Chapter V

Summary and Conclusions
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Sugar is the world's largest primary product (FAO, 2008) and is an important component of the daily diet of the human beings. Over 127 countries are producing 166.74 million tons of raw sugar in the world annually, out of which 130.02 million tons is contributed from sugarcane alone. Sugarcane is an important commercial crop accounting for about 75% sugar production in the world. Among the sugarcane growing countries India ranks as one of the top producers of cane sugar. It is third largest crop in terms of value next to rice and wheat. About 100 diseases of sugarcane caused by fungi, bacteria, viruses, phytoplasmas and nematodes have been reported from India and other countries. In recent years sugarcane yields have been severely reduced by Sugarcane grassy shoot (SCGS) disease caused by phytoplasma like organisms in several countries and in many of the sugarcane growing states of India.

Phytoplasmas are limited to the phloem of the host plant or the insect vector and they cannot be cultured in vitro and are transmitted from plant to plant through phloem-feeding insects. Based on morphological resemblance and 16S rRNA gene sequence analysis, phytoplasmas are designated members of the class Mollicutes. Members of this class are characterized by a small AT rich genome, lack of a cell wall and fastidious growth requirements. Phytoplasmas cause little leaf, phyllody (leaflike reproductive organs), stunting, yellowing, virescence, witches' broom (proliferation of shoots) and dieback in general to host plants. In sugarcane, SCGS phytoplasmas cause yellowing, growth stunting or grassy growth and during heavy infestation there will no cane formation, which is the main storehouse of sugar. Little is known about the underlying molecular mechanisms for the symptoms evoked in the host plants due to this infection. Furthermore it is particularly frustrating, as phytoplasma cannot be cultivated and obtaining their pure DNA is a daunting task for genome analysis to unravel the mechanism of pathogenicity. Further to investigate the potential of a biological system to interact with external perturbations, the genomics studies will be potentially more useful by arraying the resulting changes in the gene expressions of the host system and will provide means to develop long term strategies to combat against such invasions. Towards
the goal of elucidating the molecular responses of sugarcane to SCGS infection and obtain good quality phytoplasma DNA for genome analysis.

In this study a differential filtration approach was used to isolate and enrich the SCGS phytoplasma and their genomic DNA. Scanning electron microscopy and PCR analysis using SCGS phytoplasma specific 16S rDNA gene was used as detection system. Ratio of pathogen to host plant DNA was found to be $1: 25 \times 10^3$ and $1: 37 \times 10^5$ in infected tissue and enriched fraction respectively. The method that has been developed is very simple compared to conventional method and easily accessible to the general laboratories at the same time allowed the enrichment of phytoplasma DNA from host plant nuclear and organelle DNA contamination. The present technique offered 100-fold enrichment of phytoplasma particles and 148-fold increase in sensitivity for their detection, hence more suitable for systematic analysis of phytoplasma specific genes, which will enable the researchers in this field to understand the etiology of phytoplasma infection and allow to develop strategies for its detection and control.

Phytoplasmas contain a minimal genome and lacks many important genes such as genes coding for ATP synthases and sugar uptake and use, making them dependent on their host. For their successful perpetuation and survival in hosts they may need an association with other life forms such as viruses or insects. In this study a simple PCR and RT-PCR technique was used to study the coexistence of phytoplasma (Sugarcane grassy shoot phytoplasma, SCGSP) and virus (Sugarcane streak mosaic virus, SCSMV). Specific primers used in this study, consistently amplified a 1,155 bp fragment of the phytoplasma 16S rRNA gene and a 1,197 bp fragment of the SCSMV specific coat protein gene. Forty samples with or without SCGS symptoms from commercially cultivated varieties (CoC 671, Co 86032) were screened, 72.5% (29 samples) were phytoplasma-positive. Among the 29 phytoplasma-positive samples, 62.07% (18 samples) were also SCSMV-positive. This is the first report on a phytoplasma/virus association with sugarcane grassy shoot syndrome.
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Till date most of the phytoplasmas were differentiated on the basis of biological properties such as disease symptoms on plants, plant host range and specificity of insect vector. These procedures are time-consuming, often subjective and unreliable. Substantial progress has been made in recent years towards improved differentiation and classification of phytoplasmas by using serological and molecular techniques. However these antibodies, DNA probes and PCR based evaluation of rRNA genes (rDNAs) are not suitable for differentiating closely related phytoplasmas and determine their phylogenetic or taxonomic characterization. Hence differentiation of phytoplasmas occupying distinct biological niches but displaying less than 3% divergence in 16S rRNA genes must be achieved by comparing genetic loci other than 16S rRNA genes for developing the efficient and species-specific detection tools. Towards identifying and isolating SCGS phytoplasma gene fragments AP-PCR based profiling method was successfully employed using 100 deca-mer primers. Finally ribo-profile scoring revealed 308 scorable transcript-derived fragments (TDFs), among these six were up and 68 were down regulated while 70 TDFs were found to amplified from phytoplasmal origin. After re-amplification and dot blot analysis seven potential phytoplasmal TDFs were subjected towards developing SCGS specific detection tool. Further phytoplasmal detection efficiency of four SCGS specific TDFs Opa11P3, Opc3P4, Opc8P3 and Opk3P2 was determined in comparison with 16S rDNA universal primers. Among the four SCGS specific fragments Opa11P3 has found highest detection efficiency 98% followed by 76.8%, 73.2% and 73.2% by Opc3P4, Opc8P3 and Opk3P2. The SCGS detection efficiency of Opa11P3 specific primers was 13% more as compared with universal 16S rDNA based detection system hence will be a robust and efficient SCGS specific detection tool at earlier stages in sugarcane rather than existing universal detection system and will be of immense use in quarantine checkups.

