Tropical Plant Research
An International Journal
© Society for Tropical Plant Research

ISSN (E): 2349 – 1183
ISSN (P): 2349 – 9265
5(1): 01–07, 2018
DOI: 10.22271/tpr.2018.v5.i1.001

Research article

Methylation status of ACCase promoter affects seed vigor-viability trait in *Oryza sativa* L.

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Abstract: Rice (*Oryza sativa* L.) varieties exhibiting phenotypic features of high/low seed vigor-viability were used to explore genetic controls associated with a varietal difference in seed vigor and viability traits. Since germination rate *i.e.* vigor is a reflection of the onset of metabolic activity of a hitherto quiescent embryo and seed viability reflects cell molecular events controlling macromolecular damage/protection in post-harvest (dry) quiescent embryo, expression of a house-keeping gene *viz.* Acetyl CoA Carboxylase (ACCase), that through its dual role controls lipid biosynthesis for germination and flavonoid biosynthesis for macromolecular protection during post-harvest aging, was studied. Our data revealed variation in ACCase gene expression among high and low vigor-viability varieties although Southern blot analysis demonstrated single copy of this gene in all the varieties regardless of their vigor-viability status. Methylation Sensitive Restriction Enzyme assay revealed methylation in CpG island of ACCase promoter in the phenotypically designated low vigor-viability varieties confirming that low seed vigor in rice varieties is due to low expression of ACCase gene brought about by its promoter methylation. Since promoter methylation is transferable through breeding lines this study opens up an opportunity for introgressing high seed vigor-viability trait into otherwise desirable varieties.
Keywords: *Oryza sativa* - Seed vigor-viability - Acetyl CoA Carboxylase - Gene copy number - Gene expression - Promoter methylation.

[Cite as: Ghosh S & Sen-Mandi S (2018) Methylation status of ACCase promoter affects seed vigor-viability trait in *Oryza sativa* L. *Tropical Plant Research* 5(1): 1–7]

INTRODUCTION

Seed vigor- seed germination performance of freshly harvested seed (Maguire 1962) and viability- assessed as seed germination performance of aged seed (Bewley & Black 1994, Sen & Osborne 1977) are important agronomic traits that exhibit varietal difference (Hodgkin & Hengarty 1978, Julliano *et al*. 1990). These traits have been shown to be interrelated through experiments on rice seed under different storage condition have demonstrated that varieties exhibiting low seed vigor at fresh harvest are poor stores exhibiting low seed viability/storability status (Chang & Tolentino 1983). From similar seed vigor studies, Milosevic *et al*. (2010) have proposed that seed vigor traits could be used as indicators of seed viability. Such empirical studies indicate a commonality in gene function where seed vigor trait through storage environment related modifications appears to manifest seed viability trait. Notwithstanding till date, there is not much information on the genetic/epigenetic control that determines the variation of these traits in the orthodox seed.

Conventionally, genetic studies on seed vigor and viability have mostly focused on identifying and mapping genes associated with different facets of germination manifested at late seedling stage through identification of QTLs such as : long root length in rice (Redona & Mackill 1996), heat stress in *Brassica oleracea* (Bettey & Finch-Savage 1998), seed tolerance to artificial ageing in Arabidopsis (Benstink *et al.* 2000), controlled deterioration test in Arabidopsis (Tesnier *et al.* 2002), seedling establishment under cold and drought stress in barley (Chloupek *et al*. 2003), tolerance to natural ageing (NA) in Arabidopsis (Emile *et al*. 2004). Such reports establish a QTL characteristic of late seedling growth without commenting on early embryonic growth that constitutes *sensu stricto* germination elaborated by Bettey (2000) and Holdsworth *et al*. (2008).

Received: 11 November 2017 Published online: 31 January 2018
https://doi.org/10.22271/tpr.2018.v5.i1.001

