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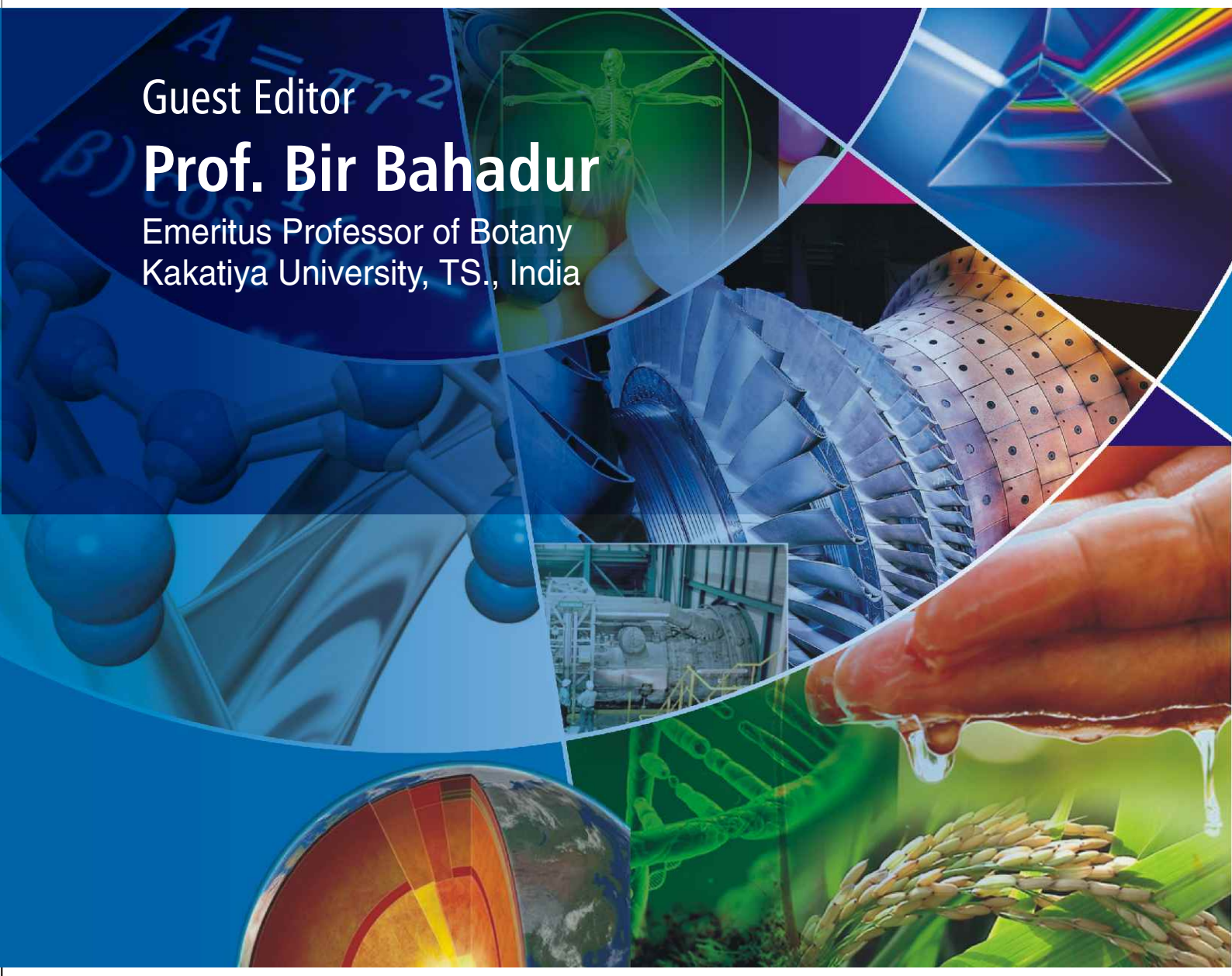

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FOREWORD

The Telangana Academy of Sciences has been engaged in the Science popularization activities including Publications, Journals, Special Issues on different occasions. The recent publication activities include "*Diabesity: The Unacknowledged Indian Knowledge"*, "*Jivanayanam lo Rasayanalu*" and"*21st Century Noble Awards in Chemistry"*. As a part of its continued efforts in this direction, the Proceedings of the Telangana Academy of Sciences entitled '' Frontiers in Biosciences " has been brought out .

This Proceedings covers diverse topics in frontline areas of Biosciences like Virus Resistant Transgenics; RNA interference as a novel tool for Engineering fungal resistance in crop plants, to name a few,with authors drawn from different institutes and universities of repute.

I hope this Proceedings will serve as a pace setter for detailed investigations in Biosciences and be beneficial for the budding researchers in the field. I appreciate efforts made by Prof. BirBahadur, Guest Editor, for bringing out such an informative Proceedings. I thank the Executive Council and the Editor of Publications for their sincere efforts in bringing outthis Proceedings .

S. Chandrasekhar
Hon. Secretary, TAS

From the Guest Editor

It gives me pleasure to bring out this Special Issue of the Proceedings of Telangana Academy of Sciences. (Biological Sciences), which includes articles by eminent and senior Botanists as well as a promising young botanist. This issue contains an array of topics of current interest that hopefully will appeal to researchers in the field of Biology &in particular to botany and allied subjects.

I take this opportunity to thank all the contributing authors for sharing their knowledge with others. I thank all the reviewers for sparing their precious time for valuable constructive comments and suggestions.

Prof. Bir Bahadur
Guest Editor

From The Editor of Publications

Telangana Academy of Sciences (TAS) which has a glorious past of about sixty years, has been active by undertaking a variety of scientific publications of high standard. It also publisheda quarterly journal covering different vital areas of life sciences, chemical, physical ,engineering, earth sciences and mathematics. On the occasion of its Golden Jubilee it has undertaken special Proceedings in different disciplines by inviting articles from eminent scientists in the respective fields. The academy also started a quarterly Newsletter highlighting the activities of the Academy, scientific accomplishments of Fellows and scientific information of special importance.

Inspite of a vast community of scientists and significant contributions, technological advancement of public utility could not be made. Further, younger generation of science need to be encouraged and motivated towards scientific developments taking place around the world. The Academy is making sincere efforts to harvest scientific knowledge of illustrious Fellows of the Academy and create a congenial atmosphere for the scientific pursuits. The Academy is also organizing seminars to activate younger generation and involve scientific discussions and attract the young minds to innovations.

In this direction, the Academy also initiatedonline publications to reach many more people. The Proceedings in Life Sciences is one such initiative. Prof. Bir Bahadur was requested to be a Guest Editor who could motivate many eminent persons in different areas of Life Sciences. He has taken a keen interest in shaping the Proceedings, The Academy acknowledges the efforts made by Prof. Bir Bahadur

S.M. Reddy
Editor of Publications, TAS

Frontiers in Biosciences

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RNA INTERFERENCE AS A NOVEL TOOL FOR ENGINEERING FUNGAL RESISTANCE IN CROP PLANTS

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ABSTRACT

The phytopathogenic fungi such as *Alternaria, Phytopthora, Magnaporthae, Fusarium*, and others cause immense loss of crop productivity. Therefore, plant disease management is very important to control the yield losses in various crop plants, and the preventive measures include the use of agro-chemicals, biological agents and transgenic crops. Although, transgenic technology is a promising one for more than a decade for developing fungal resistant plants, but so far no transgenic crop has been commercialized. Hence, there is an urgent need for novel alternative methods for the control of pathogens and RNA interference (RNAi) is one such method (siRNA, miRNA and artificial miRNA based approaches). More recently, genome editing tool based on CRISPR-Cas9 system has also proven to be a potent alternative for disease control. RNAi-mediated disease control involves the silencing of a vital gene of the target fungal pathogen through host induced gene silencing (HIGS) by expression of dsRNA specific to fungal target gene. This short review covers the basic biology of RNA silencing and the developments in RNAi-mediated control of plant fungal pathogens.

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INTRODUCTION

Plant and pathogens continuous combat have increased the survival rate of pathogens by evolving resistance against agro-chemicals that are used for their control. This has led to increased crop loss worldwide, where a total of 18% of the crops are majorly affected by diseases caused by fungal, bacterial and viral pathogens (Oerke and Dehne, 2004; Dong and Ronald, 2019), and fungal pathogens solely contribute 70% to this proportion. As the expected world population is going to reach about 10 billion in 2050 (US Bureau Census, International Database 2000), crop protection and disease management have drawn attention of plant scientists and seed companies. Although the breeders have been manipulating the crop genomes for gaining resistance against fungal pathogens by using classical breeding approaches (Miah *et. al*. 2013), still the plant and pathogen co-evolution are making the job even more difficult (Mann *et al*. 2008). In this regard, the genetic manipulations using RNA interference (RNAi) or host-induced gene silencing (HIGS) has shown to be a robust way of disease prevention by introducing fungus-specific gene silencing construct in transgenic crops (Sanju *et al.* 2016; Pareek and Rajam, 2017; Qi *et al.* 2018; Qi *et al*. 2019). This can be achieved by small interfering RNA (siRNA), micro RNA (miRNA) or artificial miRNA (amiRNA) mediated gene silencing pathways (Khraiwesh *et al.* 2008; Sanju *et al.* 2016; Yogindran and Rajam 2016; Pareek and Rajam 2017; Qi *et al.* 2018, 2019; Tetorya and Rajam, 2018). Since last more than a decade, siRNA-mediated down-regulation of target fungal genes has been exploited for crop disease management (Mann et al. 2008; Rajam 2011; Panwar *et al.* 2016; Wang *et al.* 2016; Chauhan *et al.* 2017) and more recently the amiRNA strategy is being utilised to achieve the same goal with no or reduced off-targeting effects in fungus (Thakur *et al.* 2015) and other crop pests such as *Helicoverpa armigera* and *Physcomitrella patens* (Khraiwesh *et al.* 2008; Yogindran and Rajam 2016; Saini *et al.* 2018). These technologies have been exploited for the improvement of many different crops (Debener and Winkelmann 2010; Thakur *et al.* 2012). Interestingly, the genome editing technology using CRISPER-Cas9 system has also come up as a new tool for crop protection against fungal pathogens (Borrelli *et al.* 2018; Vicente *et al.* 2019). The genome of many plants like grapevine, apple, barley, and tomato have been edited using CRISPR-Cas9 for resistance against fire blight and powdery mildew disease (Malnoy *et al.* 2016;Nekrasov *et al.* 2017; Borrelli *et al.* 2018). Similarly, this technique was also exploited for genome editing of other food crops as well (Fister *et al.* 2018; Wang *et al.* 2018). This review has focussed on the mechanism of gene silencing by RNAi and its applications as well

as the limitations for fungal resistance and improvement in crop yield, and also discussed the essential pathways targeted in the pathogens.

MECHANISM OF GENE SILENCING BY RNAi

The gene-specific silencing at transcriptional or translational levels can be achieved by RNAi mediated-pathways, i.e., siRNA, miRNA or amiRNA (Khraiwesh *et al.* 2008; Yogindran and Rajam 2016; Pareek and Rajam 2017). In eukaryotic systems, siRNAs are derived from dsRNA, which is formed by viral infections, transgenes, secondary structures in mRNA, read-through transcription, etc. after the cleavage by Dicer (RNase III-type enzyme) and are associated with a multi-protein RNA induced silencing complex (RISC) in which argonaute protein (AGO) acts as a slicer to degrade the cognate (target) mRNA (Hamilton *et al.* 1999; Chen 2004) (**Fig. 1**).

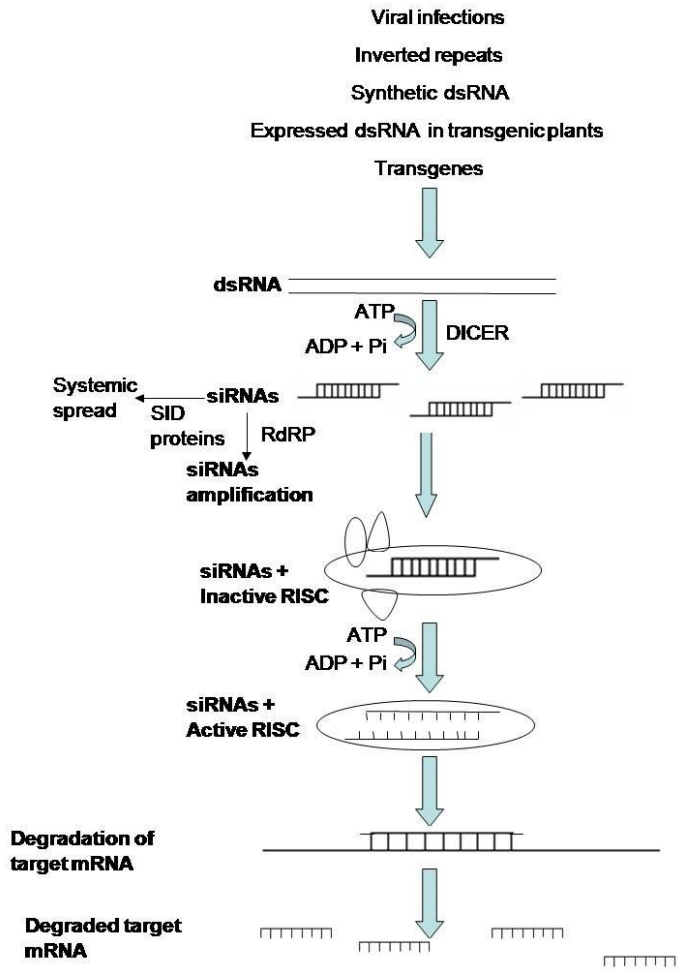

Fig. 1. RNAi-pathway for gene silencing in plants (Source: Rajam 2011).

The endogenous and exogenous siRNAs were studied in the regulation of transcripts and trangene-introduced silencing in fungus *Mucor circinelloides* (Nicolas *et al.* 2010; Cervantes *et al.* 2013) by generating *dcl1*, *dcl2* and *ago1* mutants that lead to growth and developmental defects (Nicolas *et al.* 2010; Cervantes *et al.* 2013), whereas targeting of *mcplD* (phospholipase D) and *mcyo5* (cargo transporter) has shown to reduce the virulence (Trieu *et al*. 2017). On the other hand, miRNA genes in plants are transcribed by RNA polymerase II (rarely by RNA polymerase III) to produce primary miRNA (pri-miRNA), which is then cleaved by Dicer-like 1 (DCL-1) protein to produce precursor miRNA (pre-miRNA), Pre-miRNA is again processed by DCL-1 to form miRNA-duplex (miRNA:miRNA*) that is transported to cytoplasm by Exportin-like protein. Then, miRNA-duplex is recruited in RISC complex, where the passenger or sense strand is selectively get degraded and guide or antisense strand is retained in RISC complex, and this complex can identify and down-regulate the target mRNA either by cleavage or translational blockage, depending on the extend of homology between mature miRNA and its complementary sequence on the target mRNA, which is mostly present in 3'UTR, i.e., in general complete homology leads to degradation and incomplete homology with one or few mismatches results in translation repression of the target mRNA (Bartel 2004; Bartel 2009). Although there are reportsin animals and mammalian cell lines illustrating that mRNA degradation was dependent on miRNA association with AGO (Liu *etal*. 2004; Pillai *et al*. 2005; Liu *et al*. 2005), few mismatches can be tolerated for endonucleolytic cleavage of targeted mRNA, depending on which type of AGO is associated with RISC as not all AGO carry an endonucleolytic activity due to lack of RNase-H like catalytic domain while some cannot perform endonucleolytic cleavage of mRNA even after having catalytic residues and perfect base-pairing (cause of deficit is unrevealed), but still all of them are capable of translational repression by accumulating the perfectly paired miRNA and mRNA associated with AGO in P-bodies (Liu *et al.* 2004; Song *et al.* 2004; Yekta *et al.* 2004; Liu *et al.* 2005; Pillai *et al.* 2005; Valencia-Sanchez *et al.* 2006). Unlike miRNAs, amiRNAs are not endogenously expressed in the cell, rather they are designed using softwares like web MicroRNA designer 3 (WMD3 - wmd3.weigelworld.org/) according to the guidelines, where the miRNA-duplex region of the relevant pre-miRNA is replaced with the target gene region of 21 bp (Schwab *et al*. 2006; Ossowski *et al.* 2008; Vu *et al*. 2013) and after custom synthesis or cloning in the expression vector amiRNAs are delivered to the cell for silencing of the target gene (Yogindran and Rajam 2016).

PREPARATION OF RNA SILENCING CONSTRUCTS

RNAi silencing of cognate target mRNA can be achieved by siRNA or miRNA or amiRNA. In case of siRNA mediated gene silencing, off-target free region from the target gene (usually partial sequence of 200-300 bp) was selected on the basis of high siRNA prediction score, using online siRNA prediction tools like siRNA Target Finder- Genescript (https://www.genscript.com/tools/sirna-target-finder), OligoWalk (http://rna.urmc.rochester.edu/servers/oligowalk), InvivoGen – siRNA Wizard Software (https://www.invivogen.com/s0irnawizard/siRNA.php) and WI siRNA Selection Program (http://sirna.wi.mit.edu) and cloned in the expression vectors like pSilent (fungal specific vector) or pCAMBIA (plant binary vector) in sense and antisense orientation with a spacer DNA, usually a small intron between them under the control of a suitable promoter (Singh *et al.* 2014; Pareek and Rajam 2017; Choubey and Rajam 2018; Tetorya and Rajam 2018) **(Fig. 2)** or cloning in a single orientation but with dual promoters (pSD1) to form double-stranded RNA but without hair-pin (Nguyen *et al.* 2008; Nguyen *et al.* 2011; Panwar *et al.* 2016). Then, the RNAi vectors can be used to generate transformants of fungal pathogen (Pareek and Rajam 2017; Tetorya and Rajam 2018) using glass bead method (Pareek *et al.* 2015) or *Agrobacterium*-mediated transformation (Mullins *et al*. 2001; Rho *et al*. 2001; Michielse *et al*. 2005; Pareek *et al*. 2015)and plant, using *Agrobacterium*-mediated transformations (May *et al.* 1995; Zhang *et al*. 2006; Madhulatha *et al.* 2007; Pan *et al.* 2010; Belide *et al*. 2011; Singh *et al.* 2014; Choubey and Rajam 2018) and positive transformants were selected on the selection medium containing the selection agent (usually an antibiotic). Similarly, in miRNA based construct designing, the off-targeting of the selected sequence is a crucial step, once it is done the whole sequence of pre-miRNA is cloned in the selected vector (Panwar *et al.* 2016) and because of the presence of secondary structure it will form loop after it is being expressed in the cell and can be easily recognised by host DCL1 for further processing and formation of miRNA-miRNA* duplex (Yang *et al.* 2015; Wamiq *et al.* 2018). This construct can be used for over-expression studies of particular miRNA (Yang *et al.* 2015; Wamiq *et al.* 2018), whereas the amiRNA have been exploited for down-regulation studies of target mRNAs (Schwab *et al.* 2006; Yogindran and Rajam 2016).

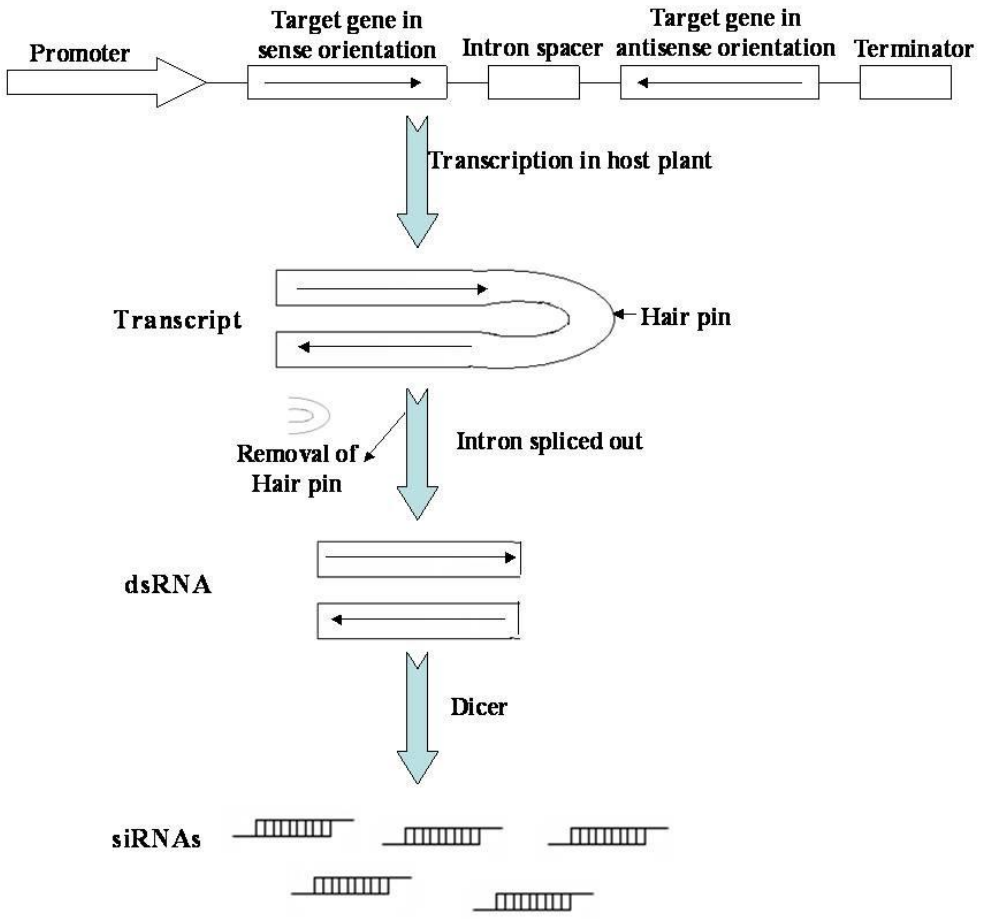

Fig. 2. Schematic representation of the production of hpRNA from a RNAi expression vector, splicing of the intron and the formation of siRNAs by dicer activity (Source: Rajam 2011).

