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Improvement of *Lactuca sativa* slat Tolerance by Plastid Transformation with BADH Gene

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ABSTRACT

Salinity is one of the major factors that limits geographical distribution of plants and adversely affects crop productivity and quality. Here high-level expression of betaine aldehyde dehydrogenase (BADH) was reported in cultured explants of lettuce via plastid genetic engineering. Lettuce (Lactuca sativa) plant was primarily experimented for tolerance of betaine aldehyde (BA) and soudium chloride (NaCl) by tissue culture technique and it was found that the wild type lettuce tolerated 10 and 75 mM from each substance respectively. Genes required in this study were amplified by polymerase chain reaction (PCR) technique using specific forward and reverse primers, and these genes were BADH, prrn promoter and many other regulatory genes. Some of these genes were isolated from their hosts and some were obtained from previous work available at Daniell laboratory. All these genes beside many techniques for ligation, extension, sequencing, orientation confirmation were used to construct the cassette vector pLS-BADH-LS which carries the gene of interest. Homoplasmic transgenic plants exhibiting high levels of salt tolerance were regenerated from bombarded cell cultures via somatic embryogenesis. Transgenic lettuce plants expressing BADH grew in the presence of high concentrations of NaCl (up to 150 mM), the highest level of salt tolerance reported so far among genetically modified lettuce, and the tolerance to betaine aldehyde was 30 mM.

Introduction

Salt stress and drought are major abiotic stresses in agriculture. The problem of soil salinity has been compounded by irrigation and excessive use of fertilizers. About 20% of the world‘s irrigated lands are affected by salinity[1]. High salinity causes ion imbalance, toxic levels of cytoplasmic sodium, and drought stress. Plants utilize a number of protective mechanisms to maintain normal cellular metabolism and prevent damage to cellular components [2]. One of the metabolic adaptations to salt stress is the accumulation of osmoprotectants. Glycine betaine and *b*-Ala betaine are quaternary ammonium compounds that accumulate in many plant species in response to salt stress[3]. Glycine betaine protects the cell from salt stress by maintaining an osmotic balance with the environment and by stabilizing the quaternary structure of complex proteins[4]. Betaine aldehyde dehydrogenase (BADH) enzymes are classified as substrate-specific oxidoreductases (EC 1.2.1.8) and belong to family 10 of the large superfamily of aldehyde dehydrogenases[5]. BADH catalyse the oxidation of betaine aldehyde to betaine. Trimethylglycine was the first betaine to be characterised and is by far the most extensively studied; this compound is now referred to as glycine betaine‘ (GB) to distinguish it from other betaines. In plants, the study of betaine has almost exclusively focused on GB which is known to be particularly effective in conferring protection against abiotic stresses such as salt, water deficit, heat and chilling [6]. In general terms, a plant BADH refers to an enzyme that converts BA to GB, using an oxidising co-factor.

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Glycine betaine is widely distributed in bacteria, algae, higher plants (e.g., sugar beet and cotton) and animals, and is frequently detected in those plant species that are exposed to drought and salinity stresses [3,7]. However, not all plants accumulate GB, and it has been suggested that this is due to the lack of a functional CMO [8]. Initial enzyme activity studies and molecular cloning of a plant *BADH* were performed in spinach [9]. Subsequently, numerous putative *BADH*s have been isolated based on the homology of these genes to the spinach *BADH* gene. This has led to the classification of many putative *BADH*-encoding genes without substrate specificity and enzyme activity data for the enzyme that they encode. So far, *BADH* has already been cloned from spinach (*Spinacia oleracea* L.) [9], sugar beet (*Beta vulgaris* L.), *Atriplex hortensis*, L. barley (*Hordeum vulgare* L.), sorghum (*Sorghum bicolor*), rice(*Oryza sativa* L.), *Amaranthus hypochondriacus* L., mangrove [*Avicenniamarina* (Forsk.) Vierh., *A. centralasiatica* [10]. This gene was also expressed in transgenic tobacco *(Nictiana tabacum*), which grows normally in a medium containing 1.2% (205 mM) NaCl. Lettuce (*Lactuca sativa* NC_007578) is a member of the Asteraceae family. Thousands of species are included in this large family. The family was originally referred to as compositae because the species seemed to share a compact structure often sprouting in the shape of a head . *Lactuca* means 'milk forming', *sativa* means 'common'. Lettuce is considered 'milk forming' because of the creamy substance that is often found when you snap the stem [11]. The plant is an important commercial vegetable crop cultivated worldwide in a diverse range of environments, with USA, Spain, Italy, Japan and France as the main producer countries [12]. This study conducted to express BADH gene isolated from spinach to the edible plant lettuce