Though the sequence information made available from diverse class of Mollicutes very less sequence homology (except 16S rRNA gene) is available with casual agents reported from Asian origin. Hence, SCGS genome analysis was need of today, a severe pathogen of sugarcane. In this study a modified SSH technique was used to capture and enrich rare transcripts of SCGS phytoplasma and transcripts of sugarcane expressed as a
consequence of infection. The genomic SSH library of SCGS infected sugarcane plant was constructed 83 SCGS specific fragments represented altogether 35,696 nucleotides, which corresponded to approximately 6.7 to 2.97% of the chromosome of Sugarcane grassy shoot phytoplasma, comprising approximately 85 predicted partial phytoplasmal CDS. The functional classification revealed CDS related to cellular processes, energy metabolism, nucleotide metabolism, translation and protein synthesis, transport and binding proteins, biosynthesis, regulatory protein, protein fate, mobile and extrachromosomal element functions (transposon functions) and unclassified. Out of the 132 SCGS phytoplasma CDS, 105 CDS has shown match with known and hypothetical proteins in database and 27 CDS has not found significant hits in protein database with neither nonredundant nor phytoplasma proteins. These 20% CDS not having significant hits may be the potential species-specific CDS of Sugarcane grassy shoot phytoplasma and need to be analyzed further to assign the specific functions towards deciphering the molecular basis of virulence and develop the efficient diagnostics and control strategies.

For studying the mechanisms through which sugarcane perceives and responds to this biotic stress through identifying and isolating of SCGS responsive genes of sugarcane, which will prove an important resource to obtain transgenic plants having improved tolerance. In this study cDNA-SSH library was constructed and analyzed. SSH technique used in this study, helped us to identify the up-regulated genes in sugarcane under SCGS infection condition. Comparison of EST sequences to the nucleic acid and protein databases allowed function assignment based on homology. Genes potentially involved in cell rescue, defence, ageing and apoptosis were highly represented (13.1%). We also identified genes described to be involved in cellular communication and signal transduction pathways (6.9%), metabolism (7.2%), energy (0.7%), cell growth, division and DNA/RNA processing (2.9%), protein synthesis, regulation and fate (4%), transcription factor (1.8%), transport facilitation (1%), mobile elements (5.8%) and many genes of unknown function (48.5%). These categories represent 43.9% of the 274 gene sequences already analyzed by BLAST remaining 56.1% comprised 48.5% ESTs with unknown functions and 7.6% were new sequences having no match in database. The forward SSH approach implemented in this study allowed to explicate the transcriptional
regulatory mechanisms of sugarcane in response to SCGS infection and isolated the SoMYB18 gene belong to R2R3-MYB protein family from S. officinarum hybrid. As MYB proteins might predominantly involved in plant-specific regulatory processes and this SoMYB18 gene may play important roles in the regulation of secondary metabolism, cellular morphogenesis, cell cycle signal transduction, SCGS infection and other environmental factors. Hence needs to be thoroughly studied to make it available for developing SCGS resistant transgenic sugarcane in near future.