APPLICATIONS OF RNAI TECHNOLOGY FOR ENGINEERING FUNGAL RESISTANCE IN PLANTS

RNAi has been utilised as a potential tool for functional genomics studies in plants, besides its several applications in agriculture. RNAi has been exploited to address the biotic stresses such as bacterial (Jiang *et al.* 2009) and fungal (Rajam 2011; Pareek and Rajam 2017; Tetorya *et al.* 2018) pathogens, nematode parasites (Tian *et al*. 2019), viruses (Kumari *et al.* 2018) and insect pests (Rajam 2011; Yogindran and Rajam 2016; Israni and Rajam 2016), and abiotic stresses (Jewell *et al.* 2010)experienced by plants. RNAi has been extensively used for engineering fungal resistance in different crop plants (Duan *et al.* 2012; Younis *et al.* 2014; Panwar *et al.* 2016; Wang *et al.* 2016;Machado *et al.* 2018; Zotti *et al*. 2018). RNAi has also been exploited to decipher silencing mechanism and functional characterization of the components involved in fungus, as a model organism (Armas-Tizapantzi and Montiel-González 2016). The targeting of fungal genes by siRNA-mediated silencing has shown to

play a pivotal role in studying the growth and development as well as pathogenicity of pathogens. For example, siRNA targeting ornithine decarboxylase, a key gene invoved in polyamine biosynthesis pathway, inhibited the growth of *Aspergillus nidulans* (Khatri and Rajam 2007).Likewise, *Puccinia graminis* and *Puccinia striiformis*, which cause rust disease in wheat, can be controlled by RNAi-mediated targeting those genes which are involved in sporulation, appresoria formation or pathogenecity (Yin *et al.* 2011; Qi *et al.* 2018).*PsCPK1*gene was shown to be an effective target for resistance against *P. striiformis*(Qi *et al.* 2018). Panwar *et al*. (2018) reported that wheat rust disease was controlled by expressing dsRNA within the host plant to silence MAP-Kinase or cyclophilin of *Puccini atritica* and thereby inhibits its growth and proliferation. Similarly, RNAi-mediated targeting of *MoAP1* gene in rice has led to resistance against rust disease-causing *Magnaporthe oryzae* (Guo *et al.* 2019). Targeting of *Avra10* gene resulted in resistance against powdery mildew caused by *Blumeria graminis*in wheat and barley (Nowara *et al.* 2010). Spraying of dsRNA is another way of delivering dsRNA to the pathogen system. This was achieved by spraying dsRNA targeting three cytochrome P450 *lanosterol C-14α-demethylase* genes - *FgCYP51A*, *FgCYP51B*, and *FgCYP51C*, on the distal part of the plant (passage through vascular tissues), which caused significant reduction in disease onset and conferred resistance against *Fusarium graminearum* (Koch *et al.* 2016) and lethality to *Fusarium culmorum* (Koch *et al*. 2018). Similarly, β-1, 3-glucan (*FcGls1*) and chitin synthase (*chs3b*) genes have shown to be potent targets for down-regulation for enhancing resistance in wheat against head blight caused by *F. graminearum* (Cheng *et al*. 2015; Chen *et al*. 2016). *Fusarium* is not only involved in head blight disease, but few species may also cause Fusarium wilt disease in several crops, including banana and tomato. Targeting of vital genes involved in transcription like *TF1* or signalling pathways like *MAPK* reduces pathogenecity and invasive trait of *F. oxysporum* in banana and tomato, respectively (Ghag *et al.* 2014; Pareek and Rajam 2017). Similarly, targeting of *FOW2* (zinc finger transcription regulator), *FRP1*, *OPR, chsV* (myosin motor with chitin synthase domain) and *ODC* increased the resistance against *F. oxysporum* in tomato with the delay of onset of disease symptoms (Singh 2011; Hu *et al*. 2015; Bharti *et al*. 2017).

After wheat, rice is another most important cereal crop and is susceptible to several fungal infections like rust disease caused by *Magnaporthe oryzae* (Zhang *et al.* 2015; Guo *et al.* 2019) or sheath blight disease and brown patch caused by *Rhizoctonia solani* (Zhou *et al*. 2016; Tiwari *et al.* 2017). RNAi-mediated targeting of the *bZIP* transcription factor of *M.*

Oryzae, i.e., *MoAP1* (Guo *et al.* 2019) and rice specific *OsDCL1* (Zhang *et al.* 2015) led to resistance against *M. oryzae,* and targeting *RPMK1*-1, *RPMK1*-2 and other essential genes like RNA polymerase, importin beta-1 submit, cohesion complex subunit Psm1 and ubiquitn E3 ligase, decreased disease symptoms after infection with *R. Solani* (Zhou *et al*. 2016; Tiwari *et al.* 2017). Like siRNA-based approach, miRNA-based strategies were also used for resistance against biotic and abiotic stresses (Jewell *et al*. 2010; Chauhan *et al*. 2017). In artificial miRNA technology, the miRNA duplex sequence in the endogenous precursor is replaced with the target gene region of 21 bp (Alvarez *et al*. 2006) and such amiRNAs were explored to engineer resistance against potato late blight caused by *Phytopthora infestans* by silencing the *Avr3a* gene involved in fungal virulence (Thakur *et al*., 2015). The novel target selection in the conserved region is very important as this can lead to an effective control of more than one plant fungal pathogens., for example, ds-RNA targeting of *β2-tubulin* of *Fusarium asiaticum* confers resistance against other *Fusarium* sps., *Botrytis cinerea*, *M. oryzae* and *Colletotrichum truncatum* (Gu *et al.* 2019).

CONCLUSIONS AND FUTURE PROSPECTS

RNAi technology has shown to be efficient and promising approach to raise transgenic crops with improved agronomic traits, including crop protection against fungal diseases (Rosa *et al*. 2018). This has open up the field of studying not only functional genomics of fungal pathogens, but also developing transgenic crops resistant to fungal pathogens by knocking-down the specific and vital genes of the target pathogenic fungi (Ghag *et al.* 2014; Koch *et al.* 2016; Gu *et al*. 2019)or by down-regulating the expression of host susceptible gene (Zhang *et al.* 2015). Host-induced gene silencing ensures the delivery of dsRNA to the pathogen and thereby reduces the selection pressure on pathogen to evolve resistance (Nowara *et al*. 2010; Panwar *et al.* 2018; Zhu *et al.* 2017). Considering the current status of fungal pathogens gaining resistance against fungicides or antifungal agents (Fisher *et al.* 2018), RNAi has been shown to be a promising mechanism for crop disease management and yield improvement (Wani *et al*. 2010; Puyam *et al*. 2017; Mcloughlin *et al*. 2018). Despite of having vast applications in crop improvement, including disease management, the the efficacy of fungal resistance and its stability in RNAi lines for several generations still have to be tested. Further, the off-target effects, i.e., the silencing of the unintended genes in the host plant should also be examined by transcriptome studies.

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VIRUS RESISTANT TRANSGENICS A REVIEW

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ABSTRACT

Plant viruses are the major yield-reducing factors for crop production including plant death. One of the most effective ways to control viral diseases is through host plant approach which has been successfully accomplished through genetic engineering of crops. In this review, an attempt is made to highlight the strategies followed to produce specific virus resistant crops and the developments in production of virus resistant transgenics. The technologies followed to develop virus resistant transgenic crops *via Agrobacterium*-mediated transformation, post transcriptional gene silencing, replicase mediated resistance, nucleases to target viral gene sequences tools and genome editing are discussed. The development of virus resistant transgenic crops like cucurbits, maize, grapes, squash, sugarcane, tomato, tobacco, peanuts, watermelon including the commercially released virus resistant transgenic crops is presented.

KEYWORDS: Plant viruses, virus resistant plants, transgenics, genetic engineering, plant transformation

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INTRODUCTION

Plant viruses often lead to plant death and account for serious reduction in crop yields. There are several viruses belonging to different taxa which show diverse molecular and genetic composition (single or double stranded RNA or DNA) and the amount of genetic material varies among different types of viruses (Hull, 2014). All viruses are intracellular and obligate parasites that utilize the host plant translationary machinery to express their own genetic material. This intimacy between the host and the viruses, makes it difficult in controlling viral infection. Further, chemicals that affect the virus also affect the host cells; because of the diversity of plants infecting viruses, no single approach can be considered for universal virus disease control.

Some of the approaches to prevent losses due to virus infections are to breed plant varieties for virus resistance, chemical control of insect vectors, exploitation of the phenomenon of cross protection between viruses, growing plants that are genetically engineered for virus- resistance (VR) (Kyle, 1993). Virus resistance genes have recessive, additive or dominant modes of inheritance (Whithman and Hajimorad, 2016). In these cases, traditional breeding strategies can be often difficult and time consuming. There are several modes of virus transmission through insects and vectors of plant viruses include aphids, whiteflies, thrips and leafhoppers which spread the virus in a non-circulative and non-persistent manner, making the chemical control strategy non-effective. Cross protection is a strategy- first demonstrated by Mc Kinney with tobacco mosaic virus (TMV) (Mc Kinney, 1929). He discovered that plants already infected with one strain of TMV were resistant to infection with a second closely related TMV strain. Cross protection is a phenomenon that worked successfully with many different viruses of diverse virus taxa, where it was known that cross protection has practical applications for virus disease control in agriculture. If a plant could be first inoculated with a mild strain of the virus, the plant might be resistant to super-infection later by more severe strains of this same virus. Cross protection proved to be a very effective strategy against the poty viruses, papaya ring spot virus (PRSV) and zucchini yellow mosaic virus (ZYMV). The main limitation of this strategy is that effective naturally occurring virus resistant genes are often hard to find and difficult to manage in a breeding program. Owing to these limitations with traditional breeding techniques and control strategies, genetic engineering techniques were employed for incorporating virus resistance in several economically important crop plants.

The first genetically engineered virus resistance was TMV resistance in tobacco (*Nicotiana tabacum*) (Abel *et al.,* 1986). During the past three decades of research in genetically engineered virus resistance, scientists have developed more accurate and efficient strategies for engineering resistance, and have observed the economic and environmental benefits using genetically engineered virus resistance. The strategies employed and insights gained have reached to a point where it is now possible to effectively engineer resistance against any virus. There are different strategies to develop virus resistance (VR) transgenics. Sanford and Johnston (1985) published the concept of parasite or pathogen derived resistance (PDR). They suggested that gene engineering technologies can be used to confer PDR by cloning the appropriate parasite gene modifying its expression and transforming it to the host genome. When the gene product is expressed in the host, this could yield faulty signals which could disrupt the normal replication and infection cycle of the parasite, thus resulting in the resistance phenotype. This PDR concept was especially appealing as a resistant strategy. Based on the PDR, there are different strategies to follow for development of VR-transgenics. These are coat protein mediated resistance (CPMR), replicase protein, movement protein, viral protein, helper protein, antisense RNA/DNA, ribozyme, satellite and defective interfering RNAs and gene editing. Among these, CP-MR is the most widely followed approach to develop virus resistant plants.

VIRUS-DERIVED RESISTANCE

The first VR transgenic plants (VRTs) using VDR was produced in the 1980's by expressing TMV coat protein gene in transgenic tobacco plants (Abel *et al.,* 1986). The virus depends on the host machinery for its replication. The genome of plant virus is either ssDNA, dsDNA, ssRNA or dsRNA. Most of the plant viruses have positive sense ssRNA genome that replicates by virus encoded RNA dependent RNA polymerase and forms the dsRNA replicative intermediary (Varma and Ramachandram, 2001). The viral genome is encapsulated in particles having icosahedral or spiral symmetry formed by compact arrangement of coat protein subunits of specific pattern, and the viral genome could be either monopartite, bi or multipartite, irrespective of the number of genome molecules. Each genome has open reading frames (ORF's) to produce structural and non-structural proteins for various functions like replication, cell to cell movement, vector transmission and encapsulation. The events that follow infection include disassembly of virus particles, synthesis or transcription of mRNA, translation of proteins encoded by the viral genome of

various functions, maturation of virus particles, systemic infection and vector transmission. Both coding and non-coding regions of the viral genome have been used for developing VRT'S. Coat protein gene is the most commonly used transgene for developing VRT'S against viruses belonging to different groups followed by replicase protein and movement protein genes.

METHODS OF TRANSFORMATION

For plant genetic transformation, the two most important methods employed are the vector-mediated transformation using *Agrobacterium tumefaciens* and direct gene transfer through particle bombardment method. In case of VRTs, the transformation method most frequently followed was the vector-mediated method. Di *et al*. (1996) reported that development of virus resistant transgenic soybean (*Glycine max*) through *Agrobacterium*-mediated transformation of cotyledonary nodes with *Bean pod mottle virus* (BPMV) coat protein precursor (*CP*-P) gene. Lin *et al*. (2012) developed transgenic watermelon resistant to multiple infections, by deploying a single chimeric transgene comprising a silencer DNA from the partial DNA of the N gene of watermelon silver mottle virus (WSMoV) fused to the partial coat protein (*CP*) gene sequence of CMV *via Agrobacterium*-mediated transformation. Mehta *et al.* (2013) incorporated transgenic resistance in peanut (*Arachis hypogaea* L.) to peanut stem necrosis disease caused by TSV by transferring coat protein (CP) gene of TSV through *Agrobacterium*-mediated transformation of de-embryonated cotyledons and immature leaves of peanut cultivars Kadiri 6 (K6) and Kadiri 134 (K134). Anderson *et al*. (1992) transformed *Nicotiana tabacum* cv. Turkish Samsun plants with a modified and truncated replicase gene encoded by RNA-2 of cucumber mosaic virus strain upon transformation *via A. tumefaciens*. Yan (2007) reported introduction of the inverted repeat of the coat protein gene of *Peanut stripe virus* (PStV) into *Nicotiana benthamiana* plants via *A. tumefaciens* mediated transformation. The coat protein (*CP*) gene of the G5H and G7H strains of *Soybean mosaic virus* (SMV) were cloned, sequenced and transformed into the tobacco plant (*Nicotiana tabacum* cv. Havana SR1) via *Agrobacterium*-mediated transformation (Sohn *et al*., 2004). Bevan *et al*. (1985) expressed *Tobacco mosaic virus* coat protein driven by *cauliflower mosaic virus* 35S promoter in tobacco leaf explants by transformation through *Agrobacterium.*

Cheng *et al*. (1996) reported development of transgenic papaya through *Agrobacterium*-mediated transformation method based on the wounding of cultured

embryogenic tissues with carborundum in the liquid phase. These embryogenic tissues were obtained from cultured immature zygotic embryos collected 75-90 days after pollination. Vasavi *et al*. (2018) developed transgenic sunflower resistant to necrosis disease using TSV-coat protein gene via *Agrobacterium*-mediated transformation. Clarke *et al*. (2008) reported *Agrobacterium*-mediated transformation of poinsettia (*Euphorbia pulcherrima*) internode stem explants for RNA silencing mediated resistance to poinsettia mosaic virus using *Agrobacterium* strain LBA 4404 harbouring virus derived hairpin RNA gene constructs.

Development of virus resistant wheat cultivars could make a great contribution to increase wheat production (Fahim *et al.,* 2010; 2012; Jimenez-Martinez *et al.,* 2004; Cruz *et al.,* 2014). Towards this direction, a spring wheat cultivar and several Chinese wheat varieties were transformed by microprojectile bombardment of immature embryos with plasmids designed to engineer virus resistance. Tougou *et al*. (2007) and Tougou (2009) generated soybean plants resistant to *Soybean dwarf virus* (SDV), which causes serious damage to soybean production in Northern Japan by transforming somatic embryos *via* microprojectile bombardment with constructs containing sense coat protein (*CP*) gene and inverted repeat-SDV coat protein (*CP*) genes of *Soybean dwarf virus* (SbDV).

GENE CONSTRUCTS

The strategies for engineering virus resistance in plants have been outlined and will be discussed in detail. This section deals with the different types of gene constructs that are being developed for deployment in various crop plants. The evolution is towards producing virus specific biomolecules in plants and recovering a higher and higher percentage of VR plant lines. Initially, a large number of virus sequence nearly 1 kb of the translatable sequence was used. Subsequently, non-translatable virus sequence was used that only express RNA. Following these developments, miRNA genes which only contained approximately 21 nucleotides of virus sequence embedded into a plant miRNA gene were used. Each change has been more specific targeted design, requiring the use of less foreign genes to plants for each virus resistance generated. The future of virus resistant transgenics is the possibility of genome editing using CRISPR/Cas (Bortesi and Fischer, 2015; Noman *et al.*, 2016) may be used to alter the specificity of existing plant miRNA genes, so that they produce miRNA that targets a virus of interests. Such a genome editing approach could require changing only approximately 20 nucleotides of the entire plant genome to generate a new virus resistance gene. There is a possibility of getting effective virus resistance

transgenics through this technique; which would represent a new milestone in the evaluation of engineered virus resistance through RNA-mediated gene silencing mechanisms.

Lin *et al*. (2012) reported transgenic watermelon resistant to multiple infections, by development of a single chimeric transgene comprising a silencer DNA from the partial DNA of the N gene of watermelon silver mottle virus (WSMoV) fused to the partial coat protein (*CP*) gene sequence of CMV. Anderson *et al*. (1992) transformed *Nicotiana tabacum* cv. Turkish Samsun plants with a modified and truncated replicase gene encoded by RNA-2 of cucumber mosaic virus strain. The replicase gene had been modified by deleting a 94 base pair region spanning nucleotides 1857-1950; the deletion also caused a shift in the open reading frame, resulting in a truncated translation product which was ~75% as large as the full length protein. A transgenic approach to engineering MSV-resistant maize was developed and tested by Sheperd *et al.* (2007). The pathogen derived resistance strategy was adopted by using targeted deletions and nucleotide-substitution mutants of the multifunctional MSV replication-associated protein gene (*rep*). Various *rep* gene constructs were tested for their efficacy in limiting replication of wild-type MSV by co-bombardment of maize suspension cells together with an infectious genomic clone of MSV and assaying replicative forms of DNA by quantitative PCR. Vasavi *et al.* (2018) reported cloning of the TSV-CP gene (717 bp) in sense orientation (pTCP-S) at restriction sites *Bam*HI and *Sal*I and in anti-sense orientation (pTCP-AS) at *Bam*HI and *Xba*I sites (Bag *et al.,* 2007). The different types of genes deployed and the gene constructs developed for imparting virus resistance through transgenic approaches are presented in Table 1 and the VRTs produced using different mechanisms are elaborated in the following sections.

COAT PROTEIN MEDIATED RESISTANT TRANSGENICS (CP-MR)

Beachy (1999) reported that transgenic plants which accumulate the tobacco mosaic virus (TMV) coat protein are protected from infection by TMV, and by closely related tobamoviruses. The phenomenon is referred to as coat protein-mediated resistance (*CP*-MR), and bears certain similarities to cross protection, a phenomenon described by plant pathologists early in this century. The studies of CP-MR against TMV have demonstrated that transgenically expressed *CP* interferes with the disassembly of TMV particles in the inoculated transgenic cell. Transgenic crops using CP-MR are developed in crops like tobacco, soybean, peanut, common bean, maize, sugarcane, tomato, watermelon, wheat,

sunflower, papaya, squash and potato. In 1986, Abel reported the first genetically engineered virus resistance. He demonstrated resistance to TMV in transgenic tobacco plants engineered to express the TMV coat protein (Abel *et al.,* 1986). They tested transformed tobacco plants that accumulated TMV-CP and challenged several transgenic CP-expressing lines (CP+) lines with various quantities of TMV. CP+ plants showed greater resistance to TMV infection which was demonstrated in two ways (i) some of the CP+ plants showed a lower percentage of TMV infected plants than did the non-transgenic controls, and (ii) those that became infected developed symptoms later after inoculation than did the non-transgenic plants. By increasing the inoculum level, this delay in the appearance of symptoms was reduced. None of the plants were immune to TMV challenge. Srivastava and Raj (2008) had developed transgenic tobacco for the first time against an Indian isolate of CMV subgroup IB. Two transgenic lines exhibited complete resistance against two closely related strains of CMV. Vasudevan *et al*. (2008) had developed two transgenic lines of *Nicotiana benthamiana* expressing *Turnip crinkle virus* coat protein gene. In most of the studies, the infected progenies of putative transgenics showed mild and delayed symptoms, reduced titer or failed to show any visible symptoms even after 4 weeks of post inoculation with the corresponding virus.

Virus resistant transgenic soybean lines (*Glycine max.* Merrill.Cv. Fayette) were developed with *Bean pod mottle virus* coat protein precursor (*CP*-P) gene, and 30% of the R_2 plants from one event showed complete resistance to BPMV infection and were confirmed through ELISA (Di *et al.*, 1996). Tougou *et al*. (2007) and Tougou (2009) transformed soybean somatic embryos with constructs containing coat protein (*CP*) gene of *Soybean dwarf virus* (SbDV) in sense and anti-sense orientations. After inoculation of SbDV by the aphids, most of the T_2 plants of the transgenic lines remained symptomless, contained little SbDV-specific RNA by RNA dot-blot hybridization analysis and exhibited SbDV-*CP*-specific siRNA.

Coat protein gene of *Peanut stripe virus* (PStV) was introduced into *Nicotiana benthamiana* (Yan *et al.,* 2007). By observation of symptoms and ELISA detection, 87% transgenic plants were found immune to PStV in T_0 generation, 60-100% T_1 plants of different resistant lines were immune to PStV, and T_2 plants of the most resistant line were immune to PStV. Specific siRNA was detected in transgenic plants, but not in non-transgenic plants. In this experiment, the double-stranded RNA (dsRNA) strategy was successful in inducing high frequency of transgenic tobacco plants resistant against PStV. Mehta *et al.*

(2013) incorporated transgenic resistance in peanut (*Arachis hypogaea* L.) to peanut stem necrosis disease caused by TSV by transferring coat protein (CP) gene of TSV. Integration of the transgene in T_1, T_2 and T_3 generations were analyzed and six transgenic plants in T_3 generation remained symptomless and resistance to the TSV infection. Bag *et al*. (2007) reported generation of transgenic peanutlines with sense and anti-sense coat protein (*CP*) gene of *Tobacco streak virus* (TSV).

The first transgenic maize conferring resistance to maize streak virus (MSV) was reported by (Shepherd *et al.*, 2007). The mutated MSV replication associated protein gene was used to transform maize which showed stable expression up to the fourth generation. Transgenic T_2 and T_3 plants showed a significant delay in symptom development, a decrease in symptom severity and higher survival rates than the non-transgenic plants after MSV challenge. Yong *et al*. (2011) reported that to overcome the low efficiency of agronomic protection from maize dwarf mosaic disease, a susceptible maize inbred line was transformed harboring hpRNA expression vectors containing inverted-repeat sequences of different lengths targeting coat protein gene (*CP*) of *Maize dwarf mosaic virus* (MDMV). Gan *et al.* (2014) reported that the resistance to sugarcane mosaic virus was developed in maize using hp construct to the CP gene.

Gilbert *et al*. (2005) demonstrated that genetic transformation of sugarcane (*Saccharum* spp.) holds promise for increasing yields and disease resistance. One hundred plants derived from cultivars *CP* 84-1198 (n=82) and *CP* 80-1827 (n=18), consisting of independent virus resistant lines VR 1 (n=14), VR 4 (n=24), VR 14 (n=4), and VR 18 (n=58) were evaluated. Transgenics derived from CP 84-1198 had significantly greater tonnes of sucrose per hectare (TSH) and significantly lower SCMV disease incidence. Plants from the VR 18 line had significantly greater economic indices and lower SCMV disease incidence. The large variability in yield characteristics and disease resistance encountered in the study demonstrated the necessity for thorough field evaluation of transgenic sugarcane while selecting genetically stable and agronomically acceptable material for commercial use. Zhu *et al*. (2011) reported development of *Sugarcane yellow leaf virus* resistant plant with an untranslatable coat protein gene. Six transgenic lines had at least 103-fold lower virus titer than the non-transformed susceptible parent line. The resistance level, as measured by virus titer and symptom development, was similar to that of a resistant cultivar (H78-4153).