Materials and Methods

Media preparation:-

Lettuce regeneration medium was prepared as described in manufactured procedure and poured in plates, after seed germination for 20 days. Young and fully expanded leaves (~4 cm2) which grown aseptically were taken and spliced to small pieces (0.5*0.5) cm and cultured adaxial side down in tissue culture plates and incubated for 16:8 hrs photoperiod (light:dark)at 25°C. Different concentrations of NaCl were prepared, (25, 50, 75, 100, 125, and 150 mM) mixed with LRM with plant hormone in concentration efficient for direct regeneration and the explants of lettuce were cultured to examine the tolerance to salt. Also the same procedure was applied to betaine aldehyde but the concentration used were (1, 5, 10, 25, and 50 mM) .All plates with different concentration of NaCl and BA were cultured with explants of healthy lettuce leaves 0.5 cm2 and incubated at 25°C.

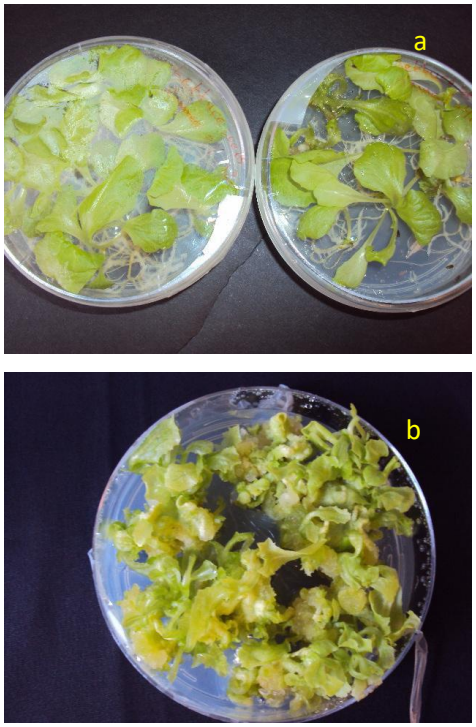
Vector construction:-

The pUC-based *L. sativa* long flanking plasmid (pLS-LF) was used to integrate foreign genes into the intergenic spacer between tRNA-Ile and tRNA-Ala genes of the plastid genome inverted repeat region. A transformation cassette for the generation of transplastomic *L. sativa* plants that express BADH from the T7 gene translational control region was transferred to pLS-LF from pZERO (Invitrogen, Carlsbad, CA). The cassette included the following published *N. tabacum* plastid regulatory sequence elements: ribosomal operon promoter (P*rrn*), *psbA* 3' UTRs. The *BADH* gene was included conferring salt tolerance and was expressed via a GGAGG ribosome binding site. All digest products (vectors and inserts) were separated by electrophoresis through 0.8% agarose-TAE (400 mM Trisacetate, 10 mM EDTA) gels containing 1ug mL-1 ethidium bromide. Gel fragments were isolated using under UV illumination

and DNA were eluted from gel using the QIAquick gel extraction kit. Plasmid products of T4 ligase mediated reactions were transformed into *E. coli* according to standard protocols . The expression cassette was digested with SnaBI. pLS-LF was digested with PvuII and treated with alkaline phosphatase prior to ligation with the SnaB1 digested cassette. Recovered plasmids were digested with restriction enzymes to determine correct orientation of the inserted cassette in pLS-LF. Nucleotide sequence of the intermediate plasmid was confirmed. The complete detailed of construction of vector and isolation of genes will be mentioned in the result section.

