

Studies on the Effect of Starvation on Prolonged Callus Cultures and Alterations in *SERK* Gene Expression during Somatic Embryogenesis in *Momordica charantia* Linn.

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Momordica charantia Linn. commonly known as bitter melon is an annual climbing herb of Cucurbitaceae family. It is widely cultivated in Asia and South East Asia throughout India, Bangladesh and Thailand (Behera et al. 2011). It has been studied extensively for its medicinal properties in treating a number of diseases and traditionally used as antidiabetic and blood purifying agent (Joseph and Jini 2013). In somatic embryogenesis, embryo-like structures are derived from somatic cells other than gametes by evading the normal fertilization process, hence the offspring produced are genetically identical to their parent tissue (Feher et al. 2003). The switching of a somatic cell to an embryogenic state involves few changes in the composition of the cell wall. Periodic renewal of the culture medium is required, for maintaining the nutritional balance in the embryogenic cells (Raemakers et al. 1996). In somatic embryogenesis regulation, there is involvement of various genes and proteins at the molecular level and acquisition of embryogenic competence in somatic cells. Amongst all the genes, a novel gene named *Somatic Embryogenesis Receptor Kinase (SERK)* was first isolated by Schmidt et al. (1997) from carrot embryogenic cells, known as *DcSERK*. *SERK* gene acts as a molecular marker for somatic embryogenesis and its involvement in signal transduction and response against biotic and abiotic stress (Santos and Aragão 2009).

In this study, seeds of *Momordica charantia* Linn. variety *Charantia* of Murshidabad district, West Bengal were used to perform all experiments. Germination experiment was repeated for five times with 10 seeds each. Seeds of *M. charantia* were decoated and imbibed in sterile distilled water for overnight imbibition. Surface sterilization was done following the protocol standardized earlier in our lab by Paul et al. (2009).

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The leaf explants excised from the germinated plantlets were then cultured in MS medium supplemented with 0.5 mg/l NAA and 5 mg/l BAP. The callus cultures were maintained according to Talapatra et al. (2014). For studying somatic embryogenesis, histological sections were done with the calli of first passage and prolonged second passage and histology was performed as per Talapatra et al. (2014). Histochemistry was performed with only prolonged callus cultures. Polyclonal primary antibodies against SERK protein were commercially designed from IMGEX Biotech, Bhubaneswar using protein sequence of MtSERK. The peptide sequence NLRTNLQDPNNVLSWD was considered as the best epitope, which belonged to the leucine zipper region of SERK protein. FITC- conjugated goat anti-rabbit secondary antibody was used for primary antibody recognition. Localization of SERK protein in the callus sections of *M. charantia* was done by following the protocol designed by Paciorek et al. (2006).

Total RNA was extracted from 100 mg each of 10-day old leaf, 21-day old first passage callus, and 80-day old prolonged second passage callus cultures using PureLink RNA Mini kit with minor modification (Talapatra et al. 2014). For relative mRNA expression pattern of *SERK* gene in leaf (Control), first passage and prolonged callus cultures of *M. charantia*, reverse transcription-PCR (RT-PCR) was performed using gene-specific primers of *McSERK* gene such as forward (5'-GCTGTCCGTGGTACTATCG-3') and reverse primers (5'-TGGGGAGGTCATCTTGGA-3') respectively. The primer sequences and RT-PCR conditions were designed by Talapatra et al. (2014). For the normalization of the target gene expression, β -*actin* was used as an endogenous control and sequences of forward and reverse primers are: 5'-CATTCTCCGTTTGGACCTTG-3' and 5'-AGTTCTCTTTTCATGTACGCG-3' respectively. The thermal reaction for RT-PCR amplification of β -*actin* was the same as that of *SERK*. For the analysis of relative expression of *McSERK* transcript, real-time PCR was performed. First strand cDNA was synthesized from total RNA extracted from leaf, first passage, and prolonged callus cultures using RNA to cDNA kit. The *SERK* gene-specific primers were designed from sequence of *M. charantia* transcriptome (Accession No. JX863894.3) using Primer BLAST. The real time primer sequences of *McSERK* used were 5'-AGAACATACTTGGTAGGGG-3' (forward) and 5'-ATACAAGCAGTCGTTTCAGTA-3' (reverse). The qPCR conditions for *McSERK* gene expression were maintained same as per Talapatra et al. (2014). β -*actin* gene was used as an internal control to normalize the fold change of *SERK* expression. The relative gene expression was performed using $2^{-\Delta\Delta CT}$ method according to Livak and Schmittgen (2001).

