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RESEARCH ARTICLE

Characterization, Transformation and Stable Expression studies of Sucrose Synthase Gene promoter in *Nicotiana Tabacum*

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Abstract

The present study is focused on stable transformation using *GUS* expression cassette under selected sucrose synthase gene promoter in *Nicotiana tabacum* via *Agrobacterium* mediated transformation. The availability of a broad spectrum of promoters helps to regulate the temporal and spatial expression patterns of transgenes. The Agrobacterium mediated genetic transformation of plants is an important technique used to transfer foreign gene into the host plant genome. The expression of transgenes is regulated by the promoter attached upstream to the gene. Promoters are important in the control of the overall expression profile of a gene. The selected *Sucrose Synthase gene* promoter was previously cloned in plant expression vector (pGA482). Further, *GUS* expression cassette was verified through restriction analysis and PCR amplifications. The PCR positive tobacco transgenic plants for *Sucrose Synthase* expression cassette was obtained and stained to check the *GUS* expression levels.

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Introduction:-

Sucrose synthase (*SuS*) is a key enzyme for sucrose metabolism in plant cells providing carbon for respiration and synthesis of cell wall polymers and starch. Since *SuS* is important for plant cell growth, its structure, localization and features are useful for defining the relationships between nutrients, growth, and cell morphogenesis. Sucrose synthase catalyzes the reversible transformation of Sucrose into Fructose and UDP-Glc. Sucrose synthase is found throughout the plant kingdom, and is located mainly in transport and sink tissues. Expression of sucrose synthase varies in different cells, tissues, and plants. Many factors have been found to regulate this expression, such as sugar availability, oxygen concentration, drought, salinity stress, and low temperature. The main activity of *SuS* is restricted to tissues that metabolize Sucrose (Baroja-Fernandez *et al*., 2003; Konishi *et al*., 2004) and monitoring of its activity provides useful information about the transport and consumption of carbohydrates during the development of plant cells and tissues (Wittich and Willemse, 1999). Because of its critical function *SuS* is localized in different cell structures, such as the cytoskeleton (Winter *et al*., 1998), cell membranes (Matic *et al*., 2004) and the tonoplast (Etxeberria and Gonzalez, 2003). The protein is present in two distinct forms. The soluble form of *SuS* is generally involved in the respiration process. The second form of *SuS* is associated with plasma membranes or cell walls and is generally involved in the synthesis of cell wall components providing metabolites for callose synthase and cellulose synthase (Amor *et al*., 1995).

Classes of Sucrose Synthase

Plant sucrose synthases can be divided into four main classes based on their amino acid sequences. Class I comprises enzymes from monocotyledonous plants. In this class, the enzymes can be subdivided into another two subgroups based on homology either to maize SUS1. Classes II, III, and IV are made up of enzymes from dicotyledonous plants. Sucrose synthase proteins in Class II share low amino-acid sequence identity with those in

Class III and Class IV (Komatsu *et al*, 2002; Shaw *et al*., 1994; Sturm *et al*, 1999). However, in general, sucrose synthase proteins are quite similar in structure and function (Koch *et al*., 1992, 1996; Komatsu *et al*., 2002; Shaw *et al*., 1994; Winter and Huber, 2000). Therefore, *SuS* promoter is a good example of constitutive regulation of the *SuS* protein and higher levels of expression in the tissues involved in sugar transport including xylem, phloem and sink tissues rather than other plant tissues.

