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Identification of Serk Gene from Bract Derived Embryogenic and Non - Embryogenic Calli of Four Diploid Banana Cultivars from South India

Smitha P D (Corresponding author)

Assistant professor, Department of Botany, Sree Narayana College,

Kollam. E-mail: smithadivakaran2009@gmail.com

Binoy K R

Assistant Professor, Department of Botany, Govt. College, Kariavattom

Ashalatha S Nair

Professor, Department of Botany, University of Kerala,

Kariavattom, Thiruvananthapuram 695581, India.

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Abstract

Somatic embryogenesis receptor kinase (SERK) gene is known to be a marker of somatic embryogenesis in several plant species. The present study reported the presence of SERK gene from bract derived embryogenic calli bearing somatic embryos. The analysis of the expression pattern of the SERK gene during embryogenic cell formation and somatic embryogenesis revealed that SERK expression continued during pro embryogenic mass formation. In the present study the amplified product of cDNA from the somatic embryos has molecular size 1459 bp. The non- embryogenic callus also showed the presence of faint bands. In all the samples the amplified product from β - actin primer showed bands of 650 bp with similar intensity in both the embryogenic and non- embryogenic samples.

Keywords: SERK gene, Diploid, Embryogenic, Non- embryogenic calli

1. Introduction

Somatic embryogenesis is the process where by differentiated somatic cells acquire competence and proliferation as embryogenic cells. Initiation of embryogenic pathway is restricted to certain responsive cells that have the potential to activate genes involved in generating embryogenic cells (Quiroz - Figueroa *et al.* 2002). Once these genes are activated, an embryogenic gene expression program replaces the established gene expression pattern in the explant tissue (Quiroz - Figueroa *et al.* 2006). During somatic embryogenesis, somatic cells are induced to form totipotent embryogenic cells capable of regenerating into complete plants. Such developmental switching involves a series of events associated with the molecular recognition of internal signals and external stimuli (Chugh & Khurana, 2002). The perception and response to these events set off various signal cascades, and the downstream pathways followed during the transition of single cells to somatic embryo eventually results in specific gene expression and somatic embryogenesis (Zimmerman, 1993; Chugh & Khurana, 2002; Feher *et al.* 2003). Analysis of gene expression during somatic embryogenesis provide information for better understanding of the process. Additionally somatic embryo models are useful for studying cell differentiation process in plants and for understanding the functional aspects of genes already implicated in somatic embryogenesis (Quiroz - Figueroa *et al*. 2006). It is important to identify a reliable molecular marker system that can be used for improvement of embryogenic competence cultures of banana. Moreover, isolation and cloning of disease resistance (R) genes are needed for introducing resistance to fungal pathogens in banana (Pei *et al*. 2006). Somatic embryogenesis receptor-like kinases (SERKs) are reported to play important roles in the process of somatic embryogenesis, and they belong to the super family of leucine - rich repeat receptor like kinases (LRR- RLK) (Schmidt *et al.* 1997). A number of full - length SERK genes have been isolated, such as DcSERK from carrot (Schmidt *et al*. 1997), AtSERK1 from Arabidopsis (Hecht *et al.* 2001), ZmSERK1 from maize (Baudino *et al.* 2001), and OsB1SERK1 from rice (Song *et al*. 2008).