Transgenic tomato with resistance to *cucumber mosaic virus* has been tested under field conditions to assess the level of resistance and agronomic importance (Tomassoli *et al.*,

1999). Trials of transgenic tomato were performed in different areas in Italy and the target virus in the majority of tests was spread naturally by the indigenous aphid populations. The resistance observed in multi-year and multi-location experiments is of commercial value for several of the most resistant lines. Engineered resistance was transferred to Italian varieties by breeding for tomato production in Italy and elsewhere. Lin *et al*. (2011) reported that the resistance to the geminivirus tomato leaf curl Taiwan virus (*AC2* gene) and TSWV (*N* gene) was developed in tomato using chimeric transgenic approach.

Liu *et al*. (2016) reported an effective transformation protocol designed for a female parent of watermelon mediated by *Agrobacterium*. Seventeen putative transgenic plants expressing artificial microRNAs that target *Cucumber mosaic virus* (CMV) 2a/2b genes were recovered, of which only seven of them were succeeded in obtaining T_1 generation seeds and the transgenic lines displayed resistance to CMV infection.

Transgenic wheat engineered with the viral coat protein gene for resistance to *wheat streak mosaic virus* through the biolistic method is reported. Sivamani *et al*. (2002) obtained 11 independent transformed plants of which five were analyzed for gene expression and resistance to WSMV strains. Northern hybridization analysis indicated a high level of degraded *CP* mRNA expression.

In India, sunflower (*Helianthus annuus* L.) is mainly affected by tobacco streak virus which causes major loss to sunflower production to the tune of 90%. Pradeep *et al*. (2012) transformed sunflower (cv. CO-4) and tobacco (*cv*. Petit Havana) plants with TSV-CP gene. Infectivity assays with TSV by mechanical sap inoculation demonstrated accumulated lower levels of TSV when compared with non-transformed controls. Vasavi *et al. (*2018) reported genetic engineering of sunflower through the deployment of the coat protein gene of TSV (*TSV-CP*). Out of 102 positive T_0 events, 20 events were carried to T_1 generation from which five events (*CP*-S-237, *CP*-S-247, *CP*-S-481, *CP*-S-648 and *CP*-S-753) were advanced till T_4 generation. One event 481 showed resistance to necrosis disease and was adequately characterized. The plants grew to maturity in *TSV-CP* transgenics while control plants (untransformed) showed mortality within 1–2 weeks following inoculation and the event was selected for transfer of the *TSV-CP* gene into agronomically superior genotypes (Table 1).

TABLE 1 VIRUS RESISTANT TRANSGENICS IN DIFFERENT CROPS

Crop	Gene	Level of protection	References

Soybean	Bean pod mottle virus coat protein gene	30% of R_2 plants showed complete resistance	Di *et al*., 1996
	CP-Soybean dwarf virus	T_2 plants remained symptoms less	Tougou *et al*., 2007
	Inverted repeat-SDV *CP* gene	One progeny exhibited SDV resistance two months after inoculation	Tougou 2009
	Mungbean yellow mosaic (India) virus coat protein gene	Two transgenic lines exhibited MYMIV resistance after inoculation	Kumari *et al.,* 2018
Tobacco	Replicase gene	Virus could be detected even after prolonged incubation	Anderson *et al*., 1992
	Tomato spotted wilt virus (TSWV) nucleocapsid gene	Double haploid lines showed very high levels of resistance	Liu *et al*., 2003
	Soybean mosaic virus (SMV-*CP*) gene	Symptoms were delayed	Sohn *et al*., 2004
	CP gene of peanut stripe virus	87% of transgenic plants were found immune to PStv in T_0 generation, 60-100% in T_1 generation, 90% in T_2 generation	Yan *et al*., 2007
	CP gene sequence of CMV	Two lines exhibited complete resistance and remained symptom less	Srivastava *et al*., 2008
	CP-Turnip crinkle virus	30-100% of protection, but showed delayed symptoms	Vasudevan *et al*., 2008
Tomato	Cucumber mosaic virus gene	23 homozygous lines	Tomassoli *et al*., 1999
	RNA silencing - tospovirus	82% of transformed plant lines showed resistance against TSWV, GRSV, TCSV, WsMOV	Bucher *et al.,* 2006
	Coat protein and pre-coat protein of Geminivirus	T_2 generation plants exhibited higher tolerant to ToLCNDV	Vu *et al.,* 2013
Maize	Mutated MSV	T_2 and T_3 plants showed	Shepherd *et al*.,

	replications-associated protein gene	significant delay in symptom development	2007
	CP-Maize dwarf mosaic virus	Six plants had confirmed resistance to MDDV	Yong *et al*., 2011
Potato	CP- Potato virus Y	ShRNA traansgenic plants exhibited resistant to PVY infection	Tabassum *et al.,* 2016
	Replicase gene - Tomato leaf curl New Delhi virus - potato	AC1 hairpin loop approach showed higher resistance in transgenic plants	Tomar *et al.,* 2018
Peanut	*TSV-CP* gene of tobacco streak virus	T_3 generation transgenic plants remained symptomless to TSV infections	Mehta *et al*., 2013
Papaya	*CP*-Papaya ring spot virus	100% protection through post-transcriptional gene silencing	Tennant *et al*., 2001
	CP-Papaya ring spot virus	100% protection against papaya ring spot virus	Ferreira *et al*., 2002
Poinsettia	RNA gene construct	Transformation frequency was 2.1%	Clarke *et al*., 2008
Sugarcane	Sugarcane mosaic virus-coat protein gene	Large variability in yield characteristics and disease resistance	Gilbert *et al*., 2005
	Sugarcane yellow leaf virus -*CP* Insert	98.1% kanamycin resistant progeny developed	Glynn *et al*., 2010
	CP- ScYLV	Delayed symptoms	Zhu *et al*., 2011
Grape vine	*CP*-gene of grape vine fan leaf virus	Transgenic crop expressing *CP* gene under field conditions of heavy disease pressure	Vigne *et al*., 2004
Wheat	*CP* gene of Wheat streak mosaic virus	Slow disease development	Sivamani *et al*., 2002
	CP gene - Barley yellow dwarf virus	Transgenic lines showed moderate resistance against BYDV after inoculation	Jimenez-Martinez *et al.,* 2004

	Nuclear inclusion protein - WSMV	Transgenic individuals were symptom free up to at least 14 dpi	Fahim *et al.,* 2010
	Polycistronic amiRNA - WSMV	T_2 generation transgenic plants showed resistance to WSMV	Fahim *et al.,* 2012
	CP gene - WSMV	T_5 generation transgenic plants exhibited resistance to WSMV	Cruz *et al.,* 2014
Water melon	Partial N gene of water melon silver mottle virus fused with coat protein gene sequence of CMV	R_1 showed resistance to CMV and WMV resistance through post-transcriptional gene silencing	Lin *et al*., 2012
	Cucumber mosaic virus (2a/2b) genes	Transgenic lines showed delayed symptoms AND resistance to CMV infection	Liu *et al*., 2016
Sunflower	Coat protein gene of Tobacco streak virus	One event (No 481) was selected for transfer of the *TSV-CP* gene into agronomically superior genotypes.	Vasavi *et al.,* 2018

GENETICALLY ENGINEERED VIRUS RESISTANCE IN PAPAYA

The most successful example of an engineered VR crop is papaya (*Carica papaya*). Papaya ring spot virus (PRSV, a member of genus potyvirus) infection is the cause of the most destructive disease of papaya and is a major disease problem world-wide. PRSV infection of papaya causes a severe reduction in fruit production. PRSV, like other potyviruses, is transmitted through aphids and is difficult to control.

Gonasalves (1980) began had done pioneering work to apply CP-MR approach to generate PRSV resistant papaya. After years of effort, they succeeded in producing transgenic papaya expressing PRSV coat protein gene. (Fitch *et al.,* 1993; 1992). In the field trials conducted at Hawaii in 1992, all the control plants were infected with PRSV, but the transgenic papaya was completely protected (Lius *et al.,* 1997). Following this, several studies were carried out for development of VRT papaya.

Tennant *et al*. (2001) reported that R_1 lines of transgenic papaya line 55-1, which expresses a single coat protein (*CP*) gene of the mild strain of the *papaya ring spot virus*

(PRSV) HA from Hawaii are resistant only to PRSV isolates from Hawaii. Two transgenic papaya cultivars were subsequently derived from the line 55-1 which included UH SunUp (SunUp) which was homozygous for the *CP* gene insertion and UH Rainbow hemizygous for the CP gene as it was an F_1 hybrid of a cross between SunUp and the non-transgenic papaya cultivar Kapoho. Steady state RNA analysis and nuclear run on experiments suggested that the resistance of the transgenic papaya is RNA-mediated via post-transcriptional gene silencing. Ferreira *et al*. (2002) also reported that virus coat protein transgenic papaya provides control to *papaya ring spot virus* in Hawaii.

In 1992, PRSV infection appeared in Puna, the major papaya production region in Hawaii. The virus spread rapidly with in this region and caused significant reductions in market yield. Field trial results with VR engineered papaya showed a yield of 125,000 pounds per acre per year for transgenic papaya and 5000 pounds per acre per year of the non-transgenic produce (Gonsalves *et al.,* 2004). These transgenic papaya lines produced millions of dollars of value to Hawaii papaya growers. In 2009, Hawaiian papaya export valued at over 14 million dollars, of which 75% of the Hawaiian papaya grown accounted for engineered VR (VIB, 2014). In 2012, the Hawaiian papaya export market value was over 23 million dollars (VIB, 2014) (John *et al.,* 2017).

Transgenic papaya has also been valuable to growers in other parts of the world. In 2006, transgenic PRSV resistant papaya expressing the *PRSV-CP* gene from transgene was deregulated in China. An estimated 3,500 ha of genetically modified papaya were grown in China 2007. By 2012 this had risen to 6,275 ha, representing more than 60% of the land area used for papaya in China (VIB, 2014). As of 2017, engineered VR papaya is still a major percentage of papaya grown in both Hawaii and China.

VIRUSES IN COMMON BEAN

Common bean (*Phaseolus vulgaris* L.) and vegetable crops such as tomato, peppers and cucurbits (Cucurbita spp.) are damaged by whitefly-transmitted begomo-viruses worldwide (Kenyon *et al.,* 2014; Leke *et al.*, 2015). Bean golden mosaic virus (BGMV) and the related bean yellow golden mosaic virus are among the biggest constraints on bean production in Latin America. After two decades of work, the Brazilian Agricultural Research Corporation (EMBRAPA) was able to produce a transgenic line of common bean showing high and stable levels of resistance to BGMV (Aragao *et al.*, 2009). The transgenic line EMBRAPA 5.1 was approved for cultivation in 2011, and field trials for registration of several

new cultivars developed from EMBRAPA 5.1 by breeding were initiated in 2012 (Aria *et al.*, 2016) (Table 2). The resistance generated is expected to enable recovery of bean production in BGMV-affected areas, increase yields and quality besides reducing the need for pesticide applications for vector control in Brazil.

GENETICALLY ENGINEERED VIRUS RESISTANCE IN SQUASH

The CMV and potyviruses such as ZYMV and WMV are the important virus diseases of squash in the United States and are transmitted by aphids (Kyle, 1993). As the control of these viral diseases by insecticidal sprays is ineffective, there is a demand to develop virus resistant plants.

Tricoli *et al.* (1995) applied the CPMR strategy to develop multivirus resistant linesin summer squash. Expression vectors consisting of a single virus CP gene sequence joined to a plant functional promoter were constructed. Recombinant DNA molecules of either two/three different expression cassettes were assembled and transferred in to squash cells by *A. tumefaciens*-mediated transformation. Transgenic plants expressing CP genes of ZYMV, WMV, and CMV showed resistance to all three viruses. Multivirus resistant squash was deregulated in the United States in 1994 and 1996 and were the first VR transgenic plants to be commercialized in the market.

Transgenic plants that showed resistance to multi viruses are some remarkable achievements possessing a tremendous practical advantage for breeding programs. Field trials reported that engineered VR squash resistant to all three viruses had a remarkable 50-fold increase in marketable yield over susceptible control lines (Fuchs *et al.*, 1998). In 2006, these resistant genes were in about 12% of all US squash production and were still providing effective protection against ZYMV, WMV and CMV infection. The study of Johnson *et al.* (2007) estimated that the benefit of VR technology to growers at 24 million dollars. Engineered VR squash continues to be grown in the United States until today (John *et al.,* 2017) (Table 2).

Gaba *et al*. (2004) reviewed the status of virus transgenics in the cucurbit family that includes a number of valuable crop species (melon, cucumber, squash/pumpkin, watermelon). Published data on field tests of transgenic cucurbits was presented which showed that much progress has been made with multiple virus-resistant cucurbit crops which can be productive without chemical control of insect virus vectors. A detailed analysis

has been made of world-wide and US field test applications for cucurbit crops. World-wide, most field test applications were for melon (54%), followed by squash (32%). World-wide most field test applications were for virus resistance (84%), and most applications (77%) were in the USA. Two transgenic multiple virus-resistant squash crops have been deregulated. Additionally, the analysis showed that there are transgenic multiple virus-resistant crops in all major cucurbit species for which several companies have applied for field tests. This would imply that such crops are ready to be marketed should conditions permit, which would have an impact world-wide in the reduction of ecological damage due to chemical control of the insect viral vectors.

GENETICALLY ENGINEERED VIRUS RESISTANCE IN POTATO

Virus diseases such as, potato virus Y (PVY, a member of the genus Potyvirus; family Potyviridae), potato virus X (PVX, a member of the genus Potexvirus; family Alpha-flexiviridae) and potato leaf roll virus (PLRV, a member of the genus Polerovirus; family Luteoviridae) are the most common and devastating potato viruses worldwide. The aphid-transmitted PVY and PLRV are reported to cause significant yield losses. The contact-transmitted PVX becomes significant with PVY co-infection, which induces synergistic interactions of the viruses leading to high accumulation of PVX. Transgenic potatoes expressing the PVY-CP gene were found to be highly resistant to PVY (Kaniewski *et al.,* 1990). Virus-derived resistance was engineered to both PVY and PVX in potato cv. Russet Burbank, representing only the second example (after tobacco) of genetically engineered virus resistance in crop plants (Lawson *et al.*, 1990; Kaniewski *et al.*, 1990). 'Russet Burbank' was also engineered for resistance to PLRV, PVY and Colorado potato beetle (*Leptinotarsa decemlineata*) in the late 1990s and approved for marketing under the name NewLeaf™ (Thomas *et al.*, 2000; Lawson *et al.*, 2001) (Table 2). Resistance to PLRV in 'NewLeaf' reached commercial production in the USA, but the engineered variety remained on the market only a couple of years before it was withdrawn due to the decision by major potato processing industries to abstain from using transgenic potatoes (Thornton, 2003). Most of the potato events developed for virus resistance are stacked events combining resistance for coleopteran insects.

Transgenic potatoes expressing the PLRV replicase gene were also generated (Kaniewski and Thomas 2004). After screening more than 1000 transgenic potato lines in the

field, approximately 300 were found to be PLRV resistant. The best three lines were selected in terms of yield, field resistance and product quality were eventually commercialized (Kaniewski and Thomas 2004).

TABLE 2 TRANSGENIC VIRUS-RESISTANT CROPS APPROVED FOR CULTIVATION

Crop and event	Genes introduced	Year and country approved for cultivation
Bean (*Phaseolus vulgaris*) EMBRAPA 5.1	BGMV ac1 (sense and antisense	2011, Brazil
Papaya (*Carica papaya*) 55-1- Rainbow, SunUp 63-1 Huanong No. 1 X17-2	 PRSV-CP PRSV-CP PRSV-rep PRSV-CP	 1997, USA 2011, Japan 1996, USA 2006, China 2009, USA
Plum (*Prunus domestica)* **C-5** (**ARS-PLMC5-6)**	ppv cp	2007, USA
Squash (*Cucurbita pepo*) CZW3 ZW20	CMV-CP WMV-CP ZYMV-CP WMV-CP ZYMV-CP	1996, USA 1994, USA
Tomato (*Lycopersicon esculentum*) PK-TM8805R (8805R)	CMV-CP	1999, China
Sweet pepper (*Capsicum annuum)* **PK-SP01**	 cmv cp	 1998, China
Potato (S*olanum tuberosum* L*)* RBMT15-101-New LeafTM Y Russet Burbank potato RBMT21-129- New LeafTM Plus Russet Burbank potato HLMT15-15- Hi-Lite NewLeaf™ Y potato	 PVY-CP PLRV-orf1 PLRV-orf2 PVY-CP	 1998, USA 1999, Canada 1998, USA 1999, Canada 1998, USA

HLMT15-3- Hi-Lite NewLeaf™ Y potato	PVY-CP	1998, USA
HLMT15-4**6**- Hi-Lite NewLeaf™ Y potato	PVY-CP	1998, USA
RBMT21-152- New Leaf™ Plus Russet Burbank potato	plrv_orf1 plrv_orf2	1998, USA
RBMT21-350**-** New Leaf™ Plus Russet Burbank potato	plrv_orf1 plrv_orf2	1998, USA; 1999, Canada; 2001, Australia, Japan, Mexico, New Zealand; 2004, Philippines, South Korea
RBMT22-082- New Leaf™ Plus Russet Burbank potato	plrv_orf1 plrv_orf2	1998, USA; 1999, Canada; 2001, Australia, Japan, Mexico, New Zealand; 2004, Philippines, South Korea
RBMT22-186- New Leaf™ Plus Russet Burbank potato	plrv_orf1 plrv_orf2	1998, USA
RBMT22-238**-** New Leaf™ Plus Russet Burbank potato	plrv_orf1 plrv_orf2	1998, USA
RBMT22-262**-** New Leaf™ Plus Russet Burbank potato	plrv_orf1 plrv_orf2	1998, USA
SEMT15-02**-** Shepody NewLeaf™ Y potato	pvy_cp	1998, USA; 1999, Canada; 2001, Australia, Mexico, New Zealand; 2003, Japan, Philippines; 2004 South Korea
SEMT15-07**-** Shepody NewLeaf™ Y potato	pvy_cp	1998, USA
SEMT15-15**-** Shepody NewLeaf™ Y potato	pvy_cp	1998, USA; 1999, Canada; 2001, Australia, Mexico, New Zealand; 2003, Japan, Philippines; 2004 South Korea
TIC-AR233-5	pvy_cp	2018, Argentina

Viral genes or protein-coding sequences used as transgenes: ac1- replicase-encoding gene of bean golden mosaic virus; rep- replicase-encoding sequence of papaya ring sport virus; orf1- replicase-encoding sequence of potato leaf-roll virus (PLRV); orf2- helicase domain-encoding sequence of PLRV; CP- coat protein-encoding sequences of cucumber mosaic virus (CMV), papaya ring spot virus (PRSV), plum pox virus (PPV), potato virus Y (PVY), zucchini yellow mosaic virus (ZYMV) or watermelon mosaic virus (WMV). **Source: International Service for the Acquisition of Agri-Biotech Applications (ISAAA)**

REPLICASE PROTEIN

Replicase protein gene is the second widely used gene to confer resistance against plant viruses and the strategy was referred to as replicase protein-mediated resistance (Golemboski *et al.*, 1990). RPMR gives nearly immune type and highly specific resistance for the virus from which the transgene was isolated. Potato lines combining RPMR to PLRV and BT resistance to Colorado potato beetle have also been developed (Thomas *et al.*, 1995; 1998). RPMR also showed promise for developing VRTPs resistant to gemini viruses, which are emerging aT a fast rate and are major growth limiting factors in a wide range of crops in various parts of the world (Varma and Malathi, 2001). The transformed *N. benthamiana* plants either remain symptomless or produced delayed and attenuated symptoms (Hong and Stanley, 1996).

A transgenic approach to engineering MSV-resistant maize was developed and tested by Shepherd *et al.* (2007; 2007a). A pathogen derived resistance strategy was adopted by using targeted deletions and nucleotide-substitution mutants of the multifunctional MSV replication-associated protein gene (*rep*). Transgenic lines containing a mutated full-length *rep* gene displayed developmental and growth defects, those containing a truncated *rep* gene were fertile and displayed no growth defects, making the truncated gene a suitable candidate for the development of MSV-resistant transgenic maize.

Anderson *et al*. (1992) transformed *Nicotiana tabacum* cv. Turkish Samsun with a modified and truncated replicase gene encoded by RNA-2 of cucumber mosaic virus strain. The replicase gene had been modified by deleting a 94 base pair region spanning nucleotides 1857-1950; the deletion also caused a shift in the open reading frame, resulting in a truncated translation product ~75% as large as the full length protein. Transgenic lines when challenged with either cucumber mosaic virus virions or RNA at concentrations up to 500 pg/ml or 50 pg/ml, the resistance was absolute, as neither symptoms nor virus could be detected in uninoculated leaves, even after prolonged incubation (120 days after inoculation). These data suggest, therefore, that such a "replicase-mediated" resistance strategy may be applicable to other plant and animal viruses.

MOVEMENT PROTEIN

Cell to cell transport of viruses in plants is brought by movement proteins (MPs) (Mushegian and Koonin, 1993) or coat protein (Dolja *et al.*, 1995) or helper component proteinase (HC-PRO) protein (Cronin *et al.,*1995) encoded by viruses. This approach offers an attractive possibility to confer broad spectrum resistance to related and unrelated viruses. A defective TMV MP expressed in transgenic tobacco plants had shown to confer varying resistance to a number of viruses that are not members of tabamovirus group, including AMV, CMV, PCSV, TRV, and TRSV (Cooper *et al.*, 1995). Transgenic potato lines expressing mutant PLRV pr 17 movement protein exhibited resistance against the unrelated viruses PVY, and PVX (Tacke *et al.,* 1996). Transgenic plants expressing the putative movement protein gene from TSWV were resistant to infection by TSWV strains only (Prins and Goldbach 1998).

Liu *et al.* (2001) reported that both the movement protein (MP) and coat protein (*CP*) of maize streak virus (MSV) are required for systemic infection. Towards understanding the roles of these two proteins in virus movement, each was expressed in *E*. *coli* and interactions of the MP with viral DNA or *CP* were investigated using South-Western, gel overlay and immune precipitation assays. Unlike the *CP*, the MP did not bind to viral DNA but it interacted with the *CP in vitro* and an MP-*CP* complex was detected in extracts from MSV-infected maize, indicating the potential for interaction *in vivo*. Microinjection showed that the MP could prevent the nuclear transport of an MSV *CP*-DNA complex in maize and tobacco cells. These results were consistent with a model in which the MP diverts a *CP*-DNA complex from the nucleus (where viral DNA replication takes place) to the cell periphery, and in co-operation with the *CP*, mediates the cell-to-cell movement of the viral DNA. In this respect, the MSV-MP and *CP* have a functional analogy with the BC1 and BV1 proteins, respectively, of the *Begomovirus* genus of the *Geminiviridae*.

SATELLITE AND DEFECTIVE INTERFERING RNAS

Some viruses have specific satellite RNA molecules (sat-RNA), which are considered viral parasites being dependent on the helper virus for multiplication (Matthews, 1991). Transgenic tobacco expressing sat RNAs of CMV or TRSV on challenging inoculation exhibited attenuation of disease symptoms (Harrison *et al.*, 1987; Gerlach *et al.*, 1987). This strategy has not gained much acceptance as resistance is often incomplete and minor mutations in sat-RNA into a disease exacerbating sat-RNA as has been shown for CMV sat-

RNA. The first demonstration of this approach in plants was for ACMV, a member of the Gemini virus group (Stanley *et al.*, 1990). DI RNA has been shown to reduce disease severity due to CyRSV in transformed *N. benthamiana* plants (Rubino *et al.*, 1992).