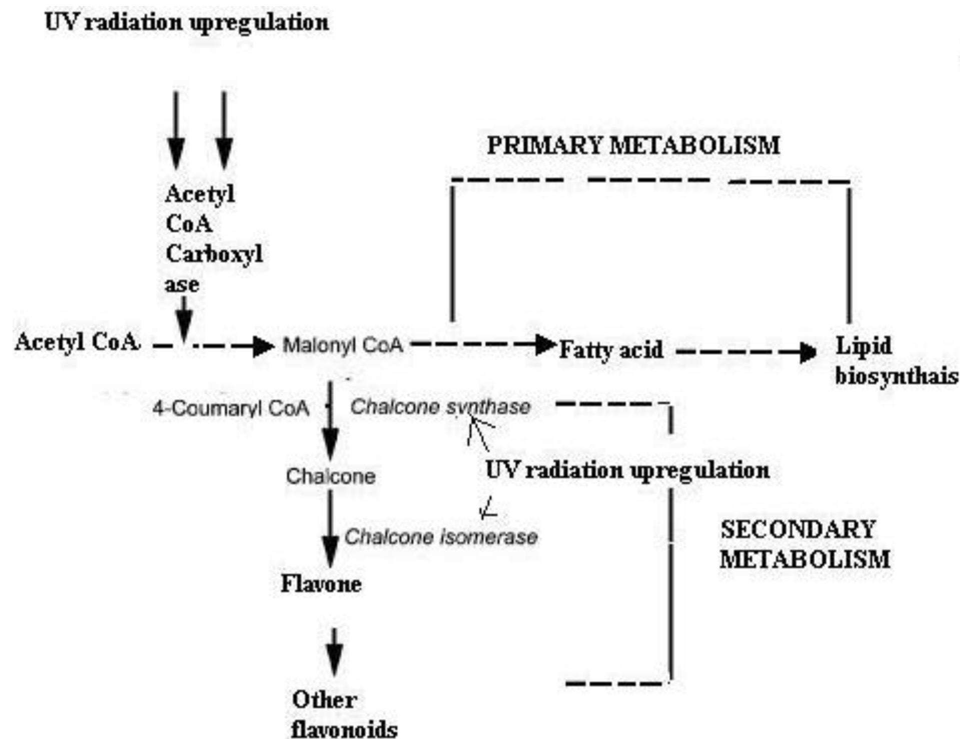

Figure 1. Interrelation between primary and secondary metabolic pathways in field grown plants.

Reports on genomics and proteomics study at *sensu stricto* germination include studies of Caligaris *et al.* (2012) who have identified a gene transcript (At3g08030) a member of the highly conserved family (DUF642) of cell wall-associated proteins in Arabidopsis that have been proposed as a marker for seed aging that determines seed viability status. Cademan *et al.* (2006) have studied cell molecular changes during early and late germination times of Arabidopsis using microarray for global transcript analysis plant. Weitbrecht *et al.* (2011) have demonstrated a massive change in Arabidopsis transcriptome during very early times of seed imbibitions *i.e.* when the hitherto quiescent embryo initiates growth-related cell molecular activity representing *sensu stricto* germination. Rajjou *et al.* (2004) have reported a proteomics analysis relating to accelerated Arabidopsis seed aging. Analyzing gene activity during Arabidopsis seed development Papi *et al.* (2000) have identified a seed-specific transcription factor *viz.* DAG1 in Arabidopsis. Catusse *et al.* (2004) have reported proteome and transcriptome profiling also in Arabidopsis for understanding the molecular mechanism underlying the seed germination and vigor trait. Subtractive cDNA approach has also been undertaken by Linkies *et al.* (2010) in *Lepidium sativum* to understand the seed germination process. While such studies have provided interesting information on seed vigor - viability (*i.e.* storability) these have not identified vigor-viability associated major/candidate gene. Using precisely (metabolomics based) selected high and low vigor varieties of rice seed Talai & Sen-Mandi (2010) have identified a 900bp DNA Marker associated with high vigor trait. This has been shown to be useful in germplasm screening for identification of high seed vigor-viable varieties. The authors have demonstrated a correlation (using NCBI information) of this marker with the house keeping gene ACCase that affects cellular metabolism through synthesis of lipids, necessary for cell wall synthesis in germinating embryonic axis and cellular protection in mature (dry) orthodox seed by flavonoids synthesized during seed maturation on field grown soybean plants (Mazza *et al.* 2000). Shyam-Choudhury & Sen-Mandi (2012) have reported higher flavonoid content in mature high vigor seeds of rice. These reports together with reports of Baudry *et al.* (2004), working on Arabidopsis, indicate a possibility for ACCase enzyme as being a common controlling factor for seed vigor and viability traits. Diversion from primary metabolism to the secondary metabolic pathway (Logemann *et al.* 2000) through Malonyl CoA via the Phenyl Propanoid pathway for production of flavonoids occurs under natural UV radiation (Fig. 1). Mazza *et al.* (2000) have reported biosynthesis of flavonoids sunscreens in field grown (maturing) soybean crops. Flavonoids thus synthesized in field grown plants, remaining stored through post-harvest dry storage, confer environmental protection (Shyam-Choudhury & Sen-Mandi 2012). Being non-enzymatic, flavonoids serve for protection against spontaneous UV radiation, a major factor causing cell molecular damage and thus loss of vigor viability