***Agrobacterium* mediated transformation**

Optimization of gene transformation, using *Agrobacterium tumefaciens* has been central to the cascade of advances in the area of transgenic plants. *Agrobacterium tumefaciens* is a Gram-negative soil pathogen and naturally infects the wounded sites in many of the dicotyledonous plant species. Under natural conditions the infection results into the formation of crown gall tumors (Smith and Townsend, 1907). The bacterium transfers a part of DNA known as transfer DNA or mobile DNA segment (T-DNA) into the nucleus of infected cells along with virulence proteins coding DNA. It is then stably integrated into the host genome and transcribed (Nester *et al.,* 1984; Binns and Thomashaw, 1988). The bacterium genetically transforms several dicots, some monocots and gymnosperms (DeCleene & DeLey, 1976). It may even transform various fungal species as well as human cells (Bundock *et al*., 1995; de Groot *et al*., 1998; Gouka *et al*., 1999; Kunik *et al*., 2001). The *Agrobacterium* mediated gene transformation of plants is an important means due to its natural capability to transfer foreign gene into the host plant genome. The *Sucrose synthase* gene promoter is abundantly expressed in dicot plants. This study was focused on stably transforming expression cassette of selected *SuS* promoter sequence in *Nicotiana tabacum* via *Agrobacterium*-mediated transformation and to perform expression analysis via Histochemical *GUS assay*.

Material and Methods:-

***Agrobacterium*-mediated tobacco transformation**

The *SuS* plant expression construct was transformed in tobacco through *Agrobacterium* mediated transformation using LBA4404 as a carrier strain. The experiment was performed under ultra-sterile conditions and the chemicals used in this experiment were appropriately sterilized by filter sterilization. Plasmid DNA of the pGA482 clone having *SuS* promoter cassette was isolated from overnight cultures and plasmid miniprepkit (Fermantas).The isolated plasmids was transformed into electrocompetant cells of agrobacterium strain LBA4404 strain via an electric shock adjusted at1.44K.V by electroporation method. The transformation were spread on LB (Lauria Bertani) agar plates containing Rifampicin (25µg/ml) and Kanamycin(50µg/ml) and were incubated at 28°C for 48 hr. Single colonies were selected and cultured in LB broth containing antibiotics with relative concentrations required for *Agrobacterium* growth. After 48 hours plasmid were isolated by using classical alkaline lysis technique and analyzed by PCR of respective genes. The confirmed clones were then preserved in 30% glycerol for future use.

Selection and regeneration of transformed callus

The glycerol stocks were streaked on LB agar plates containing Rifampicin and Kanamycin antibiotics to get fresh single colonies. From the four *Agrobacterium* clones, single colonies were cultured in 100ml flasks having 30ml LB media containing respective antibiotics at the appropriate concentrations. The cultures were incubated at 28°C with 140 rpm for 48 hours. The same day, leaf disks were obtained from four weeks old tobacco plantlets grown *in vitro.* The leaf disks co-cultivated with *Agrobacterium tumefaciens* were collected, washed 4-5 times with MS liquid medium containing Cefotaxime (250µg/ml) to remove extra growth of *Agrobacterium* and blotted to remove excessive water. About 4-5 leaf disks were placed per petri dish on the shoot selection medium for induction of callus formation and selection. Leaf disks on selection medium were incubated at 25 ± 0.5˚C under 16 hour constant light for two weeks. After 7-10 days, tiny plantlets were started to emerge and non-transformed cells ultimately died because of antibiotic stress. When plantlets developed 3-5 leaves, they were shifted to the jars containing selection media, sealed with cling film and incubated in plant growth room at 25 ± 0.5°C. The putative transgenic and control tobacco plants were routinely observed and noted for their morphological appearance during developmental stages.

Molecular analysis of putative transgenic plants

Young leaves from putative transgenic plants and negative control tobacco plant were selected for DNA isolation by CTAB method. The isolated DNA was re-suspended in 50µl of ultrapure sterile H_2O and stored at -20°C. Positive control plasmid DNA for each construct was isolated by using miniprep plasmid isolation kit (Fermantas). The primers for transgene analysis were designed at specific sites inside the promoter and gene for amplification of junction regions near promoters and genes specific to each construct. PCR reaction setup and profile for amplification of Promoter-Gene junction regions from isolated DNA of transformed tobacco plants are given in

table 1 and 2 respectively. The PCR reactions for transgene analysis were analyzed by electrophoresis on 1% agarose gel containing 0.05% EtBr along with standard 1kb DNA ladder.