2. Materials and Methods

Male flower bunches were obtained from adult field - grown banana after maturation of fruit bunch. The bracts with associated hands of male flowers were removed in a step-wise manner until they become too small to be removed by hand. The remaining portion having an approximate size of 4-5 cm length was immersed in 1% (v/v) Labolene® for 6 min and kept under running tap water for 30 min. The explants were surface sterilized in 0.1 % (w/v) mercuric chloride for 4 min followed by three rinses in autoclaved double-distilled water, 5 min for each rinse. Two or three outer protective bracts and corresponding groups of male flowers were sequentially discarded. ~ 1 cm square pieces from the basal region of inner bracts were excised, the male flowers with a single cut and the bract was inoculated on MS medium supplemented with TDZ (0.045-9.00 μM), 2- 4,D (0.45- 4.5 μM), 0. 30gl^{-1} sucrose, 0.7gl^{-1} agar. The $_{p}$H of the media was adjusted to 5.8 before autoclaving at 120 ℃ for 18 min. The cultures were maintained at a temperature of 25±2 ℃ with a photoperiod of 16h/day under 50μmol m^{-2} s^{-1} light intensity provided by fluorescent lamps. RNA was isolated from embryogenic calli regenerated on MS medium with TDZ (0.45 μM), non - embryogenic calli regenerated on MS medium with 2, 4-D (0.45 μM) and tissue isolated from bract meristem

(0.5 gm each) as control from the four diploid *Musa acuminata* cultivars cv. Matti, cv. Sannachenkadali, cv. Chingan and cv. Njalipoovan. The samples were frozen with liquid nitrogen and powdered using a mortar and pestle. Small portions of powdered sample were added to a vial containing 3 ml of extraction buffer (150 Mm Tris base hydroxymethyl - hydrochloride), 2% (w/v) SDS, 100 Mm EDTA adjusted to pH 7.5 with saturated boric acid and 30 µl of β -mercaptoethanol (1%, v/v) . This suspension was quickly mixed using a cut tip to avoid RNA damage and transferred to eppendorff tubes (750 µl per tube), precipitated with 66 µl 5M potassium acetate and 150 µl absolute ethanol, vortexed for 1 min, added 850 µl chloroform - isoamyl alcohol (49:1,v/v), centrifuged at 16,000 g for 20 min and the supernatant collected into an eppendorff tube. The suspension was again vortexed for 10 seconds and centrifuged at 16,000g for 20 min at room temperature. The supernatant were recovered and transferred to new tubes and added 850 µl phenol-chloroform-isoamyl alcohol (25:24:1, v/v/v) per tube, vortexed for 10 sec and centrifuged at 16,000g for 15 min at room temperature. The supernatant was recovered, transferred to new tubes, along with 850 µl chloroform-isoamyl alcohol. The tubes were vortexed for 10 seconds and centrifuged at 16,000 g for 15 min at 4°C. The supernatant was recovered, 12M $LiCl_2$ was added to a final concentration of 3 M, and the tubes with RNA samples were gently mixed by inversion and left to stand overnight at -20 ℃. The samples were centrifuged at 16,000g for 20 min at 4 ℃, the pellets were washed twice with 70% ethanol (500 µl each) and centrifuged again at 16,000g for 10 min at 4 ℃. The pellets were dried at room temperature and resuspended in 10 µl DEPC (Diethyl pyrocarbonate) treated sterile distilled water. The suspension was pooled in a new tube and stored at - 80 ℃ until use. Quantification of RNA samples were done by using Bio-photometer (*Eppendorf's*, Germany). RNA sample (2 µl) was dissolved in 48 µl DEPC treated water and absorbance at 260 and 280 nm was taken. The ratio between 260/ 280 was calculated to assess the purity of samples.

First strand cDNA synthesis was performed from the total RNA isolated from selected tissue samples. Reaction mixture was prepared according to the manufacturer's protocol (*Fermentas*, Lifescience). Each reaction mixture containing DNAse treated RNA sample (20 ng), oligo dT primer (0.5 µg) and the volume adjusted to final (11.5 µl) with DEPC treated water. A quick spin was given for the mixture for 5 sec and incubated at 65 ℃ for 5 min, chilled on ice and given another short spin to collect droplets sticking on the tube. The mixture was kept on ice and 4 µl 5X reaction buffer, 0.5 µl of 20u of Ribo Lock TM ribonuclease inhibitor, 2 µl of 1mM dNTP mixture, 40u of M - MuLV reverse transcriptase (2 µl) were added and the final volume adjusted to 20 µl. The reaction mixture mixed gently by a short spin. This reaction was incubated at 37 ℃ for 60 min and was terminated by heating at 70 ℃ for 10 min. This mixture was stored at -20 ℃.