The sterilized seeds of *M. charantia* were aseptically transferred in jam jars containing agar-sucrose media for germination of the seedling which germinated within 10-12 days (Fig. 1A). Out of 10 seeds, 8-9 seeds were germinated after 10 days. Germination percentage was 90% (88 ± 8.36). After 21 days of the first passage, the callus mass formed was green in color and globular structure of somatic embryos started forming in the callus cultures (Fig. 1B). Callus cultures in the second passage were kept undisturbed for prolonged period in the same media to observe the effect of starvation on inducing

somatic embryos. Callus formed were green and pale yellowish in color, few cells incapable to survive died and turned brownish (Fig. 1C). Number of globular structure per cm² of callus were found more in the prolonged second passage (35.8 ± 3.37) and less in first passage (9.8 ± 1.32). The histological study of the callus sections confirmed the initiation of somatic embryo and formation of globular structures in first passage with more embryogenic region. The cells had densely stained cytoplasm, small vacuole and nuclei (Fig. 1D). An initiating early stage somatic embryo was observed in the first passage callus section (Fig. 1E). In the prolonged cultures, non-embryogenic region was

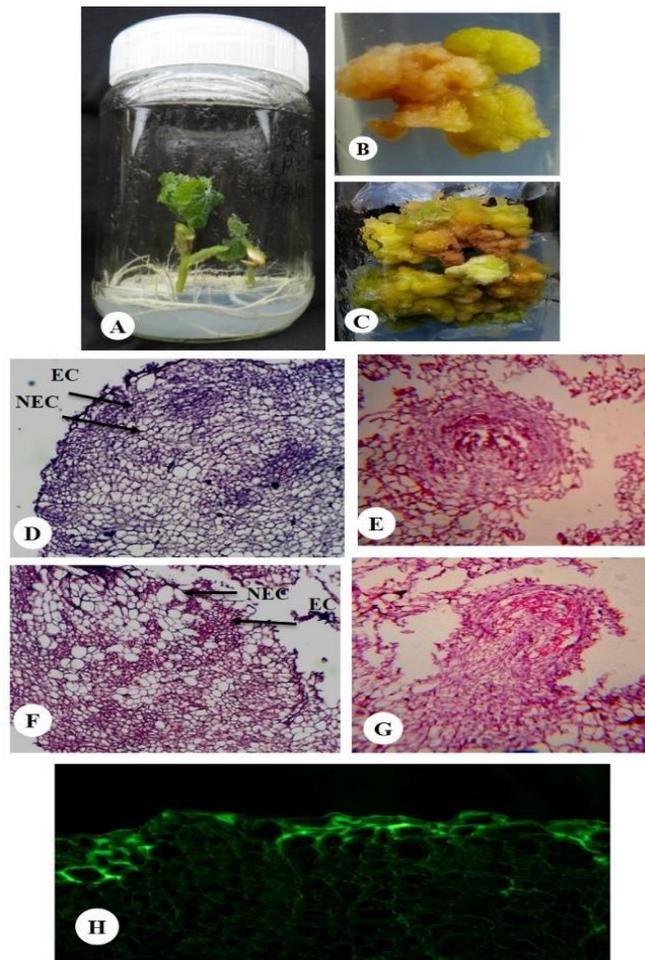


Fig. 1. (A) Germinated seedlings of *Momordica charantia* seeds after 10 days. Somatic embryogenesis of *M.charantia* in (B) First passage at 21st day and (C) Prolonged second passage at 80th day. Histological sections of (D) first passage callus culture, (E) initiating embryo in early stage (F) prolonged second passage callus culture showing embryogenic cells (EC), non-embryogenic cells (NEC) and (G) Globular shape somatic embryo. (H) Localization of SERK protein in histochemical sections of 80-day old prolonged second passage culture of *M. charantia* in the embryogenic cells.

characterized with the presence of large, elongated and vacuolated cells (Fig. 1F) and a globular somatic embryo (Fig. 1G) was observed in the peripheral region of the second passage callus tissue section. During *in vitro* culture, the formation of somatic embryo formation is a rare developmental process as somatic cells undergo dedifferentiation of cells, cell division activation and various changes in the expression pattern of associated genes (Feher 2015). Confocal microscopic analysis showed localization of *Momordica charantia* SERK protein in the embryogenic cell present in the peripheral regions of the tissue sections of 80-day old prolonged second passage callus culture (Fig. 1H). A number of studies reported that SERK is localized in the plasma membrane of embryogenic cells. Higher the fluorescence intensity indicates a higher accumulation of SERK protein in the callus tissue (Kedong et al. 2011).