POST-TRANSCRIPTIONAL GENE SILENCING (PTGS)

Stam *et al.* (1997) reported that in genetically modified plants, the introduced transgenes are sometimes not expressed and can be silenced. Transgenes can also cause the silencing of endogenous plant genes if they are sufficiently homologous, a phenomenon known as co-suppression. Silencing occurs transcriptionally and post-transcriptionally but the silencing of endogenous genes seems predominantly post-transcriptional. If viral transgenes are introduced and silenced, the post transcriptional process also prevents homologous RNA viruses from accumulating; this is a strategy for generating virus-resistant plants. Virus resistant plants have been developed successfully by targeting the coat protein through RNAi. Bonfim *et al.* (2007) developed a common bean resistant against geminivirus BGMV through RNAi approach. Taha *et al.* (2016) reported that the 3' end fragment of the *Rep* gene used for stable transformation on the transgenic *C. pepo* plants showed a higher level of resistance against squash leaf curl virus using PTGS tool. Lin *et al*. (2012) developed transgenic watermelon resistant to *cucumber mosaic virus* and water melon mosaic virus. To develop transgenic watermelon resistant to multiple infections, a single chimeric transgene comprising a silencer DNA from the partial DNA of the N gene of watermelon silver mottle virus (WSMoV) fused to the partial coat protein (*CP*) gene sequence of CMV was transformed *via Agrobacterium*-mediated transformation. The progeny R_1 of the two resistance R_0 lines showed resistance to CMV and WMV but not to CGMMV. Low level accumulation of the transgene transcripts in resistant plants was detected through Southern and northern analysis, indicating that the resistance was established through post-transcriptional gene silencing (PTGS). This study demonstrated that the fusion of different viral *CP* fragments in transgenic watermelon contributed to multiple virus resistance via PTGS. Transgenic bean (*Phaseolous vulgaris*) plants engineered with genes Rep-TrAP-Ren and BC1 of BGMV, a gemini virus in antisense orientation showed attenuated symptoms to the virus (Fuchs *et al.,* 1997). Cruz *et al.* (2014) obtained 11 WSMV RNAi independent transgene events and each line exhibited higher resistance towards WSMV and found stable through the T_5 generations.

CRISPR/CAS 9 SYSTEM

Genome editing by CRISPR/Cas 9 system can yield disease resistance plants in a short period. The different parts of the viral genome have been targeted and transgenic plants are developed. In the recent years, the new breeding techniques like SSN (sequence specific nucleases), ZFN (zinc finger nucleases), TALEN (transcription activator like effector nucleases), CRISPR/Cas9 have been developed for genetic improvement of plants against pathogens. Baltes *et al.* (2015) reported that the *N. benthamiana* plants exhibited higher resistance against bean yellow dwarf virus through Cas 9 technology. Studies of Zhang *et al.* (2018) demonstrated a significant reduction in viral symptoms against cucumber mosaic virus in *N. benthamiana* plants. The resistant plants were developed using CRISPR/Cas 9 system and might be considered as non-GM crops.

CONCLUSION

In this review, the various strategies being employed to generate virus resistant transgenics through genetic engineering techniques are highlighted. Among the various approaches, pathogen derived resistance through CP-MR is most widely adopted for development of VRTs. In most of the successful transgenics, durable resistance could be introduced and resistance was manifested in terms of complete lack of symptoms, delayed symptom development and low viral titer. The advent of new tools and precise techniques like genome editing and rapidly accumulating knowledge on host genome and mechanisms of resistance would accelerate generation of plants with broader and durable resistance to various viruses.

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NATURAL FOOD COLOURS: MINI REVIEW

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ABSTRACT

Colour of food makes the food from ordinary to extraordinary by its eye-catching appearance. More than attraction natural colours are safe with potential nutritional and therapeutic effects. There is growing Global market demand for food colour with an estimated USD 1.79 billion in 2016, CAGR of 5.9% and which is going to be increased to 6.1% during 2018-2030. The endogenous antioxidants and reactive oxygen species are also been encountered by these natural colours extracted from various fruits and vegetable parts. To avoid synthetic colours use in food that causes diverse diseases, the demand for natural colours has been increased with added health benefits are deliberated.

Keywords: Antioxidant, Anthocyanins, Betalains, Chlorophyll, Food colours, Market demand, Nutraceuticals

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INTRODUCTION

Food is an undeniable energy source of mankind, and there is no way of living without eating (Carocho et al., 2015). Hence this is an utmost valuable commodity for the wellbeing of the living. Colour, flavour, and texture are also important characters affecting the sensory acceptability of the foods by the consumers. From an aesthetic point of view, the development of the food with pleasant-looking appearance and flavour is a significant objective in the food processing industries. Hence the colouring of the foods is an important factor for attracting foods by the food industry. The concept of natural colours use over synthetic chemical dyes gained importance. Natural colours extracted from fruits and vegetables serve as a promising food-colourants, acts as an antioxidant that fights against harmful diseases.

Colour is the visual palette that “sets the table” to other sensory experiences. The use of synthetic dyes has gone up fivefold in the past 50 years due to low prices. The indiscriminate use of these dyes leads to behavioural problems in children etc. Now, there has been a great concern for synthetic food colours as most of these artificial colour additives have demonstrated negative health issues following their consumption, the food industry repeatedly raises the demand for natural pigments. Therefore, there is a growing interest in natural food colourants developments of plant origin which offers multiple benefits to human health (Ali et al., 2011). The expected natural colours used for shading foods ought to have been passed the security and quality standards. In perspective on ascending in consumers’ mindfulness, there is a need to create a natural colour based functional foods having nutraceutical potential. This interest is fuelled by cutting edge look into natural food-colour-shading. To consider the natural colours as a food colorant; stability, yield and cost are the principle requirements. A large portion of them are delicate to pH, warmth and daylight. It is innovatively plausible to get ready new natural food colourants from locally-known plants and microorganisms that have not been researched experimentally.

Generally, natural colours are extracted from fruits and vegetables are of four different types such as green chlorophylls, orange to yellow carotenoids, violet to purple anthocyanins and red betalains (Figure 1). The selection of the type of colour and their use depends on the consumer’s target shade, the physical or chemical attributes of the food with good retention of colour during shelf studies without any deterioration is important (Sigurdson et al., 2017).

Carotenoids are tetraterpenoids found in the chloroplast of photosynthetic organisms. They absorb light in the 400-500 nm range with yellow, orange and red colour. Anthocyanins are water-soluble phenolic groups in glycosylated forms. They are found in berries, grapes, and vegetables. Betalains are water-soluble nitrogen-containing vacuolar pigments of the order Caryophyllales with 75 known structure types with yellow to red appearance.

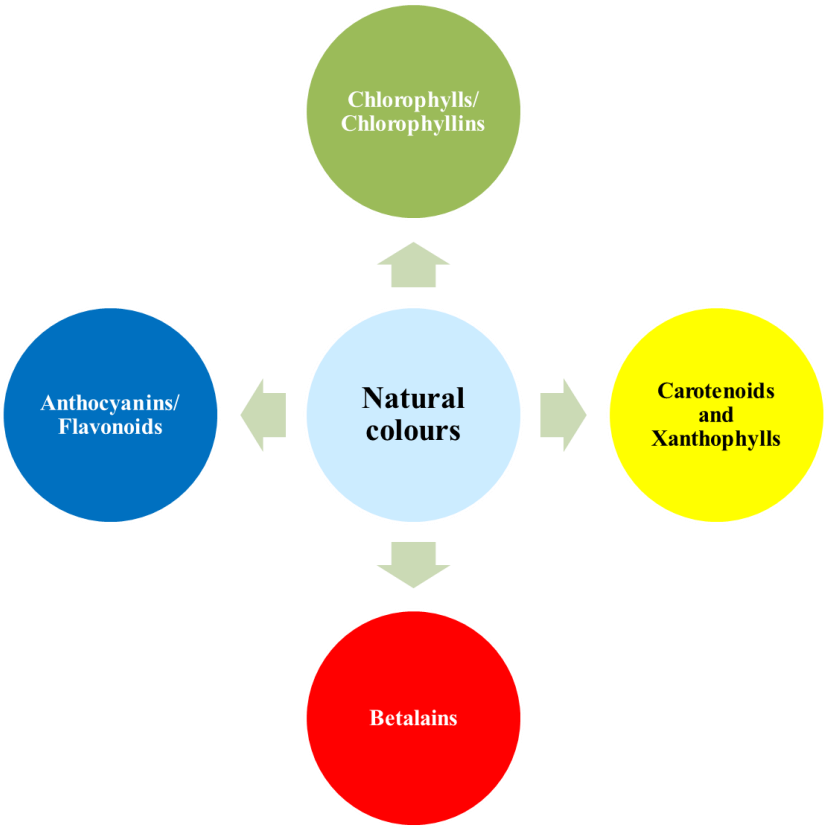

Figure 1. Types of natural colourant available.

Globally colouring food with food is a new concept in recent times, especially in food processing seasoning and colouring agent. The colours made from using the physical manufacturing process and their processing without any chemical additives or GRAS solvents from fruits, vegetables and edible plants can be considered as a food colourant (Mcavoy, 2014). Nowadays there is a growing demand in the global market for truly natural colours that are having enough raw material traceability, good stability upon the process in foods and beverages, safe label and sustainable chain at each step right from identification to commercialization. In addition to this, the recommended natural colourant should be suitable for diverse diets such as a wagon, kosher, vegetarians and halal etc.

NATURAL FOOD COLOURANTS

The history of colours in the food industry that originated in Egypt for coloured candy preparation and coloured wine as long as 400 BC.The developing food industry has a vast array of synthetic colour used in the late 1800s. In general food colourants, are classified as

three classes such as artificial (chemically synthesized), selective extracted additive colours (beta carotene and caramine) and natural colours (naturally synthesized). The use of synthetic colours led to health issues due to some toxic compounds in it. Hence the concept of natural colours that are defined as any dye, pigment or any substance obtained from plants, animals, insects, algae, mineral or other sources that capable of colouring food and cosmetics. These natural colours can be developed by the changes in factors such as pH, heat, light, storage condition and processing conditions (Shamina et al., 2007).

The extraction of food extractives ranges from a simple extraction to degrees of selective extraction up to isolation of pure pigments. The classification of these products is essential to identify when a product is no longer “a food normally consumed as such or normally used as a characteristic ingredient of food” which needs a mandatory approval. The relationship between the ratios of pigment content to the nutritive or aromatic constituents in the colouring product to that of the source material is termed as enrichment factor. Once the pigments are selectively extracted relative to the nutritious or aromatic constituents, the extract should be classified as a food colour. The differentiation between selective and non-selective extraction can be known by analysing the threshold value. The value of the threshold should be high and low enough to cover seasonal and geographical differences, and the primary extracts could be still considered to be foods or food ingredients with food colour specifications.

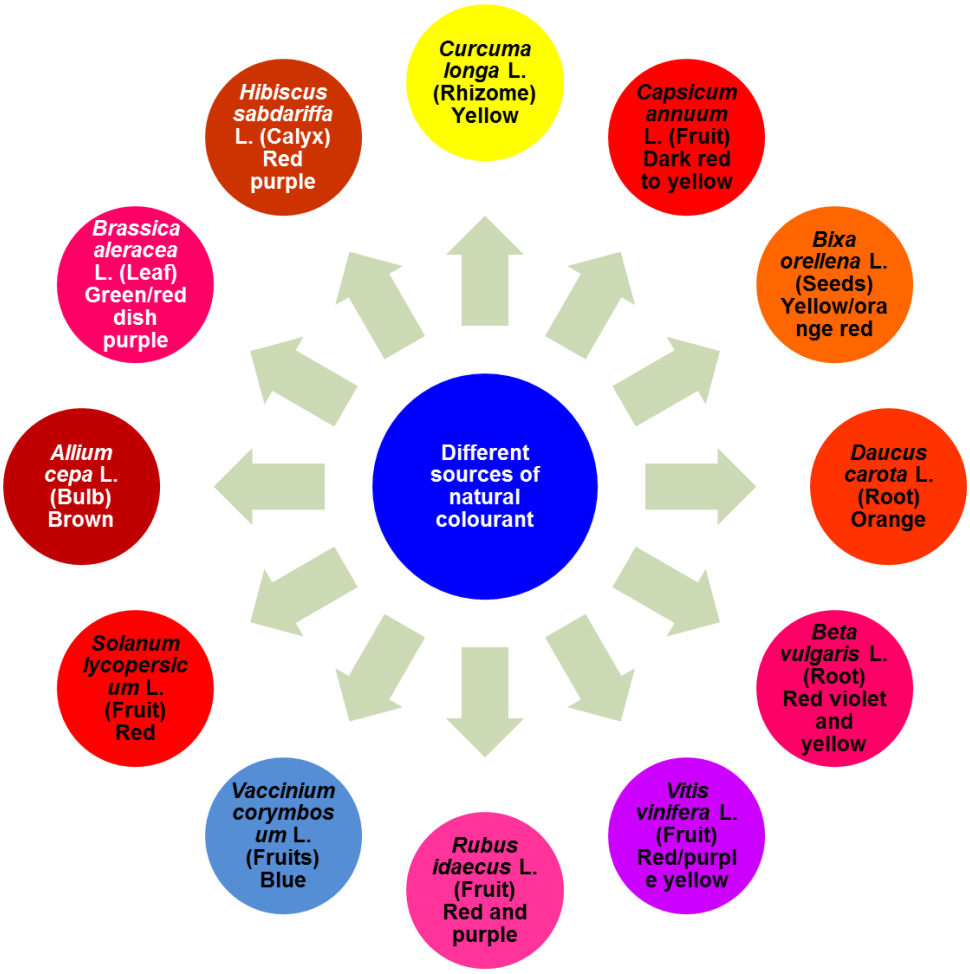

Figure 2. Different sources of natural colours explored with different colour properties.

AESTHETIC PROPERTIES

The food perception of the senses, i.e., eyes for colour, lustre, texture and appearance represents the aesthetic properties of food. They are determined by the type of additive added to make the processed food more consumers acceptable. Colour is an important parameter for the sense to accept the food to be consumed. The natural colours are eco-friendly and possess beneficial health activities for the consumers to better health of humans. Optimised quantity of the colour to foods is very important for acceptance. Natural additives with good consumer acceptability scores will increase the sensory acceptability of foods.

APPLICATIONSN FOOD

Natural colours use for colouring foods, beverages, confectionery and in baking, etc. Over, 2000 natural colours have been reported to be produced by nature plants. The main coloursare anthocyanins (E163) withcyanidin, delphinidin, malvidin, pelargonidin, peonidin, petunidintypes applied in the preparation of soft drinks, confectionary products and spreads, etc. The strawberry, grape skin and raspberries red-purple anthocyanins are

popularly used in confectionery and dessert products. The red colour from red cabbage is used to colour chewing gum and beverages. Elderberry deep red colour is used in sorbets and sauces etc. The betanin (E162) from beetroot is approved natural food colourant used in dairy, and meat products etc.

Similarly, there are well established other sources of betalains use in ice cream, spread and beverages preparation (Khan et al., 2015a and Kumar et al., 2015a). Annatto (E162B) is high in carotenoids, which may benefit eye wellbeing. Specifically, it's high in the apocarotenoid, bixin and norbixin, which are found in the external layer of the seed and help give it its vibrant yellow-to-orange shading to foods and they are having very good stability in processed foods (Giridhar andParimalan, 2010; Giridhar et al., 2014).

Chlorophylls (E140) is a vegetable pigment that occurs in the green parts of the plant that confer colour. Only chlorophyll a and b are widely used green food colours out of five chlorophyll types available. The green chlorophyll extracted from nettles, grass, algae vegetables, parsley and broccoli etc. are well used in the preparations of pasta, confectionery, medicines and vegetable oils etc. Similarly, curcumin (E100) extracted from *Curcuma longa* L. rhizome is another widespread yellow natural used as food colourant. In many European countries and the United States mandatorily clear product label specifying/containing fruits and/or vegetable juice (for colours) or spirulina extract (for colours).

Colours making, stability testing upon fortification/processing meeting regulatory guidelines and especially of the various stages come across or every natural colourant to be considered as a food colourant. The colour term refers to the EC regulations No 1333/2008 on food additives and giving elements for differentiating colours from foods with colouring properties. However, extract is a colour it should be in accordance with the EU regulations No 231/2012. Moreover, it may be a plant or animal extract showing the colour property is used as food colourant then the source material should be approved as a food with food legislation.

The colour extracted from the stigma/style of *Crocus sativus* is saffron used for flavouring and colouring food. It is sensitive to pH changes and highly unstable with physical conditions such as light and oxygen but showing very good heat stability. It is used in baked foods, meat, confectionary and medicinal value for stomach ailments (Stich, 2016).

There is a similar kind of yellow pigment extracted from the floral parts of safflower plant *Carthamus tinctorius* L. commonly known as American or Mexican saffron. The major

pigment extracted from flowers is carthamine (a quinoid form of isocarthamin) with 30% and a red pigment of about 0.5%. The yellow colour has not been approved bythe US or in EEC, but as per existing regulations permit its use in Japan that is stableto heat and light that is popularly used in beverage and baked foods.The stigma of the plant contains a yellow crocin pigment (water soluble glycoside), safranal a fragrance and a bitter substance picocrocin.

In the remote past, saffron has long been used to give a yellow hue to rice. Post-industrialization leads to rapid urbanization and changed life style that paved the way for food processing and further considered as the beginning of colouring various foods that processed industrially (Downham and Collins, 2000). The edible plant sources such as tomatoes, vegetables (spinach, red beet), fruits (grapes, blackberries, elderberries) are often considered to colour food, which were initially deliberated as additives mainly because of their high colour intensity and colour hue. All these colouring foods are mostly subjected to physical processing only. Colourings from animal source or animal-derived were also used such as Cochineal insect derived orange-red carminic acid or squid ink to impart black appearance to pasta. The colouring substances in the edible plants are always safe. Also, the colouring principle in respective extracts together associated with dietary phytochemicals to which potential health benefits is attributed (Stintzing and Carle, 2004).

FUNCTIONAL ATTRIBUTES

All natural colours have been shown to be good antioxidant and show nutraceutical properties. Anthocyanins are one of the bioactive metabolite having nutraceutical and traditional medicinal value. It is used as an appetite stimulant, choleretic agent and in the treatment of many diseases. These coloured pigments are potent nutraceuticals possessing health benefits such as antioxidant effects, antiangiogenesis, and prevention of cardiovascular diseases, anticancer, visual health, antimicrobial, antiobesity and neuroprotective function. Betalains are also structural analogs of anthocyanins with antiradical activity. They also act to fight against chronic diseases such as cancer and inflammation etc. Betalains from red beets have 1.5-2.0 folds greater free radical scavenging activity than some anthocyanins. Gomphrenin type betalains were found stronger antioxidant activity than potent ascorbic acid molecule (Kumar et al., 2015b). The *in vitro*, *in vivo* and a combination of both proving the potential health benefits of betalains with mechanisms involved, were scientifically reported (Gengatharan et al., 2015).

Based on the pharmacological properties such as anticancer, anti-inflammatory and antimicrobial activities of betalains, the betalains fortified foods served as functional foods to the consumers (Khan &Giridhar, 2015b; Khan et al., 2016). A Good number of epidemiological evidence showed that the consumption of yellow carotenoid rich foods lowered the risk of chronic diseases such as cancer and cardiovascular diseases etc. These carotenoid fortified foods are sensory accepted with new technologies developed by the long-term basis. Many carotenoids have been identified and characterized from various sources; in any case, far less are devoured and arrive at noticeable levels in serum and tissues, and just some of which can be changed over to vitamin A in humans. It has likewise been appeared to cross the blood-brain barrier and is perceivable in the mind tissue. Recently astaxanthin produced by freshwater and marine microorganisms is also considered as one of the strongest antioxidants in nature is receiving enough attention for its neuroprotective role is considered as a new perspective in the treatment of Alzheimer and Parkinson diseases (Galasso et al., 2018). In view of these cognitive benefits, astaxanthin can be considered as a BioAstin. In addition to this astaxanthin is having the ability to protect the eye from light-induced damage, photoreceptor cell damage ganglion cell damage, neuronal damage, and inflammatory damage.

In conclusion, natural food-colorants do not appear to propel now and despite the fact that we don't use it ordinary regardless it proves to be useful now and again. Food shading can truly help to emulate our mood or add some aesthetic appearance. There are numerous things we use colours for and without them our foods would be plain and lacklustre.

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ESTABLISHMENT OF PHYTOCOENOSES ON COAL MINE OVER BURDEN OF PRANAHITA GODAVARI BASIN, TELANGANA

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ABSTRACT

Coal mine industry ravaged areas are often called as "Industrial deserts" or "Lunar scapes" that does not permit normal growth of plants as such sited dumped with technogenic waste and deficit in plant required resources (moisture and essential nutrients). Restoration of such pe is cost prohibitive and require knowledge of local flora, pant taxonomy and plant biology. USA and Russia have been practicing long term research of "biological recultivation" which has been satisfactorily implemented in large tracts by seeding of perennial grasses, tree and bushy species. In this paper we are presenting a case study on how to establishment of phytocoenoses

on coal mine over burden of Pranahita-Godavari basin in the State of Telangana

Key words: biological recultivation, industrial deserts, phytostabilization, plant taxonomy

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INTRODUCTION

The coal deposits in India are primarily concentrated in the Gondwana sediments occurring mainly in the eastern and central parts of Peninsular India, The estimated Coal Reserves in the State of Telangana as on 1.4.2017 = 21464 (In million tonnes). quantity of production of coal in different states in 2016-17 amounted 662.79 million tons out of which the State of Telanga contributed 8.98% (Anonymous (2018)

Coal mining disturbs the environment and nature. Opencast mining removes the large amount of soil and rocks, vegetation and produces huge volume of solid waste in the form of tailings. The tailings show adverse effects on the growth of vegetation due to low organic content, alkalinity, sodicity, acidity, poor water holding capacity, toxicity, unfavourable pH, soil fertility, oxygen deficiency and inadequate nutrient conditions (Bell 2001, Bing-Yuan and Li-Xun 2014, Bolshakov V and Chibrik T. 2007). Approximately twenty five tons of overburden would be generated for extracting one ton of coal (Prasad et al. 2016). The overburden (tailing) is material which is removed from a specific location using excavators, draglines and glovels and dumped in another location layer by layer. Overburdens cause drastic changes in the environment and affect the human health. It can also impact on the health of people, animals and environment in which they survive. Mine waste dumps are low in organic materials, nutrients and moisture.