PCR amplification of specific gene (SERK) was carried out from the synthesized cDNA. The PCR reaction was carried out in a DNA Thermal cycler (*Master cycler gradient, Eppendorf*). Amplification condition were 200 µM dNTP (0.4 µl), 1.25u Taq polymerase (1 µl), 1X Taq polymerase buffer with $(NH_4)_2SO_4$ (2 µl), 25mM $MgCl_2$ (0.8 µl) and 5Mm primer (SERK R and F) (1 µl each) with 20 µg cDNA. The PCR amplification was done by using specific primers, SERK F (5'-ATG TCA CTG ACT AAT ATG ACA ACA CTT CAA G-3') and SERK

R (5'- TGT ACA TGG GTC TCC TTG TAC TCC AT-3') with the following PCR conditions: 94 ℃ (2 min); four cycles at 94 ℃ (30 sec); 45 ℃ (4 min); 72 ℃ (1.5 min); 28 cycles at 94 ℃ (30 sec); 63 ℃ (45 sec); 72 ℃ (1.5min); and 72 ℃ (8 min).

3. Results

To understand and compare the gene expression (SERK) during somatic embryogenesis embryogenic calli induced from bract explant inoculated on MS medium fortified with TDZ (0.45 μM) and non- embryogenic calli induced in the presence of 2,4-D (0.45 μM) and control tissue (bract meristem) were selected. Isolated RNA samples from embryogenic and non-embryogenic calli and bract meristem showed two fragment of 28S and 18S rRNA (Fig. 1). The amplified product of cDNA from all the samples has molecular size 1459 bp (Fig. 2). The amplified product from embryogenic callus showed maximum relative intensity of the segment with 31.35 ng and 30.17 ng in cvs. Matti and Sannachenkadali respectively. Minimum relative intensity (25.89 ng) was observed in cv. Njalipoovan. Non - embryogenic calli showed relative intensity between 26.90 and 29.41ng. The control sample (bract meristem tissue) showed relative intensity 24.76 ng. All the embryogenic samples exhibited higher intensity bands compared to non- embryogenic and control samples. Amplification of all the samples in presence of β - actin primer showed amplified product of 650 bp with similar intensity (Fig. 3).