Reverse transcription - PCR and densitometric data revealed that *SERK* gene expressed in all samples (Fig. 2A). Low level of *SERK* expression was detected in leaf samples in contrast to the first passage and prolonged callus cultures with an increased fold change of 1.06 and 1.2 respectively (Fig. 2B). The data showed a higher expression of *SERK* gene in the prolonged second passage cultures. β -*actin* acted as endogenous control and there was no altered change in β -*actin* expression. Real time PCR analysis observed an increase in fold change of 1.78 and 2.75 in the first passage and prolonged second passage respectively (Fig. 2C). Torres et al. (2015) reported in their study that peak expression recovery of *SERK* gene in *Coffea arabica* L. at 60th day embryogenic suspension culture was due to increased nutritional stress.

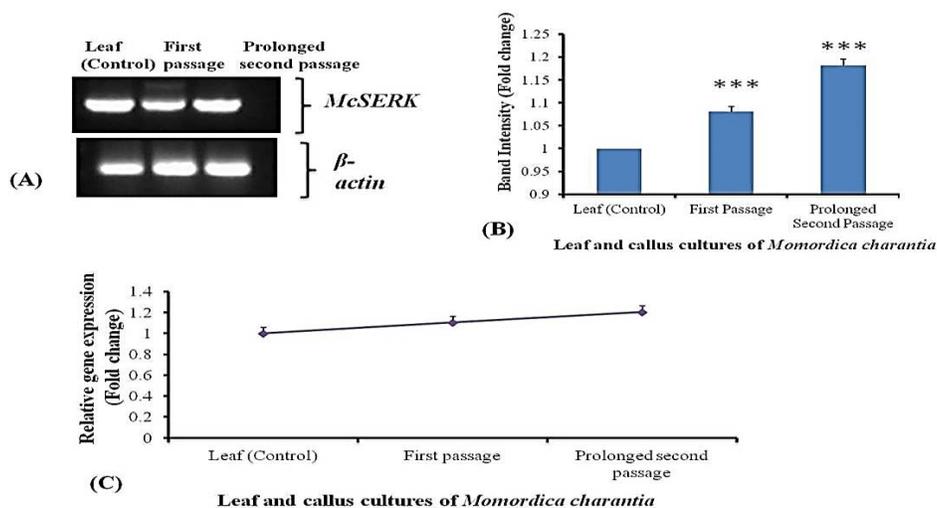


Fig 2. (A) Gel image of *McSERK* expression and β -*Actin* as endogenous control in leaf and different culture passages of *M.charantia*. (B) Graphical representation of densitometric analysis of band intensity by ImageJ representing the change in *SERK* expression. (The data are represented as mean \pm standard error of mean (SEM) (n = 5). The level of significance denotes Asterisk; *p < 0.05, **p < 0.01, ***p < 0.001). (C) Graphical representation of Real-time PCR analysis showing alteration in the relative gene expression of *SERK* gene in *M. charantia*. (The data are represented as mean \pm standard deviation (SD) (n = 3)).

In this study, starvation acted as an effective stress in the induction of somatic embryos. It was found that a little alteration in the micro and macronutrients under stress conditions altered the somatic embryo production. Densitometric analysis and real time PCR data documented higher *McSERK* expression in the prolonged callus cultures which could be clearly correlated with the localization of SERK protein in the prolonged callus cultures. The high fluorescence intensity in the immunohistochemistry analysis provided the plausible explanation for localization of SERK protein paved new way into the functional role of SERK family proteins. Hence it could be concluded that nutrient supplementation and elevated level of *SERK* expression could play a crucial role in somatic embryo propagation and minimizing stress induced by starvation.

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