenics, vaccine production, genetics and pharmaceutical biotechnology.

Dr. B. Y. Sathish Kumar, M.Sc., Ph.D., is Assistant Professo and HOD (IC), Postgraduate Department of Biotechnology, JSS College, Ooty Road, Mysore. He worked on Vector Genetics, Stress Physiology, transgenics.

Dr. Nanjundappa Haraprasad, is Professor and Head of Department biotechnology Sri Jayachamarajendra College of Engineering and Deputy Registrar JSS Science and Technology University, he worked on Medical Biotechnology, Biological Control and transgenics.

Rambod Barzigar, is a PhD Research Scholar at JSS Research Foundation, University of Mysore, He worked on transgenics and vaccine, Area of Interests in transgenics, vaccine production, genetics and pharmaceutical biotechnology.

Dr. Bashasab Fakrudin, is Professor and Head of Department of Biotechnology and Crop Improvement college of Horticulture ,UHS campus, GKVK post bangalore. He worked on Genomics, transgenics, crop improvement and genetics.

Dr. Sayan Paul, is Postdoctoral Researcher at Institute for Stem Cell Science and Regenerative Medicine (DBT-inStem). He worked on Genetics, Bioinformatics, Molecular Biology and transgenics.

Cite this article: Mehran MJ, Kumar BYS, Haraprasad N, Barzigar R, Fakrudin B, Paul S. Evaluation of *iceA1* Gene Expression of *Helicobacter pylori* Risk Factor of Gastric Cancer in Transgenic Brinjal. Indian J of Pharmaceutical Education and Research. 2021;55(4):1197-206.