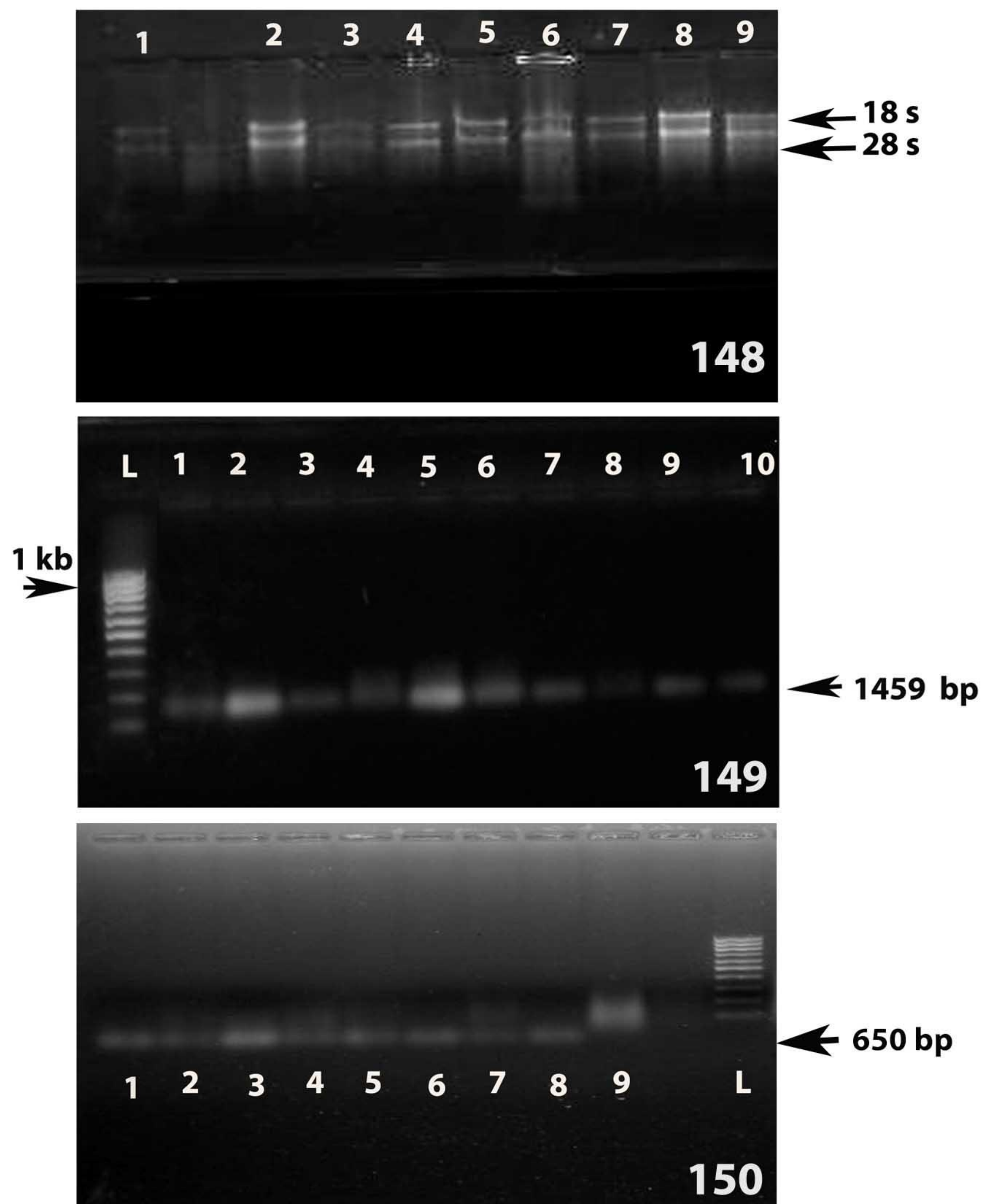

Fig 148. Isolated RNA samples with two bands of 28S and 18S rRNA, (Lane 1- Control, Lane 2-5 non- embryogenic callus from cv. Matti, cv. Sannachenkadali, cv. Chingan & cv. Njalipoovan, Lane 6-9 embryogenic callus from cv. Matti, cv.Sannachenkadali, cv. Chingan & cv. Njalipoovan, Fig 149. Amplified product of cDNA has molecular size 1459 bp, (L- ladder, Lane 1- control, Lane 2- Ec of cv. Matti, Lane 3 & 4- Nec of cv. Matti, Lane 5- Ec of cv. Sannachenkadali, Lane 6- Nec of cv. Sannachenkadali, Lane7- Ec of cv. Chingan, Lane 8-Nec of cv. Chingan, Lane 9- Ec of cv. Njalipoovan, Lane 10- Nec of cv. Njalipoovan), Fig 150. Amplified product of β – actin primer showed 650 bp with similar intensity (Lane 1- 4 Nec of cv. Matti, cv. Sannachenkadali, cv. Chingan & cv. Njalipoovan, Lane 5 -8 Ec of cv. Matti, cv. Sannachenkadali, cv. Chingan & cv. Njalipoovan, Lane 9- Control) (Ec- Embryogenic callus, Nec- Non-embryogenic callus)

