EMBRYOGENIC COMPETENCE AND *HbSERK* GENE EXPRESSION DURING SOMATIC EMBRYOGENESIS

P. Kumari Jayasree, Sajeena Saseendran and Suni Annie Mathew

Rubber Research Institute of India, Kottayam-686 009, Kerala, India

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Unlike zygotic embryogenesis, during somatic embryogenesis an induction phase is required where differentiated somatic cells acquire embryogenic potential. The transition of somatic cells to embryogenic phase is the first and crucial step in somatic embryogenesis. This process involves reprogramming of gene expression patterns as well as changes in the morphology, physiology and metabolism. These changes are dependent on the up and down regulation of certain genes which are essential for the transition. Although the mechanism is still unclear, the search for such genes led to the discovery of *SERK* gene. The objective of the present study is to determine the expression pattern of the *HbSERK* gene during the transition of somatic cells to the embryogenic state. In RT-PCR assay, no *SERK* transcripts could be detected in somatic callus before embryo induction. However, the *SERK* expression was restricted in embryogenic cells. Regarding the *HbSERK* expression in other tissues, no signal was found either in leaves or in non-embryogenic callus, but was observed in emerging shoot. Thus the present study demonstrated that in addition to the acquisition of embryogenic competence, *SERK* gene also plays a role in shoot organogenesis in *Hevea*.

Key words: Embryogenic competence, Expression phase, Induction phase, *SERK* gene expression

Plant somatic cells under favorable *in vitro* conditions acquire embryogenic competence and later get converted into embryos, which is the basis of somatic embryogenesis. These embryos after passing through a series of developmental stages result in the production of whole plants. Somatic embryogenesis pathway has close resemblance with zygotic embryogenesis. However, unlike zygotic embryogenesis which is intrinsically embryogenic, most of the somatic cells are not naturally embryogenic and hence an induction phase is required for the somatic cells to acquire embryogenic competence (Dodeman *et al*., 1997). The induction phase, when the transition of somatic into embryogenic cells has occurrs, is the first and most critical step and is a complex process and this process in plant cells remain largely unknown. It was reported that during this somatic to embryogenic transition state, cells have to de-differentiate, activate their cell division cycle and reorganize their morphology, physiology, metabolism and gene expression patterns (Feher *et al*., 2003) and the search for such genes resulted in the discovery of *SERK* genes (Schmidt *et al*., 1997).

Correspondence: KumariJayasree P. (Email: kumarijaya@rubberboard.org.in)

In *Hevea*, somatic embryogenesis is an integral part of transgenic technology for crop improvement. However, *Hevea* somatic embryogenesis pathway is a slow and lengthy process. One of the major limitations is the long time requirement in the production of embryogenic callus (Englemann *et al.*, 1997; Jayasree *et al.*, 2012). Moreover, in *Hevea* during explant culture, different callus lines with varying morphogenic potentials were induced from the same genotypes even from the same explant. Differential display analysis of these calli revealed differential expression of 28 cDNAs and among these, five could be used for early diagnosis of embryogenic potential (Charbit *et al.*, 2004). In addition, histological, biochemical and histochemical approaches were used to characterize the embryogenic callus (Michaux-Ferriere *et al.*, 1992; Jayasree *et al.*, 2012). In many plant species, *SERK* gene was identified as a potential marker of embryogenic competence. Very recently, a partial *SERK* gene with 0.6kb size from genomic DNA of *Hevea* was isolated and characterized (Jayasree *et al.*, 2016). Considering the significance of embryogenic callus as target tissue in transgenic experiments, the present work was framed to investigate the *HbSERK* gene expression during the transition phase from somatic to embryogenic state. Additionally, the tissue specificity of *SERK* expression was also analyzed.

Somatic embryogenesis was induced from immature anther following the protocol developed earlier (Jayasree *et al.*, 1999). After sterilization, anther was dissected and placed into callus induction medium consisting of modified MS basal salt supplemented with 2.0 mg L^{-1} 2,4-D and 0.5 mg L^{-1} kinetin. Primary somatic callus was cultured for embryogenic callus formation and the cultures were maintained in darkness. In order to study the tissue specificity of *SERK* gene expression, leaf tissue and emerging shoot from *ex vitro* plant and non-embryogenic callus were analyzed. All samples were collected and were frozen in liquid nitrogen at -80°C for later use for RNA isolation.