in dehydrated cells (that are incapable of enzymatic repair) of mature seed during storage. Li *et al.* (1993) have reported that flavonoid mutants of Arabidopsis are hypersensitive to UV-B radiation. Flavonoids by virtue of structure-function relationship are capable of serving as antioxidants as well as sun (ultraviolet) screen (Cockell & Knowland 1999, Kirsch 2001, Amic *et al*. 2003) in dry stored seeds (Bailly 2004, Christova-Bagdassarian *et al*. 2013). It is pertinent to mention here that most of the enzymes in this secondary metabolic pathway are upregulated by UV (Kliebenstein *et al*. 2002). Using enzyme kinetics studies Sen-Mandi *et al*. (2004) have demonstrated lower affinity of ACCase enzyme (per unit µg protein) towards its substrate in low storable/low seed vigor-viability varieties than in the high storable/high seed vigor - viability varieties. Such findings suggest that in varieties with seed exhibiting low ACCase enzyme efficiency would manifest low seed vigor due to a) low rate of lipid metabolism at *sensu stricto* germination and b) low seed viability due to low level of flavonoid biosynthesis during seed maturation thereby failing to equip such low vigor seeds with flavonoids that (remaining undegraded) protect macromolecular degradation (Stapleton & Walbot 1998) under post-harvest dry storage under spontaneous UV radiation (Shyam-Choudhury & Sen-Mandi 2012). Depending on the extent of flavonoid synthesized the variety would remain accordingly protected and thus exhibit appropriate vigor status in the variety.

This report presents our studies on understanding the cell molecular mechanism relating to differential ACCase enzyme efficiency in high / low seed vigor-viability genotypes.

Data presented includes studies on contrasting genotypes with respect to seed vigor-viability traits.

1. ACCase gene expression in different genotypes.
2. ACCase gene copy number
3. Exploration of Methylation in the CpG island of the promoter region of ACCase gene in the contrasting genotypes.

MATERIALS AND METHODS

Plant Materials

Sixteen rice varieties (*Oryza sativa* L. var *indica*) *viz*. IET-13158 (R1), Badshabhog (R2), IET-9978 (R3), IET10890 (R4), Tulsimanjari (R5), Kataribhog (R6), Pushabasmati-1 (R7), Joya (R8), Patani-23 (R9), Jogen (R10), Lalat (R11), Pankaj (R12), Basmati Aman (R13), Kalojira(R14), Matla (R15), Mohan (R16) were used in this study. All freshly harvested seeds were stored at 4°C over $CaCl_2$ until used. These varieties were used after assessing their seed vigor-viability status on the basis of: a) rapid seedling growth (studied in standard germination test) b) ADH time course study on PAGE to determine transition from anaerobic to aerobic respiration coinciding with transition from *sensu stricto* germination to visible germination (Talai & Sen-Mandi 2010) c) high level of DNA integrity (assayed on denaturing agarose gel) d) membrane integrity (determined by extent of lipid peroxidation) (Sen-Mandi & Bhattacharya 2003) e) high levels of non enzymatic anti-oxidant *viz.* flavonoids content (Shyam-Choudhury & Sen-Mandi 2012).

Genomic DNA Extraction

Total Genomic DNA was extracted from 3 day old young seedlings using Walbot (1988) method.

Southern blotting

Rice genomic DNA (10 µg) was digested with HindIII and digested DNA was separated by electrophoresis on a 0.8% agarose gel and then blotted on to a Hybond-N^+ nylon membrane filter (Amersham). The filter was hybridized to 32^P-labeled partial ACCase cDNA probe under normal hybridization and washing conditions as described by Sambrook *et al.* (1989).