Phytostabilization improve the vegetation on the coal mine overburdens by introducing tolerant plant taxa serves to reduce erosion and dust spreading in the environs. It is an eco-friendly technique that minimizes the risk of contamination and enhances the restoring the vegetation. Planting of suitable grass, seedlings on the tailings promotes the soil fertility, ecological succession and improves the aesthetics of the area (Shoeran et al., 2010) and reduces surface water run-off and creates the mycorrhizal interaction to promote the green landscape. Revegetation of desirable, suitable and locally economic important plant species would decrease the spread of contamination (Gawronski and Gawronska, 2007; Prasad et al., 2016). Due to the lack of information about the plant communities growing on and around overburdens of mining areas and surrounding polluted areas in the state as there is no such record available about the distribution of plant species, diversity, nativity, exotic and their adaptation, the present study has taken up for the documentation of the local vegetation, diversity and the importance of local flora for the stabilization and eco-restoration on the mine overburdens.

STUDY AREA

Telangana is the youngest and 29th state of India formed on 2nd June, 2014 with the capital of Hyderabad district. It lies in between latitudes 15°48’32” to 19°55’46”N and longitudes 77°09’02” to 81°18’ 51”E and spreads over an area of 1,12,077 km^2. The newly formed government has reorganized the then ten districts into 33 districts (Figure 1). It is bounded on the north and north-west by Maharashtra, west by Karnataka, north-east by Chattisgarh, east by Odisha and south by Andhra Pradesh. The state drains predominantly by Godavari and Krishna rivers. The state is dry and hot during the summer and the temperature rises up to 50°C in coal mining regions. The rainfall is high (ca. 1100 m) along the Godavari valley. In December and January, The temperature drops during nights and is very cold in December and January, in some places of the state drops up to 4°C (Suthari, 2013).

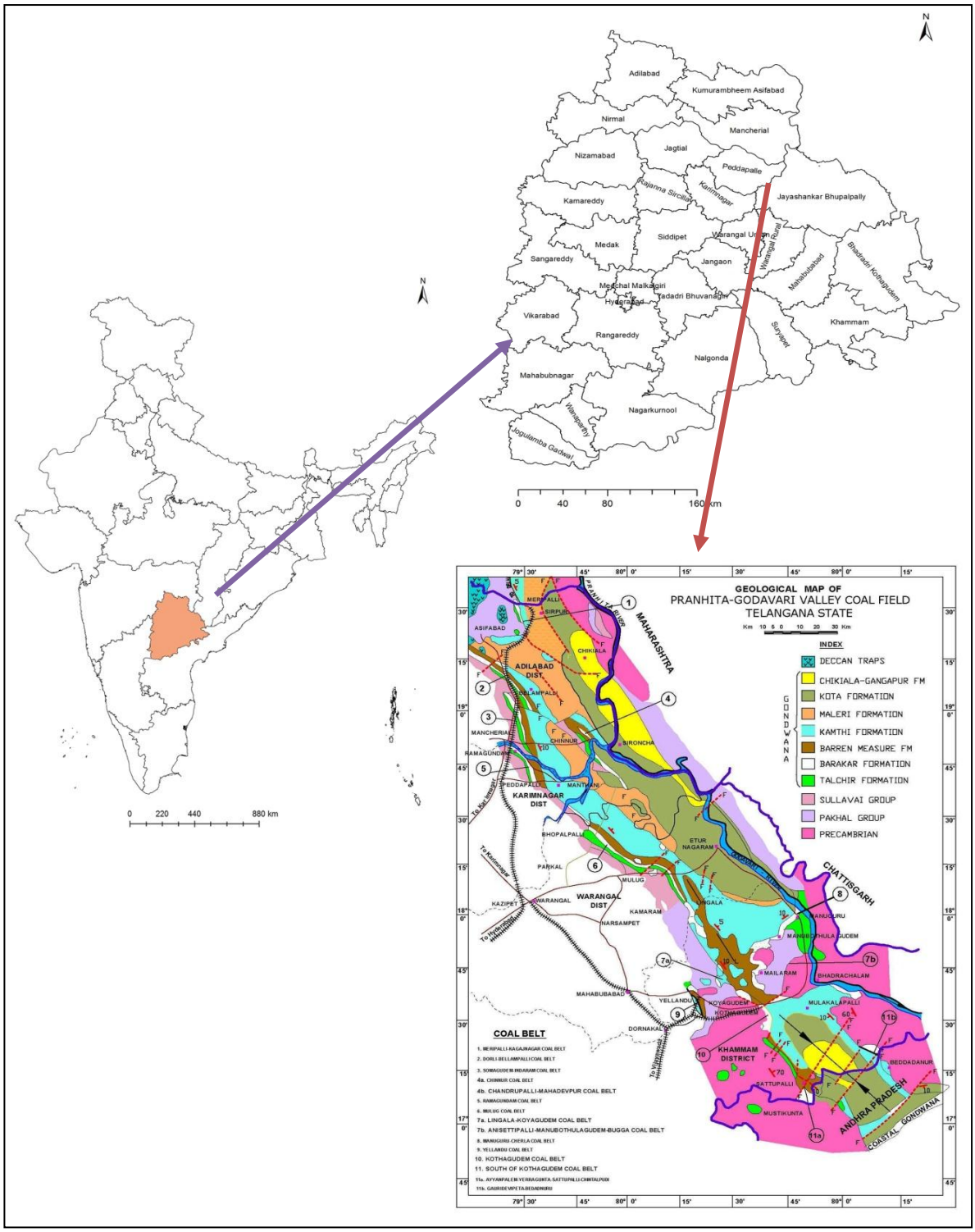


Figure 1. Map shows the locations of coal reserves in Telangana State along the gradients of Pranahita-Godavari valley, India (*image courtesy*: the SCCL).

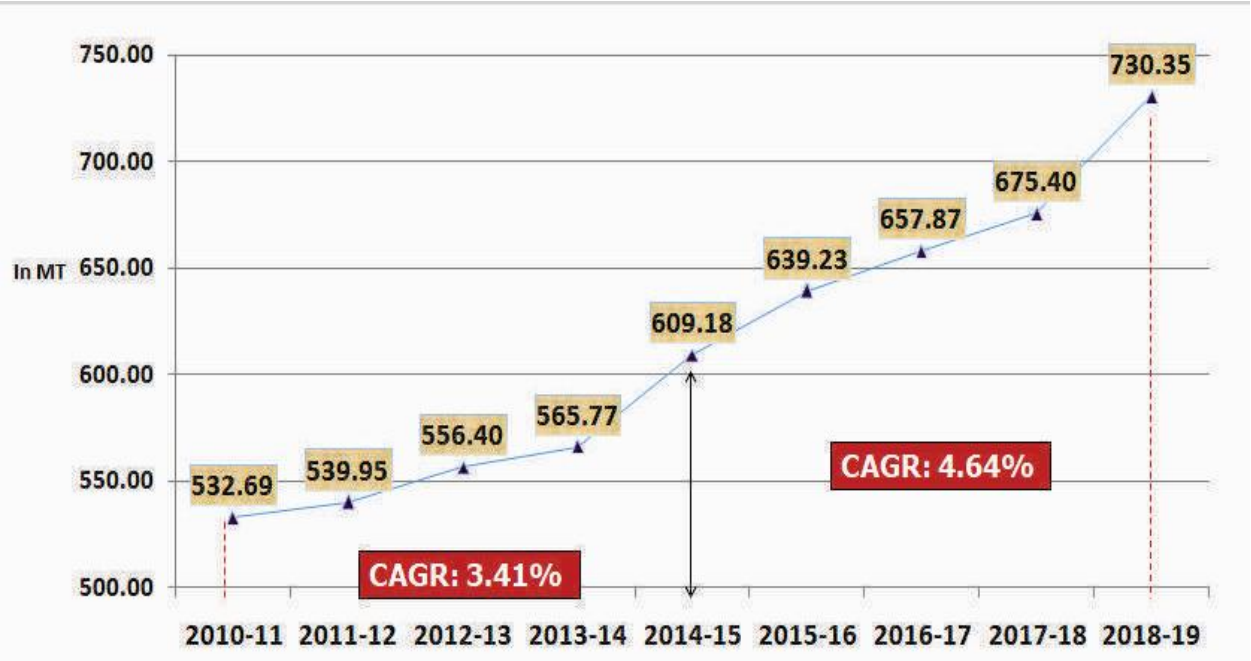


Figure 2. Coal production trend in India during 2010-2019 (CAGR: Compound Annual Growth Rate) (*Source*: The Ministry of Coal Annual Report, 2018-2019).

Telangana has rich coal deposits in Adilabad, Kumurambheem Asifabad, Mancherial, Peddapalli, Karimnagar, Jayashankar Bhupalpalli, Mulug, Bhadradri Kothagudem and Khammam districts along the Pranahita-Godavari valley and sharing 20% of the nation's coal. According to the report of GSI (2015) Godavari valley coal field area is a rich source of coal reserves as much as 22,207 million tonnes (Mt) of which only 1436.98 Mt of coal was extracted up to 2018-2019 (Anonymous, 2019).

METHODOLOGY

Extensive field surveys were conducted during 2016-2018 for the exploration of vegetation on the overburdens of mining areas of Telangana, India. A total of 42 quadrats were laid with a size of 31.62 × 31.62 m and area covered 4.2 ha to document the plant wealth. Extensive field surveys were conducted. The present study was covered the mine sites, namely, Bellampalli, Mandamarri, Mancherial, Srirampur, Godavarikhani, Ramagundam, Bhupalpalli and Sathupalli areas.

In peninsular India, Telangana is rich in coal deposits and being mined by M/s Singarenin Collieries Company Limited (SCCL). The SCCL owned the mining rights from the Hyderabad (Deccan) Co. Ltd. Best & Co. to exploit coal in 1920 and it has become a Government Company under the Companies Act, 1956. The motto of the SCCL is 'One Family One Vision One Mission'. The Pranahita-Godavari Valley is known for its coal deposits. It is producing

around 9% of the total coal production in India by operating 18 opencast and 30 underground mines in the nine districts of the State with manpower of 48,972.

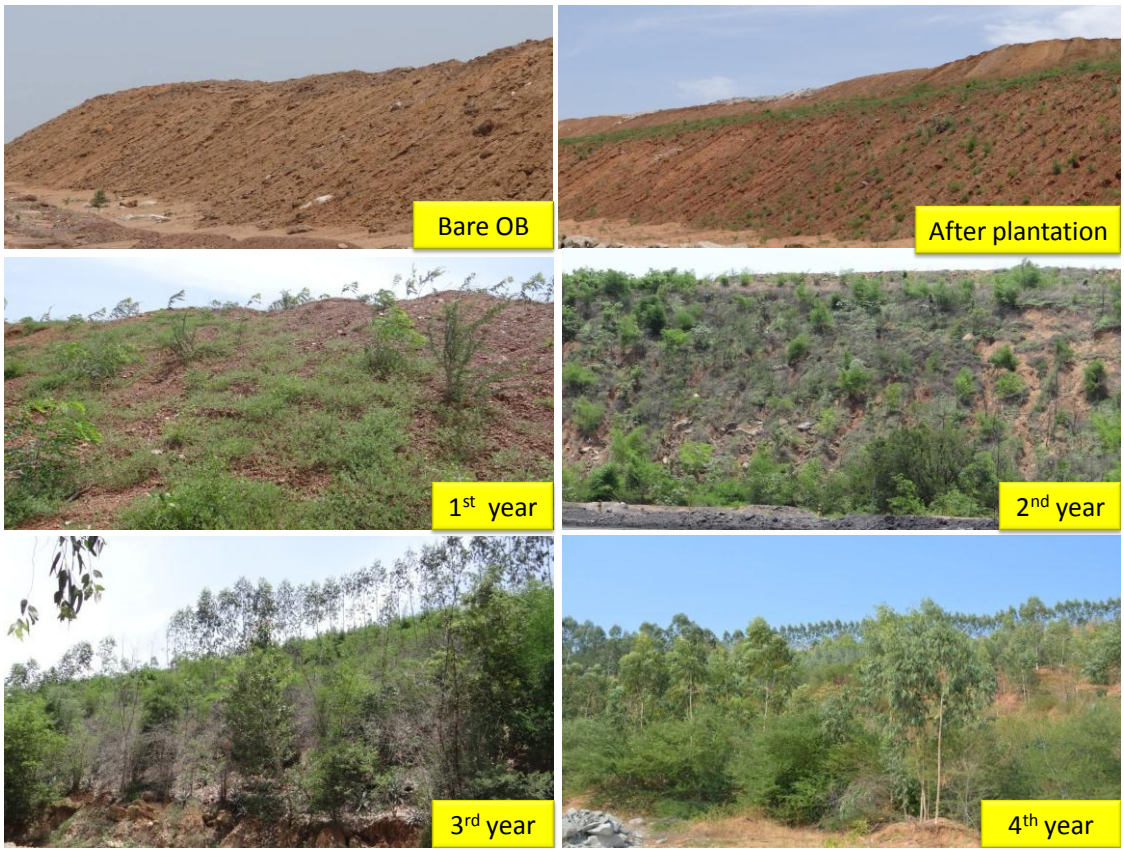
RESULTS

A total of 92 plant taxa pertaining to 79 genera and 29 families were enumerated through quadrat study and the plant species were arranged alphabetically and mentioned the availability of plants area-wise (Table 1). Of these, Fabaceae is the predominant family with 32 plant taxa, followed by Poaceae with 7, Apocynaceae, Asteraceae with 6, Convolucaee, Malvaceae with 5, Phyllanthaceae with 4 (Figure 3). Three species were recorded from Moraceae while Amaranthaceae, Euphorbiaceae, Lamiaceae and Meliaceae were placed seventh position with 2 plant taxa of each. The families such as Acanthaceae, Asparagaceae, Bignoniaceae, Combretaceae, Cyperaceae, Lauraceae, Molluginaceae, Myrtaceae, Papaveraceae, Passifloraceae, Rhamnaceae, Rutaceae, Sapindaceae, Solanaceae, Ulmaceae and Verbenaceae were spread with single species on the coal mine overburdens (Table 1).

Tailings in Bhupalpalli mining are recultivated by *Eucalyptus* plantation with various species like *Calotropis gigantea, Cajanus scarabaeoides, Echinops echinatus, Solanum surattense, Melochia corchorifolia, Pithecellobium dulce, Waltheria indica, Achyranthes aspera, Lepidagathis cristata, Indigofera linnaei*, etc. whereas the floristic diversity on the overburdens of Ramagundam mining area consists of *Calotropis gigantea, Hyptis suaveolens, Leucaena leucocephala, Alternanthera paranychioides, Atylosia scarabaeoides, Ipomoea obscura, Setaria verticillata, Dregea volubilis, Urena lobata, Cynodon dactylon, Crotalaria hebecarpa, Croton bonplandianum*, etc. (Table 1).

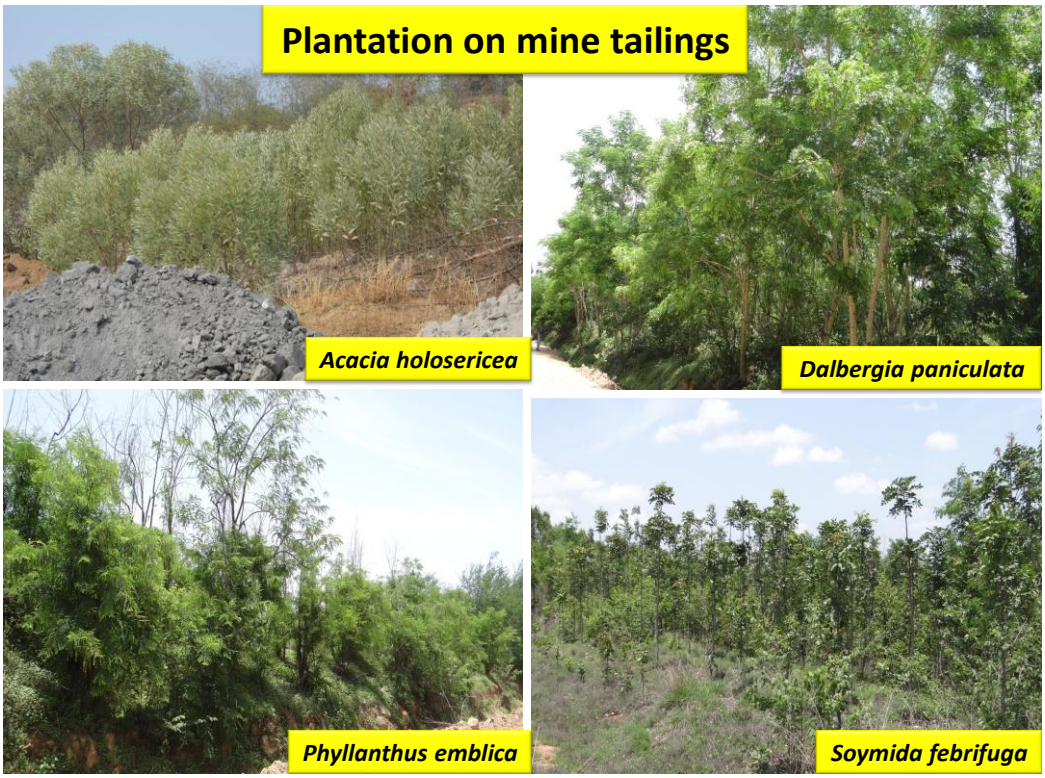
Pennisetum pedicellatum is highly dominated with its adventitious root system on the tailings of Ramagundam, Sathupalli and Godavarikhani mining regions. On the most of the tailings of Bhupalpalli, Godavarikhani, Komurambheem Asifabad, Mancherial and Bellampalli mining areas, the authorities are planted *Eucalyptus globulus, Acacia auriculiformis, Leucaena leucocephla, Bambusa bambos* and *Pithecellobium dulce* as fence. *Dalbergia paniculata, Prosopis juliflora, Azadirachta indica, Leucaena leucocephala, Gliricidia sepium, Senna siamea, Pithecellobium dulce, Vitex negundo, Eucalyptus globulus, Peltophorum pterocarpum* were planted on the overburdens of Srirampur area whereas in Sathupally, they initiated the planting of indigenous plants instead of exotics, ornamentals and introduced ones such as *Pterocarpus santalinus, Dalbergia paniculata, Ficus benghalensis, Hardwickia binata, Terminalia bellirica, T. chebula* and *Helicteres isora* along

the margin. Nearly 5000 saplings of *Pterocarpus santalinus* (red sanders) were planted 5 years back, now obtained GBH (girth at breast height) around 30 cm.



Climax vegetation through phytostabilization on the overburden of Ramagundam

Figure 3. Development of vegetation in various age series of mine dumps in Telangana.



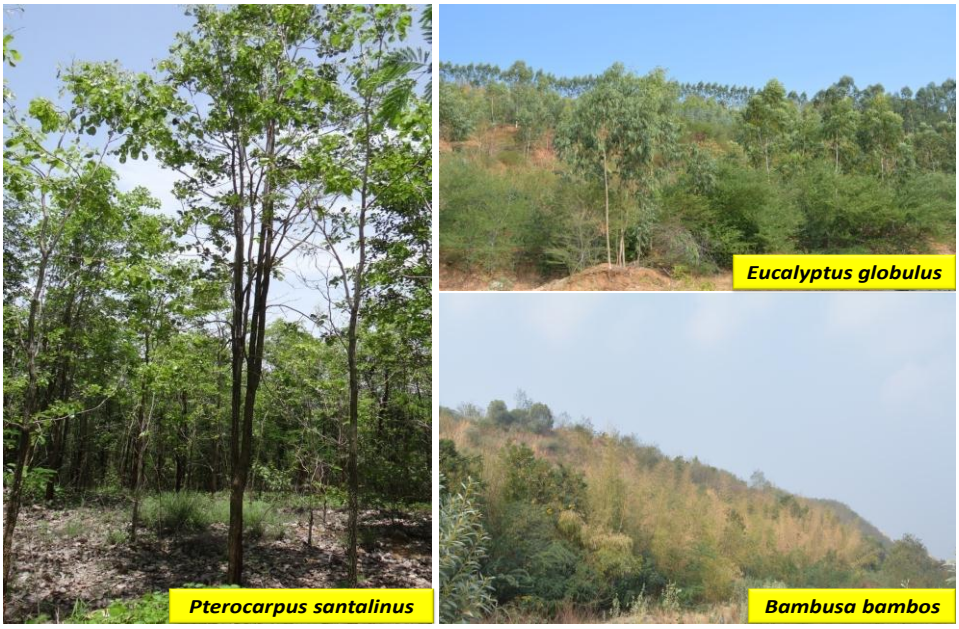


Figure 4. Various plant species as plantations on mine tailings in Telangana State.

Table 1. Local Flora on the overburdens of mining area and their occurrence and community structure.