RNA isolation was carried out according to the procedure of Venkatachalm *et al.* (1999). The isolated RNA was purified and finally dissolved in DEPC treated sterile water. The concentration of RNA was estimated spectrophotometrically and the quality was checked in one per cent agarose gel. RNA samples were treated with DNase to eliminate traces of genomic DNA. First strand cDNA was synthesized using Improm-II ™ Reverse Transcription system (M/s Promega, USA) with oligo-(dT) primer according to the direction of the manufacturer. RT-PCR was performed with primary somatic callus (20 & 40 days old) and embryogenic callus as well as with mature leaf. PCR reactions were performed with the gene specific primer (forward primer: 5′-GCT TGT ATA TCC CTA TAT GGC-3′ & reverse primer: 5′- CGA GCA AGA TCA AAA GCC C-3′) to amplify a fragment of 612 bp of *HbSERK* gene (Jayasree *et al.*, 2016). RT-PCR was also done with cDNA samples synthesized from aforementioned RNA samples using endogenous control, β-actin gene. The reaction procedure consisted of an initial denaturation at 94°C followed by 32 cycles of amplification with 94°C for one minute, 55°C for one minute and 72°C for one minute and a final extension at 72°C for ten minutes. The PCR mixture was overlaid with a drop of mineral oil and amplification was carried out in Perkin Elmer Thermal Cycler 480. Each reaction was repeated three times. The PCR amplicons were analyzed by running on a 1.5 per cent agarose gel and visualized in gel documentation system.

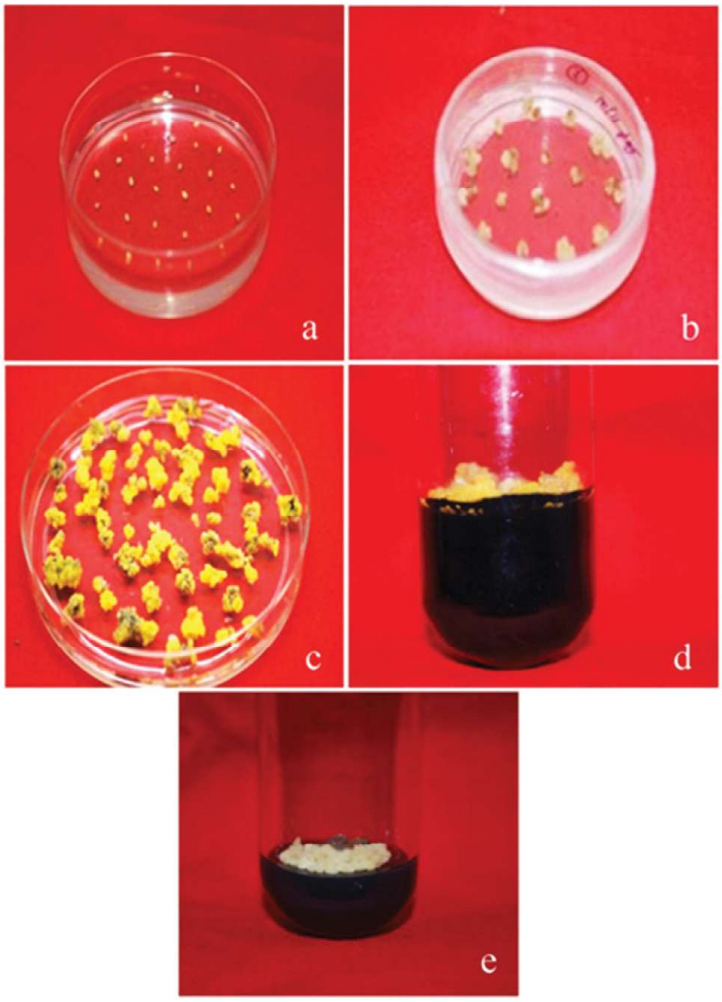

Fig 1.a-e. Establishment of callus cultures
a-Inoculated anther, b- Somatic callus (20 days)
c-Somatic callus (40 days), d-Embryogenic callus
e- Non-embryogenic callus