Analysis of ß-glucuronidase expression

Histochemical localization of *GUS* enzyme activity was carried out using 5- bromo-4-chloro-3-indolyl-b-D-glucuronide (X-Glc). All types of tissues were covered with X-gluc staining solution, and vacuum infiltrated. The samples were incubated at 37°C in dark for overnight. At the completion of incubation time, the leaves, stems, and roots were treated with different serial dilutions of ethanol (20%, 30%, 40%, 50%, 60% and 70%) to remove the pigmentation due to chlorophyll. The localized expression of *GUS* in all the tissues was intermittently monitored. The detailed observation was carried out with light microscope. Histochemically stained tissues i.e. control and positive transformants were photographed using a microscope attached to a digital microscope camera.

Results:-

***Agrobacterium*-mediated tobacco transformation**

Agrobacterium mediated tobacco transformation of *SuS* gene promoters was carried out using LBA4404 strain of *Agrobacterium tumefacians*. The construct pGASUSP was transformed independently into the *Agrobacterium* (LBA4404) electrocompetent cells by electroporation. The clones was confirmed by PCR using reverse and forward promoter specific primers to amplify the gene promoter.

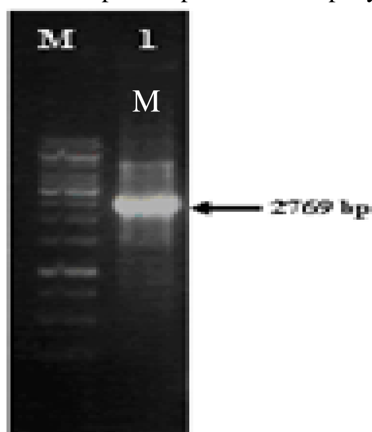

Fig 1: Confirmation of clones in *Agrobacterium*. *Lane* **M:** 1kb DNA ladder **Lane 1:** PCR amplification of *SUS* gene promoter of *Agrobacterium* clone.

Histochemical *GUS* staining

The leaf tissues from transgenic plants were stained for *GUS* activity. Staining patterns of representative leaf tissues for each stably transformed construct in different transgenic events are shown in Figure below. *GUS* assay was carried out on leaf tissues. For this, leaf tissues were dipped in staining solution to monitor color intensity of expression levels in different transgenic events for the selected promoters. The plants tissues expressing *GUS* using *2X35S* and the selected promoter were stained for 24 hours.

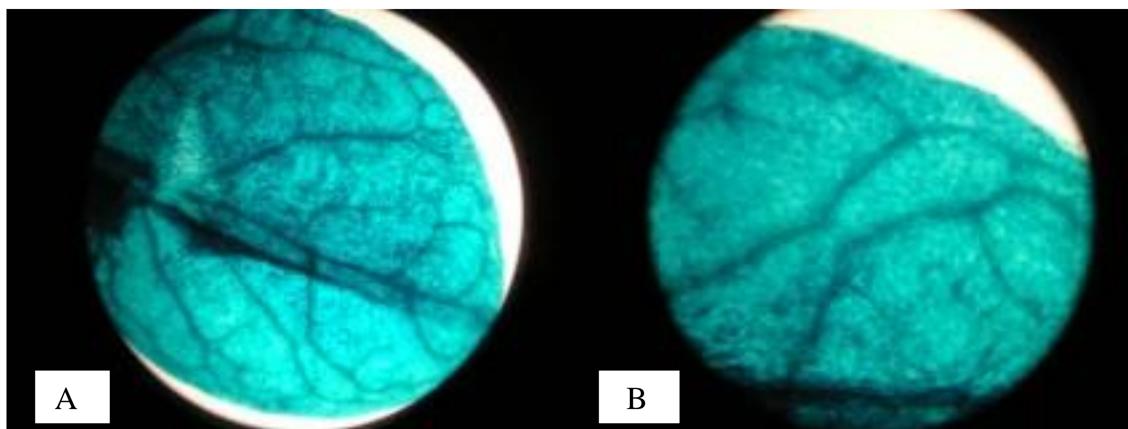

Fig 2: (A) Stable expression analysis for *GUS* activity controlled by *2X35S* promoter. (B) Stable expression analysis for *GUS* activity controlled by *SUS* promoter.