Result and discussion:

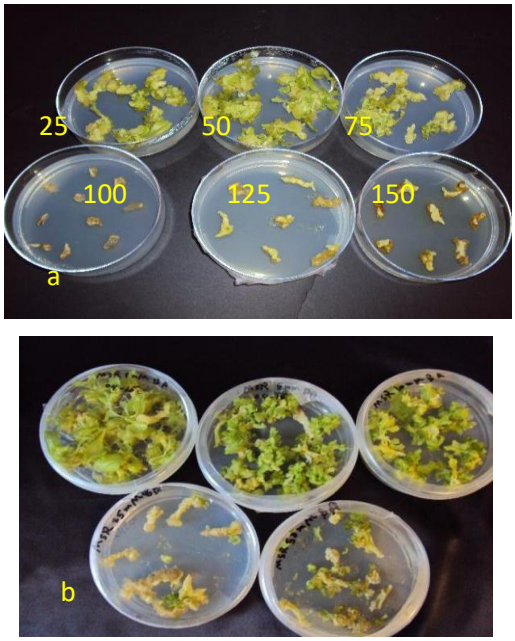
Germination occurred after 10 days growth on 1/2 strength MS medium (fig 1 A). Lettuce seeds were obtained from the store of Burnett School of Biomedical Science, University of Central Florida, USA. Results indicated that most of seeds were geminated in 1/2 strength medium containing thymine and myo-inositol at pH 5.8 after one week of incubation at 25 °C under 16:8 hrs(dark :light) photoperiod, plantlets then were successfully transferred to light box bottle and the resulted leaves were used for tissue culture and transformation experiments. Leaves formed on seedling were dissected (figure 1 B) to small pieces of (5*5 mm), regeneration ability of lettuce explants to form complete plant was tested on MS medium supplemented with NAA and BAP, indicates regeneration occurred starting with shoot formation after 10 days of culture. Shoots were transferred to another petridish for rooting and plantlets were obtained after 30 days, and thus plants were ready to be transferred to a green house .



Figure(1) Tissue culture of lettuce seeds and explants (a) lettuce seeds germination (b) lettuce explants germination

Results showed that low concentrations of NAA and BAP (0.1 and 0.2 mg/l) respectively were needed for regeneration. However, Regeneration were from leaves explants grown in such concentration of NAA and BAP occurred during the first 10 days, and the number of formed shoots increased at 20th day of culture. The frequency of adventitious shoots dying increased as the period of culture proceeded, ranging from 5 % (day 20) to 33% (day 40). Seed germination is known to be controlled by a variety of internal and external factors, and some seeds have specific requirements for germination**.** Organ-forming potential is influenced by a variety of factors including genotype, age and physiological status of the donor plant, culture medium, culture environment including light, temperature, and atmosphere, as well as the phytohormones used [13]. Lettuce explants grown on MS medium containing NAA and BAP were tested using different concentrations of NaCl (25,50,75,100,125, or 150) mM under optimum growth conditions (figure 2 A). Results obtained from this experiment indicated

that explants are able to grow in a medium containing 75 mM of NaCl but failed to regenerate in a medium supplemented with 100 mM of NaCl. It was also noticed that, growth efficiency in the presence of 50 mM of NaCl was more than those grown on 25 mM. Some explants tended to form callus in presence of NaCl rather than direct regeneration despite the presence of appropriate concentrations of plant growth regulators. Such finding was used in further experiments concerning the transgenic plant ability to tolerate NaCl. When five concentrations of betaine aldehyde were added to the growth medium to evaluate the ability of lettuce leaf explants to resist this compound, results showed that the plant was able to resist betaine aldehyde up to 10 mM and died at 25 mM (figure 2B). In contrast, the concentration 1mM concentration had no effect on the explants growth, while concentrations up to 5 and 10 mM led to callus growth rather than direct shoot regeneration. After the required genes were isolated, other steps of the project were started such as the construction of shuttle vector and cassette vector containing *BADH, prrn* promoter, all regulatory genes, and the specific restriction site. To achieve such steps, precise work regarding many PCR processes, restriction, ligation, sequencing, and transformation in *E. coli* were performed. Vector construction started with the ligation of *BADH* gene with *prrn* promoter previously isolated using *BADH* forward and *prrn* reserve primers formally designed in their isolation. The product was about 1700 bp DNA segment eluted and used to complete the shuttle vector (fig 3). *Trps*16(*rbcl*) 150 bp was amplified from genomic DNA of lettuce and used as transcription termination and enhancing the translation, and was ligated with *prrn, BADH*. All these products were ligated in pBs vector available at Professor Daniell laboratory. This vector contains ampicillin resistance gene, and therefore, it was able to transform *E. coli* with this vector to propagate new vector and to ensure the right work . The complete genetic map for vector is detailed in the figure 3.