Gene expression study by Quantitative Real Time PCR

a. RNA extraction: Embryos extracted from 72 hrs imbibed seeds were powdered in liquid nitrogen. The total RNA was extracted using TRIzol reagent. The yield and quality of total RNA were measured by absorbance at 230, 260, and 280 nm (A260/230 and A260/280 ratios) and by running samples on a 1.5% non-denaturing agarose gel electrophoresis.

b. Conversion of mRNA to cDNA: The mRNA fraction of total RNA population was converted to cDNA using random hexamer primer and Universal RiboClone cDNA Synthesis System (Promega, USA) following manufacturer’s instructions. After conversion cDNA was stored in -20°C.

c. Real Time PCR: In order to investigate the expression pattern of ACCase gene in contrasting varieties of rice

seed, quantitative RT-PCR was carried out in a 96-well optical plate using a Bio-Rad iQ5 instrument and universal cycling conditions (3 min at 95°C, 40 cycles of 10 s at 95°C and 30 s at 62°C). A melting curve was generated at the end of each run to check the specificity of amplification. Primer efficiencies and standard deviations were calculated based on a standard curve generated. First strand cDNA were used as templates in RT-PCR reaction with two primers specific (ACCase-F 5'ACTTCTATTTCCGCGTCACC3' and ACCase-R-5'TCACCCTCGTCTTCTCACAG3') to the coding sequence of ACCase cDNA. The house-keeping gene used in this reaction was UBQ5 gene which acts as an internal control. Specific primers (UBQ5-F 5'ATGATAACTCGACGGATCGC 3' and UBQ5-R 5'CTTGGATGTGGTAGCCGTTT 3') were designed according to the conserved regions of plant UBQ5 gene. The Real Time PCR amplification was performed in triplicate for target and house keeping gene for each sample.

Methylation test

Rice genomic DNA (10 µg) was digested with two Methylation Sensitive Restriction Enzymes *viz.* NauI and MbuBI simultaneously. After double digestion PCR analysis was carried out using two primers (MetF- 5' AGGAAGGAAGCTGTGTGCTG 3' and MetR-5' CAGGGAGGAGGAGGAAGAAG 3') which have complementarity to the flanking regions of the restriction sites of the two enzymes said above. The PCR cycle was run at 94°C for 2 min, 35 cycles of 94°C for 30s, 55°C for 30s, and 72°C for 1 min followed by 72ºC for 10 min. Electrophoresis was carried out in 1.5% agarose gel in TBE buffer along with a 50bp DNA ladder (Thermo Scientific).

RESULTS AND DISCUSSION

Southern hybridization using rice ACCase gene specific probe have revealed a single band of strong hybridization signal in each lane (Fig. 2) suggesting that ACCase exits as single copy in the rice genome irrespective of contrasting vigour - viability status. Data of this experiment demonstrates the presence of a single copy of ACCase gene in all the studied sixteen rice varieties studied regardless of their seed vigour - viability status. Being a single copy in both categories (*viz.* high and low vigour) suggests differential gene expression in the contrasting genotypes. This is validated by Real Time PCR studies of all the genotypes studied against ubiquitin-5 gene as the reference gene (Fig. 3). Relative-quantitative Real-Time PCR using gene-specific primers revealed high (2.5–3 times) ACCase gene expression in high vigour – viability varieties (*i.e.* R3, R4, R6, R7, R9, R12, R15, R16) compared to the low vigour-viability varieties (*i.e.* R1, R2, R5, R8, R10, R11, R13, R14) (Fig. 3). Ubiquitin 5 gene was the housekeeping gene used for normalizing gene expression data in this gene expression study. An interesting correlation with this finding is evident from our earlier report (Sen-Mandi *et al.* 2004) that has presented evidence from enzyme kinetics studies demonstrating that ACCase enzyme exhibits higher substrate affinity (*i.e.* high enzyme efficiency) in high vigour - viability varieties compared to low vigour - viability varieties. Low ACCase gene expression would result in low rate of lipid biosynthesis and thus delayed cell wall formation contributing to slow embryo enlargement and slow rate of embryonic growth at *sensu stricto* germinations that is reflected as low seed vigour. High expression of ACCase gene (Fig. 3) would be responsible for higher flavonoid (non- enzymatic antioxidants) level in high vigour-viability varieties for cell protection through adverse storage environment (*viz.* UV) in rice seeds (Shyam-Choudhury & Sen-Mandi 2012).