	Plant Species	Family	Habit	OB area
1	*Acacia auriculiformis* Benth.	Fabaceae	Tree	Ramagundam, Sathupalli, Mandamarri
2	*Acacia holosericea* G.Don	Fabaceae	Shrub	Srirampur
3	*Acacia nilotica* (L.) Delile	Fabaceae	Tree	Bhupalpalli, Srirampur, Bellampalli
4	*Achyranthes aspera* L.	Amaranthaceae	Herb	Bhupalpalli, Ramagundam, Srirampur, Godavarikhani
5	*Aegle marmelos* (L.) Corrêa	Rutaceae	Tree	Sathupalli
6	*Agave americana* L.	Asparagaceae	Herb	Bhupalpalli, Sathupalli, Bellampalli
7	*Albizia lebbeck* (L.) Benth.	Fabaceae	Tree	Ramagundam, Mandamarri
8	*Albizia odoratissima* (L.f.) Benth.	Fabaceae	Tree	Sathupalli
9	*Alternanthera paronychioides* A.St.-Hil.	Fabaceae	Herb	Ramagundam, Srirampur, Mandamarri
10	*Alysicarpus monilifer* (L.) DC.	Fabaceae	Herb	Bhupalpalli, Ramagundam, Godavarikhani
11	*Argemone mexicana* L.	Papaveraceae	Herb	Srirampur, Mandamarri
12	*Azadirachta indica* A.Juss.	Meliaceae	Tree	Bhupalpalli, Srirampur, Sathupalli, Godavarikhani
13	*Bambusa bambos* (L.) Voss	Poaceae	Shrub	Bhupalpalli, Srirampur, Sathupalli, Bellampalli
14	*Bauhinia racemosa* Lam.	Fabaceae	Tree	Sathupalli
15	*Blumea lacera* (Burm.f.) DC.	Asteraceae	Herb	Srirampur, Godavarikhani
16	*Cajanus scarabaeoides* (L.) Thouars	Fabaceae	Climber	Bhupalpalli, Ramagundam, Sathupalli, Bellampalli
17	*Calotropis gigantea* (L.) Dryand.	Apocynaceae	Shrub	Bhupalpalli, Ramagundam, Srirampur, Sathupalli, Mandamarri

18	*Calotropis procera* (Aiton) Dryand.	Apocynaceae	Shrub	Ramagundam
19	*Cassytha filiformis* L.	Lauraceae	Tree	Bhupalpalli
20	*Celosia argentea* L.	Amaranthaceae	Herb	Bhupalpalli, Bellampalli
21	*Chloris barbata* Sw.	Poaceae	Herb	Srirampur
22	*Chromolaena odorata* (L.) R.M.King & H.Rob.	Asteraceae	Shrub	Ramagundam, Sathupalli, Mandamarri
23	*Cleistanthus collinus* (Roxb.) Benth. ex Hook.f.	Phyllanthaceae	Tree	Bhupalpalli
24	*Clitoria ternatea* L.	Fabaceae	Climber	Ramagundam
25	*Crotalaria hebecarpa* (DC.) Rudd	Fabaceae	Herb	Ramagundam
26	*Crotalaria juncea* L.	Fabaceae	Herb	Bhupalpalli (escape)
27	*Crotalaria verrucosa* L.	Fabaceae	Herb	Ramagundam
28	*Croton bonplandianus* Baill.	Euphorbiaceae	Herb	Ramagundam, Godavarikhani
29	*Cryptostegia grandiflora* Roxb. ex R.Br.	Apocynaceae	Climber	Ramagundam
30	*Cynodon dactylon* (L.) Pers.	Poaceae	Herb	Bhupalpalli, Ramagundam, Mandamarri
31	*Dactyloctenium aegyptium* (L.) Willd.	Poaceae	Herb	Bhupalpalli, Ramagundam, Sathupalli, Bellampalli
32	*Dalbergia lanceolaria* subsp. *paniculata* (Roxb.) Thoth.	Fabaceae	Tree	Bhupalpalli, Srirampur, Sathupalli
33	*Dalbergia sissoo* DC.	Fabaceae	Tree	Ramagundam
34	*Dichrostachys cinerea* (L.) Wight & Arn.	Fabaceae	Shrub	Sathupalli
35	*Dodonaea viscosa* (L.) Jacq.	Sapindaceae	Shrub	Bhupalpalli
36	*Dolichandrone falcata* (Wall. ex DC.) Seem.	Bignoniaceae	Tree	Bhupalpalli
37	*Dregea volubilis* (L.f.) Benth. ex Hook.f.	Apocynaceae	Climber	Ramagundam, Mandamarri
38	*Echinops echinatus* Roxb.	Asteraceae	Herb	Bhupalpalli, Godavarikhani

39	*Euphorbia indica* Lam.	Euphorbiaceae	Herb	Srirampur
40	*Eucalyptus globulus* Labill.	Myrtaceae	Tree	Bhupalpalli, Ramagundam, Srirampur, Sathupalli, Mandamarri, Bellampalli, Godavarikhani
41	*Evolvulus alsinoides* (L.) L.	Convolvulaceae	Herb	Bhupalpalli, Godavarikhani
42	*Ficus benghalensis* L.	Moraceae	Tree	Sathupalli
43	*Ficus racemosa* L.	Moraceae	Tree	Sathupalli
44	*Ficus religiosa* L.	Moraceae	Tree	sathupalli
45	*Fimbristylis falcata* (Vahl) Kunth	Cyperaceae	Herb	Bhupalpalli, Ramagundam
46	*Gliricidia sepium* (Jacq.) Walp.	Fabaceae	Tree	Bhupalpalli, Srirampur
47	*Hardwickia binata* Roxb.	Fabaceae	Herb	Bhupalpalli, Sathupalli
48	*Helicteres isora* L.	Malvaceae	Herb	Bhupalpalli, Sathupalli
49	*Heteropogon contortus* (L.) P.Beauv. ex Roem. & Schult.	Poaceae	Herb	Ramagundam
50	*Holoptelea integrifolia* Planch.	Ulmaceae	Tree	Bhupalpalli
51	*Hyptis suaveolens* (L.) Poit.	Lamiaceae	Climber	Bhupalpalli, Ramagundam, Bellampalli, Godavarikhani, Srirampur, Sathupalli
52	*Indigofera hirsuta* L.	Fabaceae	Herb	Bhupalpalli, Ramagundam
53	*Indigofera linnaei* Ali	Fabaceae	Tree	Bhupalpalli, Ramagundam
54	*Ipomoea obscura* (L.) Ker Gawl.	Convolvulaceae	Climber	Ramagundam, Sathupalli
55	*Lantana camara* L.	Verbenaceae	Shrub	Bhupalpalli, Srirampur
56	*Lepidagathis cristata* Willd.	Acanthaceae	Herb	Bhupalpalli
57	*Leucaena leucocephala* (Lam.) de Wit	Fabaceae	Tree	Bhupalpalli, Ramagundam, Srirampur, Sathupalli, Bellampalli, Mandamarri, Godavarikhani
58	*Melochia corchorifolia* L.	Malvaceae	Herb	Bhupalpalli, Ramagundam

59	*Merremia aegyptia* (L.) Urb.	Convolvulaceae	Climber	Bhupalpalli, Bellampalli
60	*Mollugo nudicaulis* Lam.	Molluginaceae	Herb	Bhupalpalli
61	*Operculina turpethum* (L.) Silva Manso	Convolvulaceae	Climber	Ramagundam
62	*Oxystelma esculentum* (L.f.) Sm.	Apocynaceae	Climber	Bhupalpalli
63	*Passiflora foetida* L.	Passifloraceae	Climber	Bhupalpalli, Ramagundam, Mandamarri
64	*Pavonia odorata* Willd.	Malvaceae	Herb	Bhupalpalli
65	*Peltophorum pterocarpum* (DC.) K.Heyne	Fabaceae	Tree	Bhupalpalli, Ramagundam, Srirampur, Godavarikhani
66	*Pennisetum pedicellatum* Trin.	Poaceae	Herb	Ramagundam, Sathupalli, Godavarikhani
67	*Pergularia daemia* (Forssk.) Chiov.	Apocynaceae	Climber	Ramagundam, Mandamarri
68	*Pluchea tomentosa* DC.	Asteraceae	Shrub	Srirampur
69	*Phyllanthus reticulatus* Poir.	Phyllanthaceae	Shrub	Ramagundam
70	*Phyllanthus virgatus* G.Forst.	Phyllanthaceae	Shrub	Ramagundam
71	*Phyllanthus emblica* L.	Phyllanthaceae	Tree	Srirampur, Sathupalli
72	*Pithecellobium dulce* (Roxb.) Benth.	Fabaceae	Tree	Bhupalpalli, Ramagundam, Srirampur, Sathupalli, Bellampalli
73	*Pongamia pinnata* (L.) Pierre	Fabaceae	Shrub	Bhupalpalli, Sathupalli
74	*Prosopis juliflora* (Sw.) DC.	Fabaceae	Tree	Ramagundam, Srirampur, Mandamarri
75	*Pterocarpus santalinus* L.f.	Fabaceae	Shrub	Sathupalli
76	*Rhynchosia minima* (L.) DC.	Fabaceae	Climber	Srirampur, Godavarikhani
77	*Senna auriculata* (L.) Roxb.	Fabaceae	Tree	Bhupalpalli, Srirampur, Mandamarri
78	*Senna siamea* (Lam.) H.S.Irwin & Barneby	Fabaceae	Herb	Srirampur, Sathupalli
79	*Setaria verticillata* (L.) P.Beauv.	Poaceae	Herb	Ramagundam

80	*Solanum surattense* Burm.f.	Solanaceae	Herb	Bhupalpalli, Ramagundam, Srirampur, Mandamarri
81	*Soymida febrifuga* (Roxb.) A. Juss.	Meliaceae	Tree	Sathupalli
82	*Stylosanthes fruticosa* (Retz.) Alston	Fabaceae	Tree	Bhupalpalli, Ramagundam, Sathupalli
83	*Tamarindus indica* L.	Fabaceae	Shrub	Bhupalpalli
84	*Teramnus labialis* (L.f.) Spreng.	Fabaceae	Climber	Srirampur
85	*Terminalia bellirica* (Gaertn.) Roxb.	Combretaceae	Tree	Sathupalli
86	*Thyella serrata* (Choisy) House	Convolvulaceae	Climber	Bhupalpalli
87	*Tridax procumbens* (L.) L.	Asteraceae	Herb	Sathupalli, Godavarikhani
88	*Urena lobata* L.	Malvaceae	Herb	Ramagundam
89	*Vitex negundo* L.	Lamiaceae	Shrub	Srirampur
90	*Waltheria indica* L.	Malvaceae	Tree	Bhupalpalli, Ramagundam, Bellampalli
91	*Xanthium strumarium* L.	Asteraceae	Herb	Srirampur
92	*Ziziphus jujuba* Mill.	Rhamnaceae	Tree	Bhupalpalli, Ramagundam

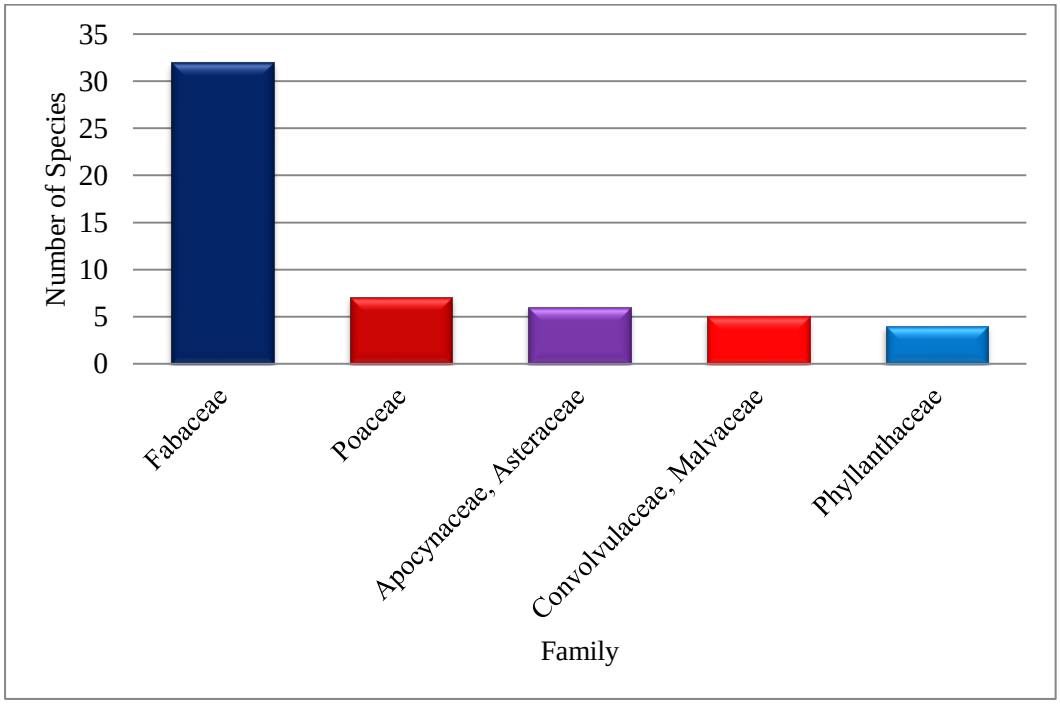


Figure 3. Top 5 predominant families found on overburdens of mining areas in Telangana State.

CONCLUSION

Through the establishment of vegetation cover, the mine tailings can be stabilized and can fulfill the objectives of stabilization, pollution control, aesthetic value and removal of threats to human beings (Freitas et al. 2014). Vegetation cover on the overburdens erosion, creates and aerobic condition in the rhizosphere and provides organic matter in the soil, increases soil aggregation and binding heavy metals (Pulford and Watson, 2003). Selection of plant species either resistant or accumulate the heavy metals should facilitate restoration of contaminated areas. The wild and indigenous plant taxa are capable to accumulate the toxic metals from contaminated areas and used as potential phytoremediators. These native plant taxa are also adapt to local environments over a period of time and helpful as biological indicators. Depends on local conditions of tailings of Sathupalli mining area, the SCCL authorities planted diversified fruit bearing, timber yielding plants and an integration of agriculture with forestry. In Telangana, experimentally, it is the first initiation of planting fruit bearing and timber yielding tree taxa on mine wastelands for both the beneficiaries such as restoration of ecosystem and for commerce.

Selection of drought tolerant and fast growing plants to grow on mining wastelands is an important step for phytostabilization. Selection of different species of grasses and leguminous plants to combine cultivation can be suggestible for ecorestoration of soil fertility. The selection of trace element tolerant species is a key factor to the success of degraded mine soils. These are commonly used for revegetation of mine tailings, and herbaceous legumes can be used as pioneer species to solve the problem of nitrogen deficiencies in mining waste lands. The selection plant in a contaminated area is a critical step to remediate the contaminated areas. Plant roots can create rhizosphere wherein the accumulation of heavy metals takes place. Planting of tree species on overburdens is an advantage due to extensive root system, generates huge biomass to contain high levels of metals in the root portion and metal tolerant characters Establishment of vegetation cover with desirable and economically important species would decrease the spread of contamination though different factors such as wind and water erosion, decrease the leaching of heavy metals and AMD into ground water.

The members of Fabaceae (Leguminosae) are widely used for revegetation and phytostabilization on overburdens of mining areas. Legume species have the ability with nitrogen fixing and growth-promoting symbiotic bacteria, arbuscular mycorrhizal fungi to enrich the low nutrient soil and fetch to establish other plant taxa. The species such as *Leucaena, Pithecellobium, Atylosia, Pterocarpus*, etc. are widely planting on tailings for stabilization of plants. The study sites were dominated by tree species which roots penetrate deeper and stronger to ameliorate the dump stability. Indigenous tree species like *Pterocarpus santalinus, Pterocarpus marsupium, Dalbergia paniculata, Ficus religiosa, Soymida febrifuga, Terminalia bellirica, Terminalia chebula, Hardwickia binata, Phyllanthus emblica, Ficus benghalensis* were found to be the best candidate species for revegetation on overburdens and provide shelter for small creatures, insects, moths, birds, etc. After revegetation of slopes and platforms (top) of overburdens, the vegetation recovery and regeneration of herbaceous layer have been increased (*pers. obs.*). Revegetation on the overburdens with indigenous plant species is highly encouraged to promote the growth of the nature, soil fertility and reduce the wind erosion and water run-off (Suthari et al., 2016). There is a need to be focused in a scientific way for the establishment of suitable native plant taxa on the overburdens depending on the nature of waste/disposal, moisture availability, soil nutrients and other climatic stresses. It is strongly advised in view of the protection of environment that environmental impact studies should be taken thoroughly and the suitable parameters should be implemented before proceeding the extraction process. One should be taken to minimize the exploitation of natural resources.

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PROBIOTICS – POTENTIAL HEALTH BENEFITS – AN OVER VIEW

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ABSTRACT

Functional foods have become a part of everyday diet and are demonstrated to offer potential health benefits beyond the widely accepted nutritional effects. Currently, the most important and frequently used functional food compounds are probiotics and prebiotics, collectively known as 'synbiotics'. The health benefits imparted by probiotics and prebiotics as well as synbiotics have been the subject of extensive research in the past few decades and explored commercially in many different products globally. *Lactobacillus* and *Bifidobacterium* are the main probiotic groups; however, there are reports on the probiotic potential of *Pediococcus, Lactococcus, Bacillus* and yeasts. Probiotic bacteria have become increasingly popular during the last two decades as a result of continuously expanding scientific evidence pointing to their beneficial effects on human health. Beneficial effects of probiotic consumption include improvement of intestinal health by the regulation of microbiota, and stimulation and development of the immune system, synthesizing and enhancing the bioavailability of nutrients, reducing symptoms of lactose intolerance, and reducing the risk of certain other diseases. Some of the identified probiotic strains exhibit powerful anti-inflammatory, antiallergic and other important properties. The primary clinical interest in the application of probiotics has been in the prevention of and treatment for GI infections and diseases. Nevertheless, there is insufficient evidence to recommend probiotics for use in other clinical conditions. Most of the studies have been marked by clinical and methodological differences, pointing to the need for well-designed clinical trials to properly assess the efficacy of probiotics. The side effects of

probiotics are generally mild, but serious infections have been reported, especially in the elderly, patients with intravenous catheters, and the immunocompromised. The present paper reviews available information and summarises the current knowledge on the effects of probiotics, prebiotics, and synbiotics on human health. It also presents a glimpse of the current scenario with special reference to probiotics and their application in the health and food areas and new trends in probiotic products and processes.

Keywords: Probiotics, Lactobacillus, Bifidobacterium, health benefits

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INTRODUCTION

"Let food be thy medicine and medicine be thy food", the age old quote by Hippocrates is the ideology of today's health conscious people. The concept of functional foods emphasizes that food not only is vital for living but also play a role in the prevention and reduction of risk factors for several diseases and are also capable of enhancing certain vital physiological functions. Functional foods also provide the body with required amount of vitamins, fats, proteins, carbohydrates, etc. (Cencic and Chingwaru 2010). Besides, the functional foods play a basic role of nutrition in the supply of necessary nutrients for growth and development of the organism, and recently some additional aspects are becoming increasingly important, including the maintenance of body homeostasis and counteracting diseases. Probiotics fill the bill of both Hippocrates quote and functional foods. Today, probiotics have become mandatory component of balanced and healthy diet, and medical prescriptions. Although there is realisation of importance and increased research interest in recent times, the use probiotics in different forms as supplement to human food is in practice since dawn of human civilization. Their health benefits are also documented in ancient literature of all civilisations. Scientific aspects of different probiotics are vigorously pursued now-a-days. Probiotic concept is strengthened by identification and health benefits conferred by related components such as prebiotics, synbiotics and postbiotics. Though general health benefits are well known, presently researches are targeted to identify particular species/strain of probiotic organism that meets the specific requirements such as ameliorating physiological disorders, prevention and control of infectious diseases, stability and viability in administered host systems. The probiotic market is expanding, especially in Japan-its birthplace-with further growth prospects in Europe and the United States and in most countries. India is a leading country in probiotic production and consumption. Biotech industries involved in this field are focussing on improved strains, low-cost bioprocess, downstream process and market suitable formulations. Despite the promising evidence, the role of probiotics in human health as well as the safety of their application needs to be further investigated.

HISTORY

The association of probiotics with human well-being has a long history. The knowledge of the beneficial effects of lactic acid fermentation on human health dates back to ancient times. Iconographic and written evidence from 3000 and 2000 BC indicated that Hindu, Egyptians, Greeks, and Romans used fermented milk products, although its origins probably lie much earlier. Indeed they are mentioned in the early sacred books of Hinduism and in the Holy Bible. In the Bible one of the first references is found in Genesis (18: 1-8) where it is said that Abraham offered to the Lord "veal, buns and sour milk." Local climatic conditions favoured the development of many of the traditional soured milk or cultured dairy products such as kefir, koumiss, leben and dahi (Panesar et al. 2009). Many of the nomadic tribes made these products as a part of their diet. A specific type of sour milk, called "leben raib", prepared from buffalo, cow, or goat milk, was consumed in ancient Egypt. A similar "jahurt" was also commonly consumed by people inhabiting the Balkans. In India, fermented milk drinks were known already 800–300 years B.C. and in Turkey in the 8th century. A milk drink called "ajran" was consumed in Central Russia in the 12th century, and "tarho" was consumed in Hungary in the 14th century (Hosono 1992).These products, many of which are still widely consumed with various labels had often been used therapeutically before the existence of bacteria was recognized (Panesar et al. 2009). The health benefits of probiotics were first described a century ago by Russian Nobel laureate Elie Metchnikoff (Metchnikoff and Schlesinger 1908), who postulated (Theory of Longevity) that the longevity of Bulgarian peasants was due to the consumption of sour milk, which contains lactic acid bacteria whose growth in the intestine displaces disease-producing organisms (Kaufmann 2008). Here is a quote from his book "Studies on Optimism": "*with various foods undergoing lactic acid fermentation and consumed raw (sour milk, kefir, sauerkraut, pickles) humans introduced huge amounts of proliferating lactic acid bacteria to their alimentary tracts*" (Miecznikow 1907).

The word "probiotic" comes from Greek and means "for life." In 1954, Ferdinand Vergin conceived the term "probiotic" in an article entitled "Anti-und Probiotika," in which several microorganisms were studied to make a list of useful bacteria and to determine the detrimental effects of antibacterial agents and antibiotics on the intestinal microbiota (Vergin 1954). A few years later, Lilly and Stillwell described probiotics as beneficial microorganisms that stimulating the growth of another

microorganism (Lilly and Stillwell 1965). Subsequently the term was further defined as non-pathogenic microorganisms which when ingested, exert a positive influence on host's health or physiology. Tissier's discovery of bifidobacteria in breast-fed infants also played a key role in establishing the concept that specific bacteria take part in maintaining health. At that time, many others were sceptical about the concept of bacterial therapy and questioned in particular whether the yoghurt bacteria (*L. bulgaricus*) were able to survive intestinal transit, colonize and convey benefits (Kulp and Rettger 1924). In the early 1920s, *L. acidophilus* milk was documented to have therapeutic effects, in particular, a settling effect on digestion (Cheplin and Rettger 1922). It was believed that colonization and growth of these microorganisms in the gut were essential for their efficacy, and therefore, the use of intestinal isolates was advocated. In Japan in the early 1930s, Shirota focused his research on selecting the strains of intestinal bacteria that could survive passage through the gut and on the use of such strains to develop fermented milk for distribution in his clinic. His first product containing *L. acidophilus* Shirota (subsequently named *L. casei* Shirota) was the basis for the establishment of the Yakult Honsha Company. Only at the end of the century, it became clear that intestinal microflora had several functions, including metabolic, trophic and protective ones (Guarner and Malagelada 2003). The health benefits derived from the consumption of foods containing *Lactobacillus acidophilus*, *Bifidobacterium* and *L. casei* are now well documented. *Streptococcus thermophilus* and *L. delbrueckii* ssp. *bulgaricus* are yoghurt starter cultures, which offer some health benefits; however, they are not natural inhabitants of the intestine. Therefore, for yoghurt to be considered as a probiotic product, *L. acidophilus*, *Bifidobacterium* and *L. casei* are incorporated as dietary adjuncts. Thus, the normal practice is to make a product with both starter organisms, *e.g. S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*, and one or more species of probiotic bacteria (Shah 2007). Following initial hiccups, research in probiotics has progressed considerably in the past two decades and has been made in the selection and characterization of specific probiotic cultures along with substantial health benefits upon consumption. Research on probiotics, in particular Lactobacilli, has grown exponentially during the last two decades as can be seen from the fact that compared to 180 research articles published during 1980–2000, more than 5700 research articles were published during 2000–2014 on probiotic Lactobacillus (Probiotic Lactobacillus PubMed 2014).

TERMINOLOGY

Probiotics: The term Probiotics is used to define living non-pathogenic organisms and their derived beneficial effects on hosts. The term probiotics was first introduced by Vergin, when he was studying the detrimental effects of antibiotics and other microbial substances, on the gut microbial population. He observed that probiotika was favourable to the gut microflora. Until recently the most widely used definition which contributed to the development of the probiotic concept in several ways was that of Fuller: "*probiotics are live microbial feed supplements which beneficially affect the host animal by improving microbial balance"* (Fuller 1989). Probiotic were then redefined by Lilly and Stillwell as *A product produced by one microorganism stimulating the growth of another microorganism*. Subsequently the term was further defined as *Non-pathogenic microorganisms which when ingested, exert a positive influence on host's health or physiology* by Fuller. The latest definition put forward by FDA and WHO jointly is '*Live microorganisms which when administered in adequate amounts confer a health benefit to the host.'*

PREBIOTICS: Prebiotics are mostly fibers that are non-digestible food ingredients and beneficially affect the host's health by selectively stimulating the growth and/or activity of some genera of microorganisms in the colon, generally lactobacilli and bifidobacteria (DeVrese and Schrezenmeir 2008) FAO/WHO defines prebiotics as a non-viable food component that confer health benefit(s) on the host associated with modulation of the microbiota. Some of the sources of prebiotics include: breast milk, soybeans, inulin sources (like Jerusalem artichoke, chicory roots etc.), raw oats, unrefined wheat, unrefined barley, yacon, non-digestible carbohydrates, and in particular non-digestible oligosaccharides (Table 1).