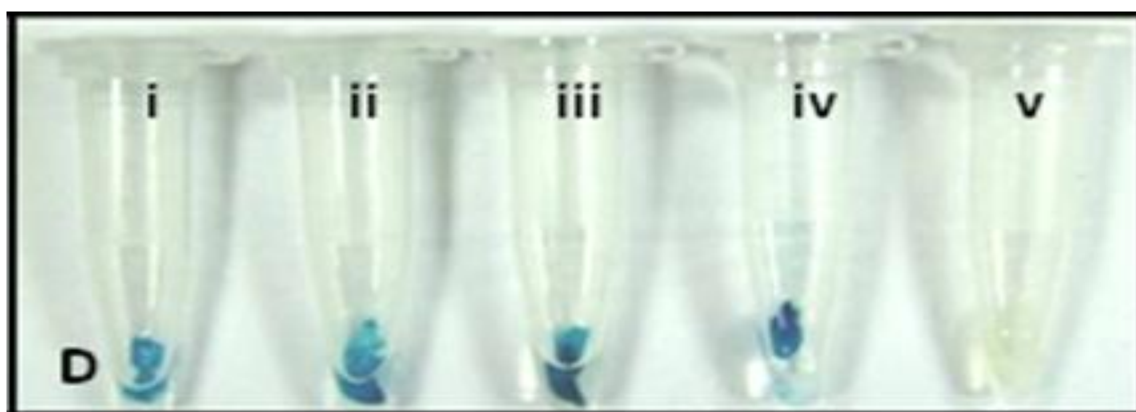

Fig 3: Panel D (i-iii) Leaf tissue stained in different transgenic events for *GUS* activity controlled by *SUS* promoter: (iv) shows *GUS* activity controlled by 2X35S (v) shows non transgenic i.e negative control.

Discussion:-

The study was focused on characterization and transformation of Sucrose synthase gene promoter for its expression analysis in tobacco (*Nicotiana tabacum* L.).The selected promoter sequence was also characterized with respect to specific motifs. The promoter is an important component in a plant transformation vector and is generally patented after its discovery and usefulness. Primary components of promoter are the *cis*-acting regulatory regions. Sucrose synthase is found throughout the plant kingdom and is located mainly in transport and sink tissues. Expression of sucrose synthase varies in different cells, tissues, and plants. Many factors have been found to regulate this expression, such as sugar availability, oxygen concentration, drought, salinity stress, and low temperature. According to some previous studies, sucrose synthase activity in juice sacs of grapefruit has a potentially important role during the early stages of fruit development, when cell division, cell wall synthesis, and respiration rates are maximal. In addition, sucrose synthase is active in transport tissues of fruit (Lowell *et al.*, 1989), to companion cells in vascular bundles (Nolte and Koch, 1993). However, analysis of function and expression of sucrose synthase have been limited. The *GUS* expression studies revealed that the *SuS* promoter actively controlled *GUS* expression in different transgenic events of the stably transformed tobacco plants. The *2X35S* promoter has been reported to exhibit strong constitutive activity in different plant species and the same was observed in our experiments. The relative comparison of the *SuS* promoter with *2X35S* promoter indicated its strong comparable expression in different transgenic events. Hence, the characterized promoter can be used for developing transgenic plants with controlled expression of desired genes.

Conclusion:-

The staining reaction showed that the *SuS* promoter was constitutively expressed in leaves. Longer incubation in the staining solution led to diffusion of the stain but did not reveal any increase of *GUS* activity or spreading of stain in additional tissues. The expression of *GUS* by *SuS* promoter was identified to be comparable with *2X35S* promoter. The staining of leaves from non-transgenic plants did not reveal the development of *GUS* stain.

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