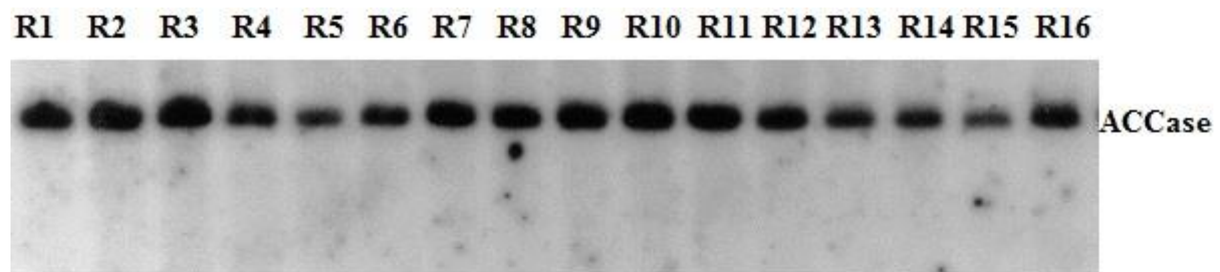

Figure 2. Southern Blot analysis showing single copy of ACCase gene in all the studied *Oryza sativa* varieties irrespective of their vigour-viability status (R1- R16).

Considering the experimental data of figure 2 and figure 3 in perspective, a possible epigenetic mechanism for controlling vigour-viability status in contrasting genotypes may be envisaged. Our studies on varietal difference in vigour viability status of rice seeds indicate promoter methylation as the mechanism responsible for these contrasting traits (Fig. 4). Analyzing the methylation status of the promoter region of ACCase gene using designed primers, complementary to the flanking region of the recognition sites of the two above said

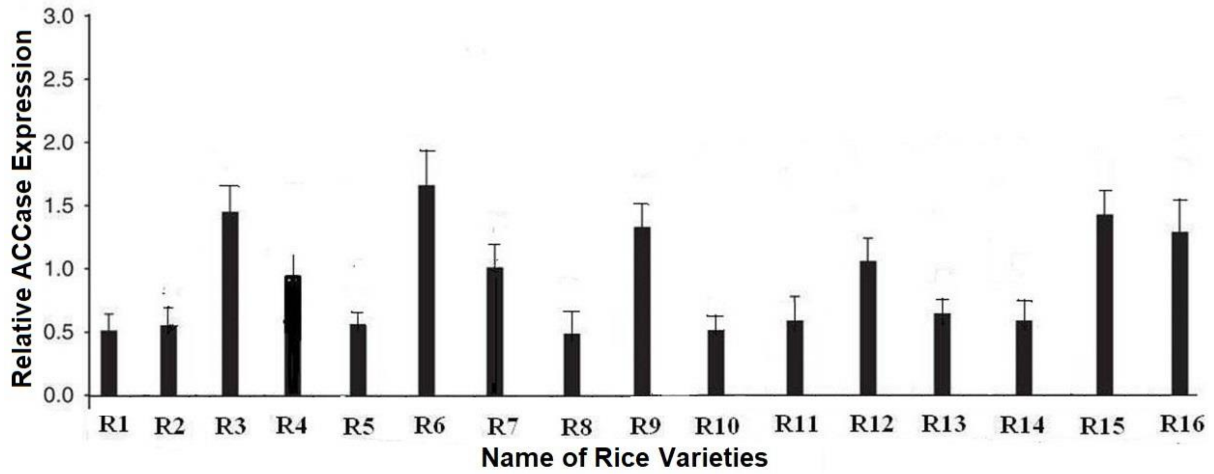

Figure 3. Relative ACCase Gene expression study in *Oryza sativa* varieties showing high ACCase gene expression in high vigour-viable varieties (R3, R4, R6, R7, R9, R12, R15, R16) and low gene expression in low vigour-viable varieties (R1, R2, R5, R8, R10, R11, R14, R15).

restriction enzymes (located within the CpG island of the promoter region of ACCase gene), revealed a visible 250 bp band (Fig. 4) in all low vigour - viability varieties (*i.e.* R1, R2, R5, R8, R10, R11, R13, R14) whereas no band is present in high vigour - viability varieties (*i.e.* R3, R4, R6, R7, R9, R12, R15, R16). Presence of a PCR product in low vigour varieties and its absence (presumably due to lack of methylation at the restriction sites of the two used restriction enzymes (*viz.* NaeI and MbuBI) in high vigour varieties establishes the occurrence of methylation at the promoter region in low vigour varieties. Using specific primer pairs complementary to the flanking region of the restriction sites of two above said enzymes, located within the CpG island of ACCase promoter region, a PCR derived DNA band of 250 bp was observed in the low vigour varieties; the band was absent in the high vigour varieties (Fig. 4). DNA methylation at the CpG island of the promoter region of the ACCase gene would cause low transcription efficiency and therefore low ACCase enzyme activity - an epigenetic effect, evident in low vigour varieties. The fact that vigour viability status in different varieties are found to remain unaltered through generations suggest that such epigenetic effects have been imprinted in the respective genomes.