4. Discussion

The most challenging aim of current somatic embryo research was to identify genes involved in the induction of embryogenesis competence and subsequent embryo development. Great progress in molecular analysis of plant embryogenesis was noticed in the last few years with the advent of modern molecular tools allowing fast and comprehensive gene expression analysis. Interaction between external stimuli and genes determining embryogenic competence and thus switching on development of embryos in vegetative tissue, was investigated at the molecular level (Feher *et al.* 2003). Understanding gene function and gene expression profile was achieved with several approaches such as analysis of the genome, the transcriptome, the metabolome and the analysis of proteome (Carpentier *et al.* 2007).The study of gene expression and its regulation "transcriptome" was achieved at the level of transcripts (RNA). In *Musa* 36,542 protein-coding gene models were reported. Among these are 89 defence-related genes encoding nucleotide-binding site leucine-rich repeat proteins were also reported (D'Hont *et al.* 2012). The *Musa balbisiana* genome has been associated with improved vigour and tolerance to biotic and abiotic stresses. The 'B' genome was 79% of the size of the 'A' genome and contains 36,638 predicted functional gene sequences which was nearly identical to the 36,542 of the 'A' genome (Davey *et al.* 2013).

The analysis of the expression pattern of the SERK gene during embryogenic cell formation and during somatic embryogenesis revealed that SERK expression continued during pro embryogenic mass formation. In the present study the amplified product of cDNA has molecular size of 1459 bp. In all the samples the amplified product from β - actin primer showed amplified product of 650 bp with similar intensity. In Maize also a gene encoding two novel members of the leucine-rich repeat receptor-like kinase (LRR-RLK) super family was isolated (ZmSERK1 and ZmSERK2). The predicted SERK protein sequence resembled a leucine rich receptor kinase protein (Chang *et al.* 1992). In *Musa acuminata* cv. Mas (AA), a SERK gene (MaSERK) was characterized and reported that open reading frame of MaSERK1 was 1,887 bp in length and encoded 628 amino acids with calculated molecular mass of 69.53 kDa (Huang *et al*. 2010). Ma SERK1 was expressed weakly in male flower clusters, but not in male flower derived non - embryogenic calli, but it was highly expressed in male flower derived embryogenic calli and embryogenic cell suspension. In the present study also SERK gene fragment showed maximum relative intensity in embryogenic tissues. In Arabidopsis AtSERK1 expression was not restricted to embryogenic cells, but it was characteristic of those cells capable of responding to hormonal signals and competent to form somatic embryos or embryogenic cell cultures (Hecht *et al*. 2001).

Somatic embryogenesis receptor - like kinase (SERK) genes encode leucine - rich repeat receptor - like kinases (LRR - RLKs), and the first SERK gene identified was reported in carrot (*Daucus carota*) suspension cultures where it was specifically expressed in cells that developed into somatic embryos (Schmidt *et al*. 1997). SERK constitute a special subgroup of receptor protein kinases associated with the process of somatic embryogenesis (Becraft, 1998). SERK genes were linked to somatic embryogenesis in a number of species including *Dactylis glomerata* (Somleva *et al.* 2000), *Arabidopsis thaliana* (Hecht *et al.* 2001), *Medicago truncatula* (Nolan *et al.* 2003) and *Helianthus annuus* (Thomas *et al.* 2004). SERK

genes were also described in relation to apomixis in *Hieracium* (Tucker *et al.* 2003) and *Poa pratensis* (Albertini *et al.* 2005) as well as zygotic embryogenesis in carrot, Arabidopsis, and wheat (Schmidt *et al.* 1997; Hecht *et al.* 2001; Singla *et al.* 2008). The best defined SERK gene in relation to somatic embryogenesis was the Arabidopsis SERK1 (AtSERK1) and over expression of this SERK was shown to enhance embryogenic competence in Arabidopsis cultures (Hecht *et al.* 2001). SERK genes existed as gene families in many species with five SERK genes in Arabidopsis. The role of MtSERK1 in cultured tissue of *M. truncatula* was previously studied (Nolan *et al.* 2003), indicated that MtSERK1 expression in culture was not only related to somatic embryogenesis, but also to organogenesis and, possibly, other forms of cellular reprogramming. There was an increased understanding of the SERK family in recent years (Song *et al.* 2008).

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