In the present work, somatic embryogenesis was induced from immature anther explant. When anther (Fig.1a) was inoculated in callus induction medium, explant got expanded and on the 20th day of culture, callus was just induced (Fig.1b). Subsequently, after 40 days of culture, profuse callus growth could be obtained (Fig.1c). This callus got proliferated by 2-3 subculture on fresh callus induction medium. Proliferated callus upon transfer to embryo induction medium changed colour from yellow to brownish in nature and by 3-4 continuous subculture at 50 days interval, highly friable embryogenic callus produced from the brown callus clumps (Fig.1d). On visual observation under microscope, this embryogenic callus showed a mucilaginous texture whereas non-embryogenic callus was characterized with non-mucilaginous, soft nature with fast proliferation (Fig.1e).

Good quality RNA could be obtained from somatic callus (20 & 40 days old) and embryogenic callus as well as from leaf

sample (Fig. 2) and cDNA was synthesized. To investigate the *HbSERK* gene expression during transition phase from somatic to embryogenic state, RT-PCR analysis was performed. The results of expression studies showed no *HbSERK* amplification in somatic callus either in early (20 day) or late (40 day) stages indicating that this gene is repressed in somatic callus. However, *SERK* expression could be detected in embryogenic callus and a fragment size of 0.4kb was amplified. When somatic callus completes the transition or somatic callus acquires embryogenic competence, the *HbSERK* transcript is expressed in the embryogenic callus, but not in somatic callus. For comparison, genomic DNA was also loaded and a fragment size of 0.6kb was produced. During RT-PCR analysis, no signals were detected with the leaf sample (Fig.3). However, with endogenous control gene, all the samples showed good amplification (Fig.4) with an approximate size of 150 bp. Analysis with different tissues showed that no *HbSERK* transcripts were detected with non-embryogenic callus. With emerging shoot cDNA, a fragment was amplified and by comparison with 21kb marker, the fragmented amplicon was *SERK* gene with a size of 0.4 kb (Fig.5) and more studies for further characterization are needed.

In *Hevea*, *HbSERK* gene was expressed only in embryogenic callus. Similarly in carrot (Schmidt *et al*., 1997), *D. glomerata* (Somleva *et al*., 2000) and *Arabidopsis* (Hecht *et al*., 2001), the acquisition of embryogenic competence is marked by an increase in the expression of *SERK* gene. In *Hevea*, *SERK* expression was not found in leaves and non-embryogenic callus. In coconut also, *SERK* expression was detected in embryogenic callus, but, not in non-embryogenic callus and somatic tissues

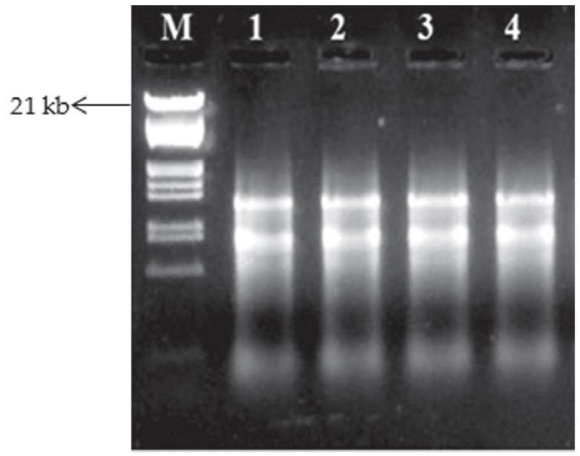

Fig. 2. Isolation of RNA from different samples
Lanes M- Marker (l DNA double digested with EcoRI & Hind III, 1-Somatic callus (20 days) 2 - Somatic callus (40 days), 3-Embryogenic callus 4 - Leaf