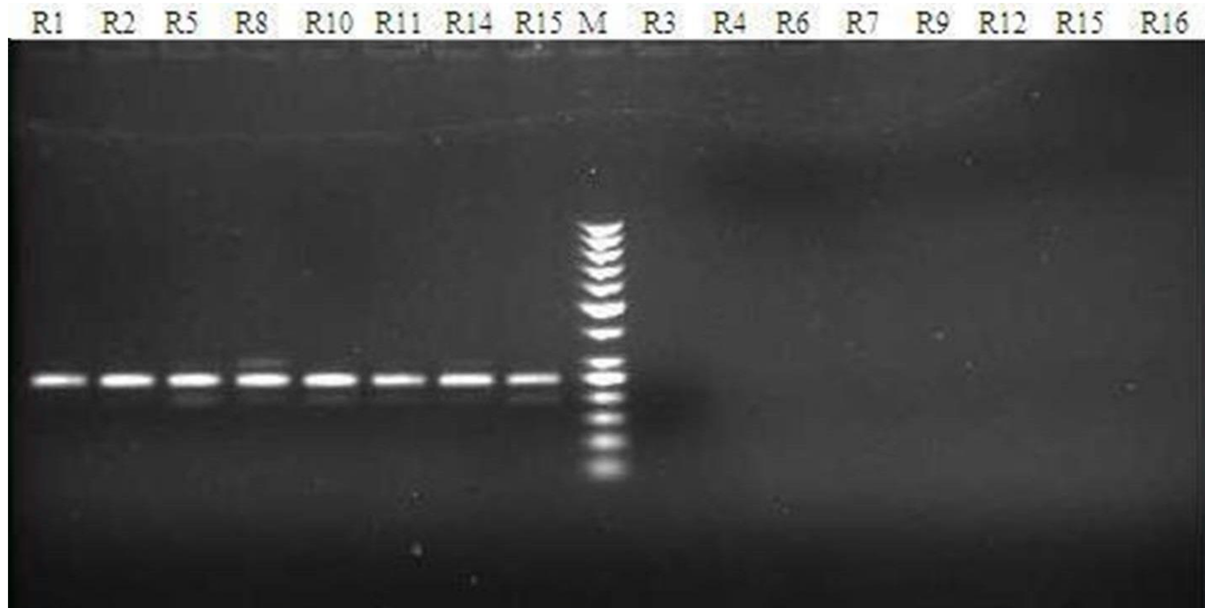

Figure 4. ACCase promoter methylation study showing a 250 bp band in low vigour-viable varieties (R1, R2, R5, R8, R10, R11, R14, R15) whereas complete absence of that band in high vigour-viable varieties (R3, R4, R6, R7, R9, R12, R15, R16). M- 50bp ladder.

Studies on precisely selected (vide 'material') rice varieties demonstrated that variation in ACCase gene expression causes variation in seed vigour-viability trait despite the fact that ACCase gene copy number is "one" in all the varieties studied. The variation in ACCase gene expression among these varieties has been

found to be due to differential methylation status at the ACCase promoter region demonstrating that promoter methylation of ACCase gene (the critical gene regulating both vigour - through lipid biosynthesis as well as viability - through flavonoid biosynthesis) affects vigour-viability traits in freshly harvested rice seeds.

ACKNOWLEDGEMENTS

Prof. Swati Sen Mandi and Dr. Subhabrata Ghosh are grateful to Indian Council of Medical Research (ICMR) for receiving Emeritusship and Research Associateship respectively [Grant No: 74/3/2011 –Pers (EMS)]. The authors wish to acknowledge Prof. Jitendra. P. Khurana, South Campus, University of Delhi for his advice on technical details on Real Time PCR study.

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