SYNBIOTICS: Gibson, who introduced the concept of prebiotics, speculated the additional benefits if prebiotics were combined with probiotics to form what he termed as synbiotics (DeVrese and Schrezenmeir 2008). A synbiotic product beneficially affects the host in improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract by selectively stimulating the growth

and/or activating the metabolism of one or a limited number of health promoting bacteria. Because the word synbiotics alludes to synergism, this term should be reserved for products in which the prebiotic compound(s) selectively favor the probiotic organism(s) (Cencic and Chingwaru 2010) (Table 2)

POSTBIOTICS: Postbiotics are defined as non-viable bacterial products or metabolic byproducts from probiotic microorganisms that have biologic activity in the host (Patel and Denning 2013). General, postbiotics include bacterial metabolic byproducts, such as bacteriocins, organic acids, ethanol, diacetyl, acetaldehydes and hydrogen peroxide, but it is also found that certain heat-killed probiotics can also retain important bacterial structures that may exert biological activity in the host (Islam 2016). Postbiotics are non-toxic, non-pathogenic and resistance to hydrolysis by mammalian enzymes, as these are non-viable bacterial products or metabolic byproducts from probiotics.

GUIDELINES FOR EVALUATION AND IDEAL CHARACTERISTICS OF A PROBIOTIC ORGANISM

FAO and WHO have jointly put forward guidelines in order to set out a systematic approach for an effective evaluation of probiotics in foods to substantiate the health claims and benefits. Some of the pre-requisites of an ideal probiotic organism are depicted in Fig. 1. The FAO/WHO guidelines on Probiotics could be used as global standard for evaluating probiotics in food that could result in the substantiation of health claims. The guidelines make it necessary to perform the following activities:

i. Strain identification.

ii. Functional characterization of the strain(s) for safety and probiotic attributes.

iii. Validation of health benefits in human studies.

iv. Honest, not misleading labelling of efficacy claims and content for the entire shelf-life.

According to the suggestions of the WHO, FAO, and EFSA (the European Food Safety Authority), in their selection process, probiotic strains must meet both safety and functionality criteria, as well as those related to their technological usefulness (Table 3).

The following criteria need to be fulfilled by an ideal probiotic (Abatenh et al 2018)

1. Probiotics must be from human origin.
2. Probiotics should be able to create a beneficial effect on host animal by increasing resistance to diseases.
3. Probiotics needed to have excessive cell viability.
4. Probiotics should be non-pathogenic and non-toxic.
5. It should be able enough to interact or send signals to immune modulator activity.
6. It must have ability to influence local metabolic activity.
7. It ought to be fit for surviving and processing in the gut condition like resistance to low pH and organic acids.
8. Probiotics must be stable, safe, effective and equipped for staying viable for periods under storage and field conditions.
9. It must have power of restore and replace the intestinal microflora.
10. It should have anti-carcinogenic and anti-mutagenic activity, cholesterol lowering effects, can maintain mucosal integrity and can enhance bowel motility.
11. It should be able to speed up, facilitate and colonize/maintain the digestive tract.
12. They must have the ability to resist gastric juices and the exposure to bile acid which seems to be crucial for oral administration.
13. Adhesion to mucosal and epithelial surfaces, an important property for successful immune modulation, competitive exclusion of pathogens, as well as prevention of pathogen adhesion and colonization.
14. Antimicrobial activity against pathogenic bacteria.
15. Bile salt hydrolase activity
16. Antibiotic resistance may help them to survive in the presence of administered drugs and other antimicrobial compounds
17. Fast multiplication, with either permanent or temporary colonization of the gastrointestinal tract.
18. Stabilization of the intestinal micro flora and non-pathogenicity.
19. Survival on passing through gastrointestinal tract at low pH and in contact with bile

PROBIOTIC MICROORGANISMS

Taking into consideration their definition the number of microbial species which may exert probiotic properties is impressive. Some of the most important representatives are listed in Table 4. As far as nutrition is concerned only the strains classified as lactic acid bacteria are of significance and among them the ones with the most important properties in an applied context are those belonging to the genera *Lactococcus* and *Bifidobacterium* (Holzapfel et al. 2001*)*. Lactic acid bacteria are Gram-positive, catalase-negative bacterial species able to produce lactic acid as main end-product of carbohydrate fermentation. *Bifidobacterium* is a genus of gram-positive, nonmotile, often branched anaerobic bacteria. They are ubiquitous inhabitants of the gastrointestinal tract. They possess a unique fructose-6-phosphate phosphoketolase pathway employed to ferment carbohydrates. Two other species playing an important role in the food industry, particularly dairy products, although not strictly considered as probiotics are *Streptococcus thermophilus* and *Lactococcus lactis* (Felis and Dellaglio 2007). Other organisms including enterococci and yeasts have also been used as probiotics. Some of these strains have been chosen based on selection criteria (Havenara et al. 1992) that are believed to be important for their efficacy such as origin of strain, *in vitro* adherence to intestinal cells and survival during passage through the gastrointestinal tract. It is important to mention that since probiotic activities are strain related, strain identification is recommended in order to establish their suitability and performance for industrial application. This is achieved by a combination of phenotypic tests followed by genetic identification using molecular techniques like DNA/DNA hybridisation, 16SRNA sequencing, and so forth (FAO/WHO 2001)

THE GENUS BIFIDOBACTERIUM*:* Bifidobacteria are microorganisms of paramount importance in the active and complex ecosystem of the intestinal tract of humans and other warm-blooded animals, as well as of honeybees (Sgorbati et al. 1995). They are distributed in various ecological niches in the human gastrointestinal and genitourinary tracts, the exact ratio of which is determined mainly by the age and diet. The indigenous microflora of infants is dominated by bifidobacteria, which are established shortly after birth. Their proliferation is stimulated by the glycoprotein components of k-casein in human colostrum and, to a lesser extent, human milk. The number of bifidobacteria decreases with increasing age of an individual and eventually becomes

the third most abundant genus (accounting for approx. 25 % of the total adult gut flora) after the genera *Bacteroides* and *Eubacterium* (Finegold et al. 1983).

THE GENUS LACTOBACILLUS:

Lactobacilli are distributed in various ecological niches throughout the gastrointestinal and genital tracts and constitute an important part of the indigenous microflora of man and higher animals. Their distribution is affected by several environmental factors, which include pH, oxygen availability, level of specific substrates, presence of secretions and bacterial interactions. They are rarely associated with cases of gastrointestinal and extraintestinal infection, and strains employed technologically are regarded as non-pathogenic and safe microorganisms. Furthermore, they have the reputation of health promoters, especially in the human gastrointestinal and genitourinary tracts (Salminen et al. 1996).

OTHER PROBIOTIC MICROORGANISMS

Although the term probiotics is more related to lactic acid bacteria as *Lactobacillus* and *Bifidobacterium,* it can be extended to other microorganisms which have not been explored. For example, *Bacillus* species have been used as probiotics for at least 50 years in an Italian product commercialized as Enterogermina®. Among this group some species that have been evaluated are *Bacillus subtilis, B. clausii, B. cereus, B. coagulans* and *B. licheniformis* . Some advantages of the bacterial spores are their resistance to heat, allowing the storage at room temperature and in a dried form. Also, these bacteria are able to reach small intestine since they survive the gastric pH of the stomach (Barbosa et al. 2005). The application of probiotic bacterial spores ranges from dietary supplements to growth promoters and uses in aquaculture (*e.g.* shrimp). Probiotic microorganisms used in animal preparations are *Enterococcus, Bacillus, Streptococcus, Lactobacillus, Aspergillus* and *Saccharomyces* (Fox 1998).

MECHANISMS OF ACTION

The mechanisms by which probiotics exert biological effects are still poorly understood, but the nonspecific terms such as colonization resistance or competitive exclusion are often used to explain their mode of action (Elo et al. 1991) (Table 5). Colonization resistance or competitive exclusion describes a phenomenon whereby the indigenous anaerobic flora limits the concentration of potentially pathogenic

(mostly aerobic) flora in the digestive tract (Vollaard and Clasener 1994). The concept of competitive exclusion was first developed during the early 1970s when it was discovered that the administration of mixed adult intestinal microorganisms conferred adult-type resistance against *Salmonella* infection to newly hatched chicks (Nurmi et al. 1992). Oelschlaeger (2010) reported that the effects of probiotics may be classified in three modes of action: (i) Probiotics might be able to modulate the host's defences including the innate as well as the acquired immune system. This mode of action is most likely important for the prevention and therapy of infectious diseases but also for the treatment of (chronic) inflammation of the digestive tract or parts thereof. In addition, this probiotic action could be important for the eradication of neoplastic host cells; (ii) Probiotics can also have a direct effect on other microorganisms, commensal and/or pathogenic ones. This principle is in many cases of importance for the prevention and therapy of infections and restoration of the microbial equilibrium in the gut; (iii) Finally, probiotic effects may be based on actions affecting microbial products like toxins and host products, *e.g.* bile salts and food ingredients. Such actions may result in inactivation of toxins and detoxification of host and food components in the gut. The same author also stated that the kind of effect(s) a certain probiotic executes depends on its metabolic properties, the molecules presented at its surface or on the components secreted. Even integral parts of the bacterial cell such as DNA or peptidoglycan might be of importance for its probiotic effectiveness. The individual combination of such properties in a certain probiotic strain determines a specific probiotic action and as a consequence its effective application for the prevention and/or treatment of a certain disease.

HEALTH BENEFITS

Some of the beneficial effects of probiotic consumption include improvement of intestinal health by the regulation of microbiota, and stimulation and development of the immune system, synthesizing and enhancing the bioavailability of nutrients, reducing symptoms of lactose intolerance, and reducing the risk of certain other diseases (Fig. 2) (Kumar et al. 2009a, b, 2010, 2011a, b; Nagpal et al. 2007, 2010, 2011; Yadav et al. 2007a, b, 2008). The primary clinical interest in the application of probiotics has been in the prevention of and treatment for GI infections and diseases (Parvez et al. 2006). Gut microbiota deviations have been associated with enhanced risk of specific diseases; therefore, modulation of an unbalanced indigenous

microbiota forms the rationale of probiotic therapy (Turnbaugh et al. 2006). Also, the development of adjuvant or alternative therapies based on bacterial replacement is becoming important owing to the rapid emergence of antibiotic-resistant pathogenic strains and the adverse consequences of antibiotic therapies on the protective flora, which enhances the risk of infection (Forestier et al. 2001). However, the use of probiotics should be further investigated for their benefits and possible side effects, if any.

INTESTINAL TRACT HEALTH: Studies proclaim consumption of probiotic bacterial species (*Lactobacillus GG, L. casei, Bifidobacterium* and *Streptococcus thermophilus*) to be useful in the treatment of many types of diarrhea (e.g. acute infantile diarrhea caused by rotavirus, antibiotic-associated diarrhea, *Clostridium difficile*-associated diarrhea, traveler's diarrhea, radiation induced diarrhea). Probiotic bacteria have been shown an important means to preserve intestinal integrity and mediate the effect of inflammatory bowel disease, irritable bowel syndrome, colitis and alcoholic liver disease. Lactobacilli may also improve intestinal mobility and relieve constipation particularly in adults.

IMMUNE RESPONSES: Evidences suggest that probiotics are considered particularly important to the elderly people. It is because they can enhance both specific and non-specific immune responses of the body by activating macrophages, increasing levels of cytokines, increasing natural killer cell activity and/or increasing levels of antibodies.

Cancer: Evidences from research demonstrate that effect of probiotic consumption on cancer appears promising. Animal and *in vivo* studies indicate that probiotic bacteria may reduce colon cancer risk by reducing the incidence and number of tumours. One clinical study showed that powder preparation of *Lactobacillus casei* [10^{10} colony forming units three times/day for one year] increased the recurrence-free period among humans with superficial bladder cancer. Results, however, are preliminarily at this stage to develop specific recommendations on probiotic consumption for preventing cancer in humans.

ALLERGY: Probiotic consumption may result in beneficial effects on certain allergic reaction by improving mucosal barrier function. In one study, yoghurt and pasteurized yogurt were fed to college-aged and elderly volunteers. A significant decrease in allergy symptoms was observed in volunteers consuming yogurt and those who did not consume yogurt. Unfortunately, no microbiological characterization of yogurt was

undertaken. Probiotic consumption may thus be a means for primary prevention of allergy in susceptible humans.

LACTOSE INTOLERANCE: Intolerance to lactose-containing food (primarily dairy products) is the most common disorder of intestinal carbohydrates digestion in almost 70% of the population worldwide. Probiotic consumption shows that the appropriate strains of lactobacilli (e.g. *Streptococcus thermophilus*, *Lactobacillus bulgaricus*) produce lactase which hydrolyses the lactose in dairy products to glucose and galactose. It seems therefore that probiotic consumption of these bacteria can exert their lactase activity in the gut lumen thus facilitating digestion and alleviating lactose intolerance.

LIVER DISEASE: In non-alcoholic steatohepatitis (NASH), generally called non-alcoholic fatty liver disease, non-alcoholic patients are found to have liver biopsy findings indistinguishable from alcoholic hepatitis. Studies in rodent models of this disease have shown that intestinal bacteria and bacterial endotoxin modulate alcohol-induced liver damage. The concepts that intestinal bacteria induce endogenous signals, which play a pathologic role in non-alcoholic steatohepatitis, suggest that novel probiotic therapy may be significantly useful in the disease.

PREGNANCY: Healthy diet during pregnancy improves the changes of the birth of healthy infant who will be at lower risk of disease later in life. Bacterial vaginosis has been generally considered as a factor that enhances the possibility of preterm labour and death of infants. Probiotics have been demonstrated to decrease risk of bacterial vaginosis and maintain balanced lactobacilli flora of vagina. Atopic dermatitis, an allergic reaction that causes severe skin rashes in upto 15% of babies, can be checked if mothers ingested probiotics (*Lactobacillus rhamnosusGG* and *Bifidobacteriumlactis*) during pregnancy and new born babies ingest them during the first 6 months of life.

BIOAVAILABILITY AND SYNTHESIS OF NUTRIENTS:

Probiotic help improving the digestibility of certain dietary nutrients such as protein and fat. Short - chain fatty acids (eg. Lactic acid, propionic acid, butyric acid) produced by lactobacilli may help maintain an appropriate pH level and protect against pathological alterations in the colonic mucosa. In addition to nutrient bioavailability, the lactobacilli have been found to increase folic acid content of yogurt, bifidus milk

and kefir and to increase niacin and riboflavin levels in yogurt, vitamin B_{12} in cottage cheese and vitamin B_6 in cheddar cheese during fermentation.

PROBIOTICS and HIV: Studies demonstrate that women with bacterial vaginosis (reduced number of lactobacilli in vaginal tract) are at significantly increased risk of human immunodeficiency virus (HIV). It has been recently shown that a human vaginal probiotic strain (*Lactobacillus reuteri* RC-14) can express potent functional viral inhibitors which may potentially lower the sexual transmission of HIV. However, these results need further confirmation before their implementation.

In addition to mentioned beneficial effects, probiotic consumption is considered helpful in health conditions like hypertension, illness-related weight loss, collaginous colitis and alcohol-induced liver damage. There are many other areas under exploration where probiotics may find their use (Table 6 & 7).

SAFETY

In theory, probiotics may be responsible for four types of side effects in susceptible individuals: systemic infections, deleterious metabolic activities, excessive immune stimulation, and gene transfer (Marteau 2001; Marteau and Seksik 2004). In practice, however, lactobacilli and bifidobacteria (and probiotics based on these organisms) are extremely rare causes of infections in humans (Boriello et al. 2003 Ishibashi et al. 2001; Salminen 1998). This lack of pathogenicity extends across all age groups and also to immunocompromised individuals (Cohendy 1906). Traditional dairy strains of lactic acid bacteria (LAB) have a long history of safe use. LAB, including different species of *Lactobacillus* and *Enterococcus,* has been consumed daily since humans started to use fermented milk as food. Probiotic species such as *Lactobacillus acidophilus* have been safely used for more than 70 years. However, the safety aspects always have to be considered and possible adverse effects should be continuously evaluated, as illustrated by recent literature. Members of the genera *Lactococcus* and *Lactobacillus* are most commonly given the GRAS status, whilst members of the genera *Streptococcus, Enterococcus* and some other genera of LA are considered opportunistic pathogens. The safety of probiotics has been considered in reviews and clinical reports which have drawn attention to isolate cases of human bacteraemia (Gasser 1994; Aguirre and Collins 1993; Saxelin et al. 1996). Surveillance studies support the safety of commercial LAB (Adams and Marteau 1995). Available data indicate that no harmful

effects have been observed in controlled clinical studies with lactobacilli and bifidobacteria (Salminen et al. 1998).

Three approaches can be used to assess the safety of a probiotic strain: studies on the intrinsic properties of the strain, studies on the pharmacokinetics of the strain (survival, activity in the intestine, dose–response relationships, faecal and mucosal recovery) and studies searching for interactions between the strain and the host. The Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food Including powder milk with live lactic acid bacteria recognized the need for guidelines to set out a systematic approach for the evaluation of probiotics in food in order to substantiate the health claims. Consequently, a Working Group was convened by FAO/WHO to generate guidelines and recommend criteria and methodology for the evaluation of probiotics, and to identify and define what data need to be available to accurately substantiate health claims. The aims of the Working Group were to identify and outline the minimum requirements needed for probiotic status. Then, guidelines were prepared in 2002 to meet this objective. These guidelines are available in: Joint FAO/WHO Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food (FAO/WHO 2002).

APPLICATION OF PROBIOTICS IN ANIMAL FEED AND AQUACULTURE

Animal feed companies and researchers have been looking for alternative products and strategies that can help to maintain animal gut health in order to prevent or reduce the prevalence of pathogens in the food chain. An alternative and effective approach to antibiotic administration to livestock is the use of probiotics, which can help to improve gut microbial balance and therefore the natural defence of the animal against pathogenic bacteria (Modesto et al. 2009; Patterson Burkholder 2003). In recent years, there has been a considerable interest in using some probiotic microorganisms and organic acids as an alternative to the use of antibiotics in feed. Multiple ways exist in which probiotics could be beneficial and these could act either singly or in combination forming a single probiotic. These include inhibition of a pathogen *via* production of antagonistic compounds, competition for attachment sites, competition for nutrients, alteration of enzymatic activity of pathogens, immunostimulatory functions, and nutritional benefits such as improving feed digestibility and feed utilization (Fuller 1989; Bomba et al. 2002; Fooks et al. 1999). It is

often reported that a probiotic must be adherent and colonize within the gastrointestinal tract, it must replicate to high numbers, it must produce antimicrobial substances, and it must withstand the acidic environment of the gastrointestinal tract. Verschuere et al. (2000) suggested a new definition of a probiotic for aquatic environments: 'a live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host's response towards disease, or by improving the quality of its ambient environment', or that 'a probiotic is an entire microorganism or its components that are beneficial to the health of the host' (Irianto and Austin 2002). Table 8 presents the application of probiotic strains in animal nutrition, during feed processing and aquaculture.

PRODUCTION TECHNOLOGY OF PROBIOTICS

In view of wide acceptance and consequently high market demand, the search for new technologies that enable high cell yield at large scale and ensure probiotic stability in food remains strong. In addition, more efficient technologies could lead to greater product efficacy and strain diversification. Some authors have presented developments in fermentation technologies for producing probiotic bacteria as well potential new approaches for enhancing the performance of these organisms during fermentation, downstream processing, and utilization in commercial products, and for improving functionality in the gut (Lacroix and Yildirim 2007; Doleyres and Lacroix 2005; Lacroix et al. 2005)(Fig.3). Until now, very few data have been reported on continuous fermentations with probiotics, although this approach could provide benefits, as recently reviewed by Doleyres and Lacroix (2005) for bifidobacteria. However, continuous fermentations can be more difficult to operate under industrial conditions, because they are highly susceptible to contamination and cell characteristics can be lost over time. This technology is worth investigating and could be used to produce cells with different physiologies and to apply various stresses under well-controlled conditions (Lacroix and Yildirim 2007). Membrane systems with continuous feeding of fresh medium where cells are retained in the bioreactor by an ultra filtration or microfiltration membrane are also an interesting technological possibility.

Different approaches that increase the resistance of these sensitive microorganisms against adverse conditions have been proposed, including appropriate

selection of acid- and bile-resistant strains, use of oxygen-impermeable containers, two-step fermentation, and stress adaptation, incorporation of micronutrients such as peptides and amino acids, and microencapsulation.

CARRIERS FOR PROBIOTICS

Probiotics are normally added to foods as a part of the fermentation process. The emphasis for prolonged survival of probiotics in the food matrix has resulted in the alteration in the functionality and efficacy of the food product. In order to exert health benefits, probiotic bacteria must remain viable in the food carriers and survive the harsh condition of GI tract, with a minimum count of 10^6 CFU g1. The nature of food carrier can affect the stability of the probiotic microorganisms during GI transit. Although dairy-based products are suggested to be the main carriers for the delivery of probiotics, other nondairy-based products such as soy and fruits are being exploited as a potential carrier of probiotic microorganisms because of the increasing demand for new flavor and taste among consumers

LEGISLATION AND SAFETY OF PROBIOTICS

The regulatory status of probiotics as a component in food has to be established on an international level. A regulatory framework should be established to better address probiotic issues, including efficacy, safety, labeling, fraud, and claims. Probiotic products shown to confer defined health benefits on the host should be permitted to describe these specific health benefits. From a legislative standpoint, probiotic-containing foods could fit into several of the four categories of foods described by the FDA; however, there is no explicit recognition of any health benefits of probiotic-, prebiotic-, or culture-added dairy foods in the United States. Donohue and Salminen (1996) provided some methods for assessing the safety of lactic acid bacteria through the use of *in vitro* studies, animal studies, and human clinical studies and indicated that some current probiotic strains are reported to fulfill the required safety standards. Salminen and Marteau (1997) also proposed studies on intrinsic properties, pharmacokinetics, and interactions between the host and probiotics as means to assess the safety of probiotics.

As there has been an increased influx of probiotic products in the Indian market during the last decade, an initiative was taken by the Indian Council of Medical

Research and Department of Biotechnology, Government of India, to formulate guidelines for the regulation of probiotic products in the country (Ganguly et al. 2011), defining a set of parameters required for a product/strain to be termed as 'probiotic'. These include the identification of the strain, in vitro screening for probiotic characteristics, and in vivo animal and human studies to establish efficacy, requirements for labeling of the probiotic products with strain specification, viable numbers at the end of shelf-life, storage conditions, etc., so as to help the consumers to safeguard their awareness.