Figure(2) (a) Effect of NaCl on the lettuce explants (b) effect of betaine aldehyde on lettuce explants.

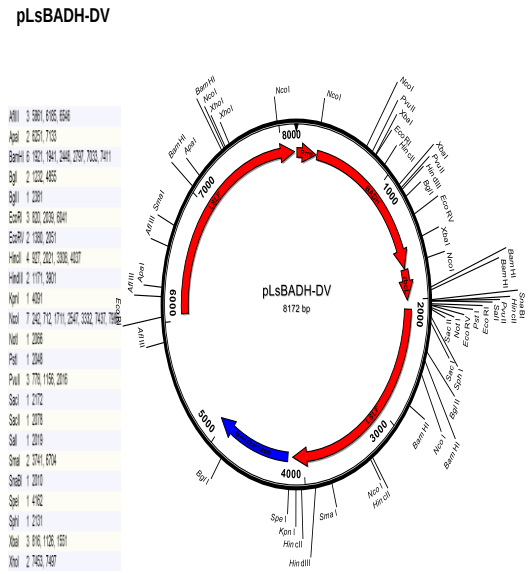


Figure (3) complete genetic map for cassette vector contain BADH gene

The constructing vector was bombarded into lettuce leaves cultured in lettuce regeneration media containing NaCl and betaine aldehyde and concentration above the MIC which indicated previously. The result indicated that explants were

able to tolerate concentration 35mM betaine aldehyde and 150mM NaCl (figure 4 a and b) and these explants were tested to ensure that they were transformed with BADH gene by PCR technology the resulted bands show the presence of BADH gene with molecular weight about 1700 bp in isolated DNA for expected transgenic plantlets figure (5).

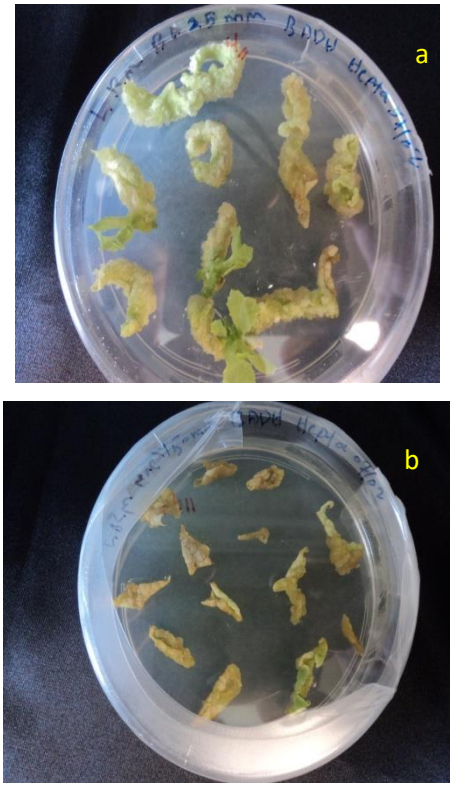


Figure (4) expected transgenic plant (a) on lettuce regeneration medium contain 35 mM Betaine aldehyde (b) on the same medium contain NaCl 150 mM