(Perez- Nunez *et al*., 2009). Similarly in pineapple, *SERK* gene was highly expressed during embryogenic competence acquisition and globular embryo formation and was very low in non-embryogenic callus (Ma *et al*., 2012). As seen in *Hevea* emerging shoot, *SERK* expression was also found both in apical and radicular

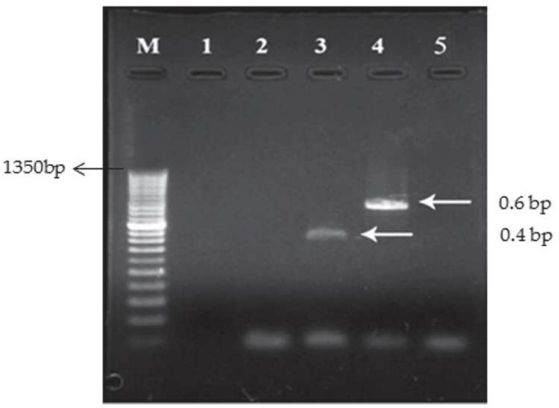

Fig. 3. Expression profile of *SERK* gene Lanes M - Marker (50 bp ladder)
1- cDNA of somatic callus (20 days)
2- cDNA of somatic callus (40 days)
3- cDNA from embryogenic callus
4- Genomic DNA from embryogenic callus
5- cDNA from leaf

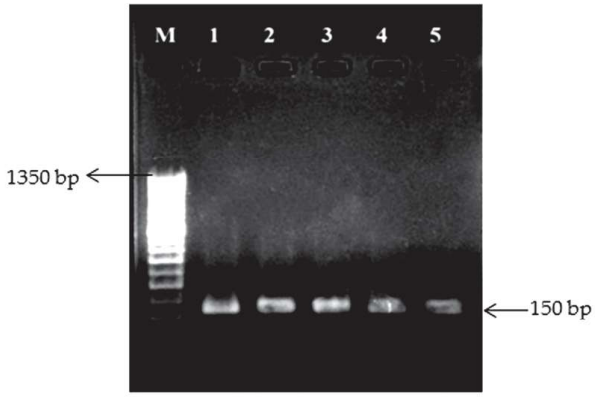

Fig. 4. Expression profile of Actin gene Lanes M - Marker (50 bp ladder),
1-cDNA of somatic callus (20 days)
2-cDNA of somatic callus (40 days)
3-cDNA from embryogenic callus
4-Genomic DNA from embryogenic callus
5-cDNA from leaf

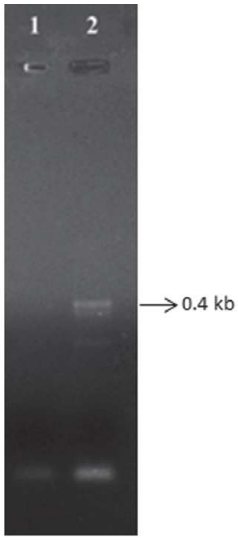

Fig. 5. Tissue specific expression of *SERK* gene
Lanes 1-cDNA of non embryogenic callus
2-cDNA of emerging shoot

meristems of *D. glomerata* (Somleva *et al*., 2000) and in apical meristem of *Hieracium* (Tucker *et al*., 2003). Similarly in medicago, its expression was found during rhizogenesis (Nolan *et al*., 2003). All these data provide the evidence that, like in other crops, in *Hevea* also,*SERK*expression is related to acquisition of embryogenic competence / induction of embryogenesis and in addition, this gene may play a broader role in other process like shoot development.

In conclusion, the expression pattern of *HbSERK* gene during somatic embryogenesis was almost similar to the patterns reported in other crops. *SERK* transcripts were completely absent in somatic callus prior to acquisition or induction phase while *SERK* expression was detected only with embryogenic callus reflecting the fact that *HbSERK* gene is involved in the acquisition of embryogenic competence. No *SERK* transcripts were detected in leaf and non-embryogenic callus. Thus *HbSERK* is a suitable marker of embryogenic competent cells in the acquisition phase. Presence of *SERK* transcripts accumulation in emerging shoot represents its additional role in shoot organogenesis of *Hevea* which need further studies.

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