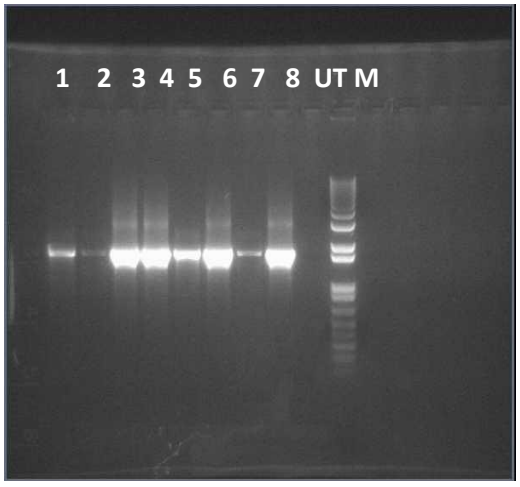


Figure (5) The transgenic plant confirmation by PCR technology using BADH specific primers lane M mleculare marker, lane UN untransformed plant, 1-8 transgenic plant

Chloroplast transformation strategies have utilized both endogenous and heterologous regulatory elements to facilitate high levels of foreign gene expression. Hybrid systems comprising a modified tobacco (*Nicotiana tabacum*) ribosomal operon promoter (*Prrn*) in conjunction with a translational control region derived from the tobacco plastid-encoded rbcL gene or from bacteriophage T7 gene 10 (g10) to express foreign genes have been utilized in numerous species [14]. Incorporation of 2900 bp foreign DNA is based on homologous recombination between the targeting region of the vector and the ptDNA. The transformation vectors are *E. coli* plasmids that do not replicate in plastids. The marker gene encoded in the vector will be stably expressed only if incorporated in the plastid genome by homologous recombination [15].

The choice of the insertion site in the plastome may have a profound effect on the level of protein accumulation. Inserting a transgene in the repeated region of the ptDNA doubles the number of transgene copies per genome, as compared with insertions in unique regions. Insertion of transgenes between genes of a heavily transcribed operon will further increase the level of translatable mRNA, typically yielding higher protein levels [16].We recommended that researcher work to apply another salt tolerance gene to other plant or the lettuce to overcome the salinity problem increased in this time.

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تحسين تحمل الملوحة لنبات الخس بطريقة التحول الوراثي للبلاستيدات بجين BADH

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الخلاصة:-

تعد الملوحة التي تقلل الانتشار الجغرافي الجيد للنبات وتؤثر بشكل مباشر في الانتاج الكمي والنوعي للمحاصيل. تناولت هذه الدراسة التعبير الجيني لجين BADHفي نبات الخس للحصول على نبات متحمل للملوحة العالية باستخدام طريقة مسدس الجينات من خلال تقنية الهندسة الوراثية للبلاستيدات الخضراء لنبات الخس. في البداية تم دراسة تحمل الزرع النسيجي للنبات لملح كلوريد الصوديوم ومادة البيتايين الديهايد NaCl, Betaine aldehyde ووجد ان النبات يتحمل 75mMو 10mM من كلا المادتين وبالتتابع. عزلت الجينات المطلوبة لهذا العمل باستخدام تقنية تفاعل استطالة السلسلة PCR لجين BADH والمعزول من نبات السبانغ المعروف بمقاومته لتراكيز ملحية عالية و Prrn وهو حفاز معروف لاستنساخ الجين وعدد من الجينات الاخرى. استخدمت تقنية تفاعل استطالة السلسلة بجانب عدد من التقنيات الاخرى مثل عملية اللحم وتحديد التتابعات الوراثية وتحديد اتجاه الجينات لصنع الناقل الوراثي المحتوي على الجين المطلوب pLs BADH Ls. عبرت النباتات المتحولة وراثيا والمتماثلة في الحصول على الجينات عن تحمل عالي لملح كلوريد الصوديوم بلغ 150mMاي ضعف تحمل النبات الاصلي وهو اعلى نسبة تحول مسجل لنبات خس مهندس وراثيا كما بلغ التحمل لمادة البيتايين الديهايد 30mM.