VALIDATION OF HEALTH CLAIMS

The Nutrition Labeling and Education Act of 1990 give the US Food and Drug Administration (FDA) the authority to regulate health claims on food labels. These claims describe the link between specific nutrients or substances in food, and a particular disease or health-related condition. The process of reviewing the scientific evidence of health claims involves the following steps: define the substance– disease relationship that is the subject of the claim, identify relevant studies, classify the studies, rate the studies on the basis of quality, rate the studies on the basis of the strength of their body of evidence, and report the studies' rank order.

FUTURE PROSPECTS: TOWARDS GENETICALLY MODIFIED DESIGNER PROBIOTICS

Genetic manipulation offers the potential to enhance the existing probiotic properties of an organism or to load an organism with probiotic properties (Steidler 2003). Elucidation of mechanisms of activity of a probiotic could enable the manipulation of organisms to create specific and targeted probiotics. Although consumer resistance to genetically modified organisms is such that GMO probiotic foods are unlikely in the near future, potential clinical applications to ameliorate or prevent chronic intractable diseases may be more readily accepted. For instance, Steidler (2003) treated mice with genetically modified *Lactococcus lactis* to deliver mouse cytokine IL-10 at the intestinal mucosa to prevent colitis, demonstrating that probiotics can be designed to produce potent bioactive chemicals. Braat et al. (2006) also constructed a biologically contained *L. lactis* to produce human IL-10 and treated Crohn's disease patients with this GM *L. lactis* in a phase-1 placebo-uncontrolled trial. A decrease in disease activity was observed with minor adverse effects, and containment of the organism was achieved through its dependency on thymidine for growth and IL-10 production.

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ECOFRIENDLY PLANT GROWTH PROMOTING RHIZOBACTERIA FOR CROP IMPROVEMENT

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ABSTRACT

Soil microorganisms are important in the geobiochemical cycles of inorganic and organic nutrients in soil and maintenance of soil health and quality. The rhizosphere of plants is inhabited by complex and dynamic communities of microorganisms, notable among which are plant growth promoting and soil supporting bacteria. Soil- Plant-Rhizobacteria interactions are complex and there are many ways in which the outcome can influence the plant health and productivity. The PGPR's are also potential biocontrol agents of several soil-borne plant pathogens. The rhizosphere provides the frontline defense of roots against attack by pathogens. The present review provides the interaction of PGPR with other microbes in the rhizosphere thus contributing for sustainable crop production.

KEYWORDS: Crop, Ecofriendly, Growth, Plant, Rhizobacteria

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INTRODUCTION

Bacteria inhabiting the rhizosphere and beneficial to plants are termed as plant growth-promoting rhizobacteria (PGPR) (Klopper et al 1980). Plant growth-promoting rhizobacteria (PGPR) or plant health promoting rhizobacteria (PHPR) (Klopper et al. 1989) were first defined by Klopper and Schroth (1978). To describe such soil bacteria that colonize the roots of plants following inoculation onto seed and that enhances plant growth. About 2-5% of rhizosphere bacteria are PGPR (Antoum and Prevost 2005) which are free-living bacteria. However, some researchers have coined a broader definition of PGPR to include symbiotic microorganisms like nitrogen-fixing rhizobia. Vessey (2003), and Gray and Smith (2005) designated rhizobia and *Frankia* species that are involved in symbiotic associations with higher plants as intracellular PGPR or symbiotic PGPR. Dinitrogen fixing associative symbiotic bacteria which do not cause any morphological modification of the host plant are also considered as PGPR.

PGPR may enhance plant growth through direct or indirect mechanisms (Klopper 1993); Lazarovits and Nowak 1997). Direct mechanisms of enhancement in plant growth include production of phytohormones, increased availability of nutrients to plants, stimulation of disease resistance mechanisms and others. Indirect mechanisms include control of plant disease, stimulation of other beneficial symbiosis, degradation of xenobiotics in contaminated soils and increasing immunity and protects from disease and abiotic stresses (Jacobsen 1997). Based their functions, PGPR may be classified as biofertilizers (increasing availability of nutrients to plants), biopesticides (controlling diseases, insect pests, nematodes etc. by production of antibiotics, antifungal metabolites etc,), phytostimulators (production of plant growth hormones) and rhizoremediators (degradation of pollutants) and others (Somers et al 2004, 2005).

In most cases a single PGPR exhibits multiple growth promoting attributes including biocontrol ability (Vessey 2003). PGPR are commonly used to improve crop yields and helps in sustainable agriculture (Fig-1). Further, they possess potential in solving environmental problems including phytoremediation to decontaminate soils and waters.

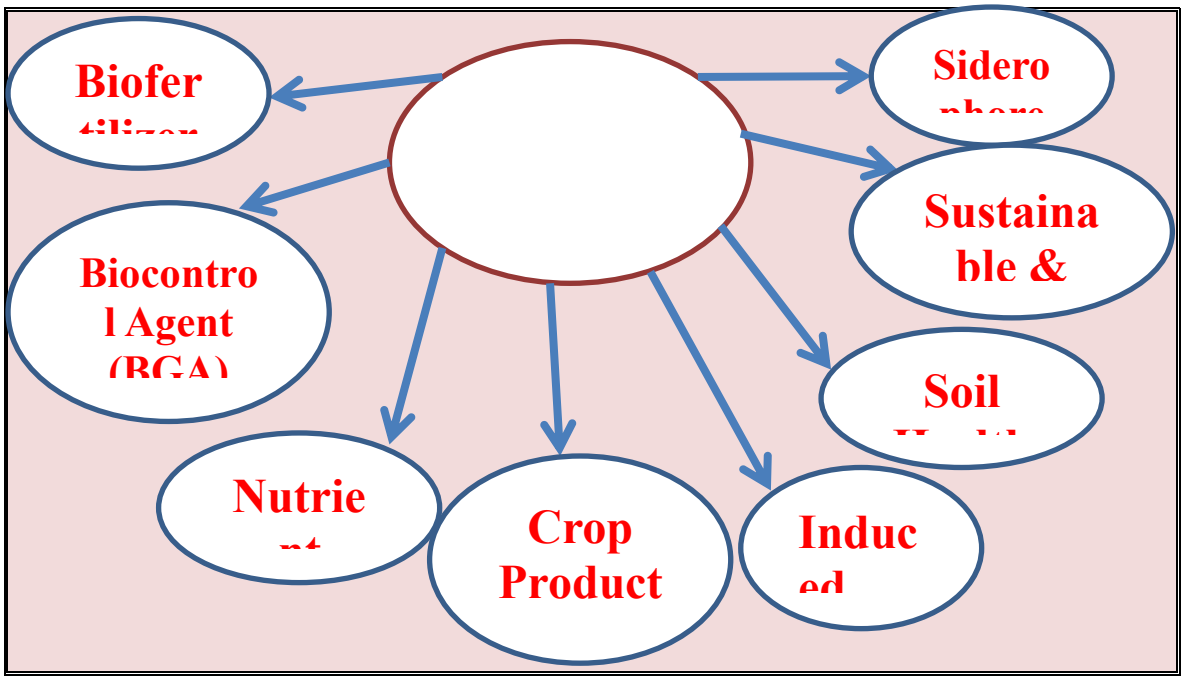

Fig. 1: Functions of Plant Growth Promoting Rhizobacteria (PGPR)

A considerable number of soil and rhizospheric fungi and bacteria collectively known as plant growth promoting microorganisms (PGPM) have demonstrated their ability to colonize plant roots and provide benefits to their respective hosts (Manoharachary and Tilak 2015; Tilak 2015a, b). among these benefits, many authors documented improved root hydraulic conductance and alleviation of abiotic stresses such as drought and salinity. It is accepted that movement through aquaporins represent a quite faster pathway of water movement across biological membranes. Groppa et al. (2012) reviewed the PGPM effects on plant water status and root hydraulic conductance, with special emphasis on the experimental data that proved or suggest an impact of PGPM on root aquaporins under both normal and water limiting conditions.

In recent years the role of the rhizosphere as an ecosystem has gained importance in understanding the functioning of biosphere and also mechanisms of action of PGPR (Barriuso et al. 2008). The earlier studies on PGPR laid emphasis on biological control of plant diseases confined to bacteria like fluorescent pseudomonads and *Bacillus* spp. in recent years, with the elucidation of many mechanisms of plant growth-promotion involving large number of plant and microbial species, knowledge about very diverse bacterial taxa has been obtained (Lucy et al. 2004).

Fluorescent pseudomonads and bacilli form major group among PGPR along with other bacteria like *Acetobacter, Actinoplanes, Agrobacterium, Alcaligenes, Arthrobacter, Azospirillum, Azotobacter, Cellulomonas, Clastridium, Enterobacter, Erwinia, Flavobacterium, Pasteuria, Serratia and Xanthomonas* etc. The rhizosphere microorganisms also include *Rhizobia* and *Bradyrhizobia*, establish symbiotic relationship with leguminous

plants, these bacteria generally, improve the plant growth through direct effects on plant by producing plant growth promoting substances thus increasing the availability and uptake of nutrients besides suppressing soil-borne plant pathogens (Dutta and Podile 2010, Tilak et al. 2010; Wu et al. 2009; Lugenberg and Kamilova 2009; Nautiyal and Tilak 2009).

Over the last decade, understanding of rhizosphere biology has progressed with the discovery of PGPR that colonizes plant roots and promote plant growth. These PGPR could compete with other rhizosphere microorganisms most effectively leading to increased plant growth (Klopper et al 1980b). application of plant growth promoting rhizobacteria has also shown to increase legume growth and development in terms of plant nodulation and nitrogen fixation under normal growth conditions along with an increase in plant yields (Angaw et al. 2011a, b; Tilak et al 2010; Vogeti et al. 2009; Podile and Kishore 2006; Gupta et al. 2003; Tilak 2015a,b).

PGPR AS BIOINOCULANT

Huge amount of literature is available on the application of bacteria for improvement of plant performance (Saxena and Tilak 1994; Malik et al. 1996, 1999; Saxena et al 2000; Gupta et al. 2003; Shende et al. 2010; Tilak et al 2010; Angaw et al 2011 a, b; Tilak 2015 a, b), but few bacteria like *Azatobacter* and *Azospirillum* have been employed in commercial production. The organisms under scrutiny for potential use in agriculture are the bacteria belonging to the genera Pseudomonads and *Bacillus* species (Nautiyal et al 2002; Nautiyal et al 2006; Tilak et al 2006; Tilak and Reddy 2006; Podile and Kishore 2006; Nautiyal and Tilak 2010). Positive response of plant growth has been recorded by promoting chitinolytic *Paenibacillus elgii* in tobacco (Das et al. 2010). In the last two decades, several examples of rhizobacteria capable of providing substantial disease control in the field have been reported (Saxena et al. 2000; Nautiyal et al. 2002, 2006). Many bacterial genera have shown their potential for biocontrol both under in vitro and in vivo conditions. The usefulness of Bacillus as a source of antagonist for many plant pathogens is well known. Several potent strains from different species of Bacillus have been tested on a wide variety of plant species for their ability to control several diseases. *Bacillus* has ecological advantages because it produces endospores that are tolerant to extreme environmental conditions such as heat and desiccation (Nautiyal et al 2006).

Fluorescent pseudomonads have revolutionized the field of biological control of soil-borne plant pathogenic fungi. During the last decade, PGPR have emerged as the largest potentially promising group involved in the biocontrol of plant diseases. They have received the much needed attention for several reasons, such as their colonization ability in roots, simple nutritional requirements, particularly their ability to use many carbon sources that exude from roots and to compete with the indigenous microflora in the rhizosphere. Apart from these qualities, pseudomonads are amenable to genetic manipulation. These characteristics make them ideal as a promosing bioinoculant. There are numerous examples of biocontrol agents that control devastating fungal plant pathogens of important crops

include florescent pseudomonads (Nautiyal 1997 a, b. Anith et al. 1998; Pal et al. 2000; Saxena et al. 2000; Nautiyal et al. 2006).

PGPR- ARBUSCULAR MYCORRHIZAE

More than 80% of all terrestrial plant species form mycorrhizal associations (Sylvia 2005). Arbuscular Mycorrhiza (AM) is the most common mycorrhizal association and has a widespread distribution throughout the plant kingdom forming mutualistic relationship with most of the vascular plants. In Cruciferae, Chenopodiaceae, Polygonaceae and Cyperaceae, either there is very little mycorrhization or no mycorrhizae. Families that do not form arbuscular mycorrhiza include Pinaceae, Betulaceae, Fumariaceae, Commelinaceae and Utricaceae. The fungal partner belongs to *Glomeromycota* forming vesicles within or between cortical cells that act as strange or reproductive organs and arbuscular that are formed within the cortical cells providing a large surface area of contact between host and fungus mycelium which is formed inside and outside the root. The genera, which form arbuscular mycorrhizal (AM) fungal associations are *Acaulospora, Ambispora, Aracheospora, Diversispora, Entrophospora, Geosiphon, Gigaspora, Glomus, Intraspora, Kuklospora, Pacispora, Paraglomus* and *Scutellospora* (Schenck and Perez 1990; Schubler et al 2001; Oehl and Sieverding 2004; Sieverding and Oehl 2006; Walker et al 2007).

Mycorrhizal association helps in increased nutrient and water uptake by absorption through improved absorptive area, translocation of elements to host tissues and their accumulation. The unique ability of mycorrhiza helps to increase the uptake of phosphorus (P) and other nutrients by plants suggesting that mycorrhizal fungi have the potential for utilization as a supplement for phosphatic fertilizers. Ectomycorrhizal fungi permeate the F and H horizon of forest floor and minerals get mobilized in these zones by hyphal network followed by their absorption before they reach sub soil system. AM fungi are known to degrade complex minerals and organic substances in soil and thus make essential elements available to host plants. Mycorrhizal association is known to offer resistance to drought, plant pathogens, tolerance to adverse conditions, release growth hormones like auxines, gibberellins, growth regulators such as vitamin B and also contribute to organic matter turnover along with nutrient cycling in forest and crop land ecosystems. Mycorrhizas are known to help in soil aggregation, soil stabilization and add strength to soil fertility. Mycorrhizas are symbiotic and hence they live hand in hand with other living organisms and are non-pollutants besides sustaining competition.

AM fungi are geographically ubiquitous and are commonly associated with plants in agriculture, horticulture, pastures and tropical forests. About 90% of vascular plants establish mutualistic relationship with AM fungi (Kendrick and Berch 1985).

The occurrence of AM fungi in roots has been reported from an exceptionally wide range of plants. beside roots, the colonization have been reported in other plant parts also example, in leaves of *Salvinia* (Bagyaraj 1984, Bagyaraj et al 1979), in senescent leaves of *Funaria hygrometrica* (Park and Linderman 1980), in decaying peanut leaves and rhizomatous tissue

of Zingiber officinale (Taber and Trappe 1982). Colonization has also been reported from scales of *Colocasia antiquorum, Elettaria cardamomum, Musa paradisica* and *Sanseviera trifaciata*, garlic and ginger (Kunwar and Manoharachary 1998, 1999).

Arbuscular mycorrhizal interactions bring about certain changes in the host metabolism and physiology. These include inhibition of increased production of cytokinins as evidenced by the presence of two gibberellins like substances in culture extracts of *Glomus mosseae* and increased nitrate reductase activity.

Mycorrhizal symbioses are important considering the fact that 70-80% terrestrial plants are mycorrhizal, thus helps in the acquisition of water and minerals, besides offering protection from diseases. The development and formation of mycorrhizae cause changes not only in host plant but also in the rhizosphere microbial community resulting interaction among rhizosphere microorganisms (Bianciotto and Bonfante 2002). Bianciotto et al (1996) suggesting that bacteria present in/on spores or hyphae of AM fungi releases extracellular soluble factors which mediate the bacterial- fungal interactions and AM fungi. These beneficial organisms serve as vehicles for colonization of plant roots by *Rhizobacteria*. *Rhizobacteria* showing beneficial effects on mycorrhizae are often termed as "mycorrhizae-helper bacteria". Bianciotto et al (2004) observed strong evidence of a vertical transmission of endobacteria through the vegetative generation of AM fungus which offers effective nutritional security to the host plant.

Studies have shown that inoculation with PGPR and diazotrophs along with AM fungi may increase plant growth and yield. Chanway and Hall (1991) estimated that associative nitrogen fixation by *Bacillus* could contribute in part to the growth promotion effect observed with *Pinus contorta* inoculated with the mycorrhizal fungus, *Wilcixina mikole*. Colonization by AM fungi may modify the root exudates pattern, which may act as chemo-attractants for the soil bacteria. In a dual inoculation study with *Glomus mosseae* and *Bacillius coagulans* was superior to single inoculants i.e *Azotobacter chroococcum* in enhancing plant biomass of *Simarouba glauca* (Sailo and Bagyaraj 2003). Wu et al. (2009) reported increased growth and nutrient uptake in maize, enhanced root colonization by the AM fungus and improved soil properties when inoculated with a biofertilizer containing N-fixer (*A.chrooccum*), P solubilizer (*B.megaterium*) and K solubilizer (*B.mucilaginous*) and AM fungus (*G. mosseae* or *G. intraradices*).

PGPR beneficial effects was also observed in *Eucalyptus diversicola* along with an unidentified bacterium resulting in 49% more shoot dry weight than the un inoculated control.

The effects of combined inoculation with PGPR, AM fungi and rhizobia have been tested by many workers. Extracellular metabolites produced by the above organisms could possibly be the reason for the synergistic effects, this is documented by the addition of cell-free culture filtrate of PGPR to the mycorrhizal and nodulated legume *Hedysarum coronarium* resulted in Maximum plant growth and nutrient uptake in comparison to washed cells of PGPR or the whole bacterial cultures (Azcon 1993).

The interactive effects of PGPR, AM fungi and rhizobia have resulted in bioremediation effect of heavy metal contaminated and polluted soils (Vivas et al. 2003 a, b). In a lead contaminated soil, co-inoculation with *Brevibacillus* sp., an indigenous PGPR strain, and a mixture of indigenous AM fungal species, enhanced plant growth, mycorrhizal infection, N and P content in clover, along with a decrease in the amount of lead absorbed (Vivas et al 2003 b).

Different mechanisms allow AM fungi and PGPR to increase stress tolerance in plants. This includes the intricate network of fungal hyphae which block pest access to roots and various biocontrol mechanisms of PGPR. Inoculation of apple-tree seedlings with *Glomus fasciculatum* and *G.macrocarpum* suppressed the apple replant disease (ARD) caused by phytotoxic micromycetes (Catska 1994). After 12 month cultivation, plant biomass (height, shoot and root dry masses) had increased by inoculation with *G.fasciculatum*. the number of colony forming units per unit soil (CFU) of phytotoxic micromycetes decreased, whereas CFU of the genus *Azospirillum* was higher. It may be assumed that the use of some AM fungi and such bacteria can replace the chemical treatment of the soil with ARD. AM fungi protect the host plant against root infecting pathogenic bacteria. The damage due to *Pseudomonas syringae* on tomato is significantly reduced when the plants are endomycorrhizal (Garcia Garrido and Ocampo 1989). The mechanisms involved in these interactions include physical protection, chemical interaction and indirect effects (Fitter and Garbaye 1994).

The rhizosphere is thus influenced by the plant roots as well as by mycorrhizal fungus. The mycorrhizosphere is the zone influenced by both the root and the mycorrhizal fungus and it includes the more specific term “hyphosphere” which refers only to the zone surrounding individual hyphae (Johansson et al 2004). Bacterial communities associated with plant roots may be affected by root –colonization with AM fungi. This may be due to metabolic products of AM fungi and their resulting changes. The hyphal exudates might have been detrimental or stimulatory effect on rhizosphere bacteria. Sood (2003) reported greater attraction of the PGPR *Azotobacter chroococcum* and *Pseudomonas fluorescens* towards tomato roots colonized by *Glomus fasciculatum* compared to non-arbuscular mycorrhizal tomato roots. Rhizosphere bacteria remain in close association with AM fungi. Endosymbiotic bacteria closely related to the genus *Burkholderia* have been found in symbiotic AM fungi *Gigaspora margarita*, *Scutellospora persica* and *Scutellospora castanea* (Bianciotto et al 2000). PGPR and AM fungi interactions have shown synergitic effects. In a petri plate system, roots of carrot (*Daucus carota* L.) inoculated with phosphate solubilizing bacteria *Pseudomonas aeruginosa* showed substantial increase in P solubilization when inoculated with *G.intraradices* (Villegas and Fortin 2001).

RHIZOBACTERIA AS BIOLOGICAL CONTROL AGENTS OF PLANT PATHOGENS

The suppression of growth of soil-borne and root-borne plant pathogens by the use of antagonistic microorganisms to reduce diseases is termed as biocontrol. National Academy of Science (USA) defined biocontrol as “the use of natural or modified organisms, genes or gene products to reduce the effects of undesirable organisms (pests), and to favour

desirable organisms such as crops, trees, animals and beneficial insects and microorganisms". Wilson (1997) defined biological control as "the control of plant disease with a natural biological process or with the product of a natural biological process." This definition allows the inclusion of biological chemicals produced by living organisms and extracted from them, host resistance (constitutive and induced) and antagonistic microorganisms (Singh et al. 2004).

The rhizosphere bacteria are the ideal biocontrol agents as they can provide the front line defense for plant roots against the attack by various root/soil-borne plant pathogens (Dey et al. 2014; Manoharachary and Tilak 2015). Disease suppression by biocontrol agents occurs due to interactions among the biocontrol agents and the members of the spermosphere and rhizosphere or phyllosphere community (Singh et al. 2004). The microbes used in biocontrol have various advantages namely: (1) these organisms are considered safer than the chemicals that do not accumulate in the food chain, (2) self replication circumvents repeated applications, (3) unlike chemical agents, target organism seldom develop resistance to target organisms (4) chemical control agents along with biocontrol agents are advocated in integraed plant disease control management and (5) properly developed biocontrol agents are not considered harmful to the population and functional dynamics of the soil microorganisms in the rhizosphere. The major disadvantages include variability of field product. Also, the effectiveness of a given biocontrol agent may be restricted to a specific location, due to the effects of soil and climate. Moreover, biological control depends upon the establishment and maintenance of a threshold population of bacteria on planting material or in soil, and a drop in viability below that level may eliminate the possibility of biological control (Weller 1988). Many soil edaphic factors, including soil temperature, moisture, pH, clay content, interactions of biological disease control microorganisms with other rhizosphere bacteria and with pathogens also affect their viability and tolerance to abiotic stresses once applied (Dey et al. 2012). Concentration of O_2 and CO_2 in the soil is also one of the major factors that affect activity of biocontrol agent in the rhizosphere (Nautiyal 1997 a, b; Goel et al. 2001).

Several rhizosphere bacteria have been demonstrated to posses biocontrol potential which include potential *Pseudomonas* spp. makes up a dominant population in the rhizosphere and seems to be one of the most appealing for the biological control of plant diseases (Fig.2). the worldwide interest in the *Pseudomonas* spp. as biocontrol agents was started in last 1970's with the studies conducted at the University of California, Berkeley, USA (Weller 1988) and several companies now have developed biocontrol agents as commercial products. Fluorescent pseudomonads posses several properties like relatively easily culturable under laboratory conditions. Production of variety of secondary metabolites which are toxic to bacterial and fungal pathogens and compatibility with community used pesticides and other biocontrol agents have made them as ideal biocontrol agents (Vidhyasekharan and Muthamilan 1995).

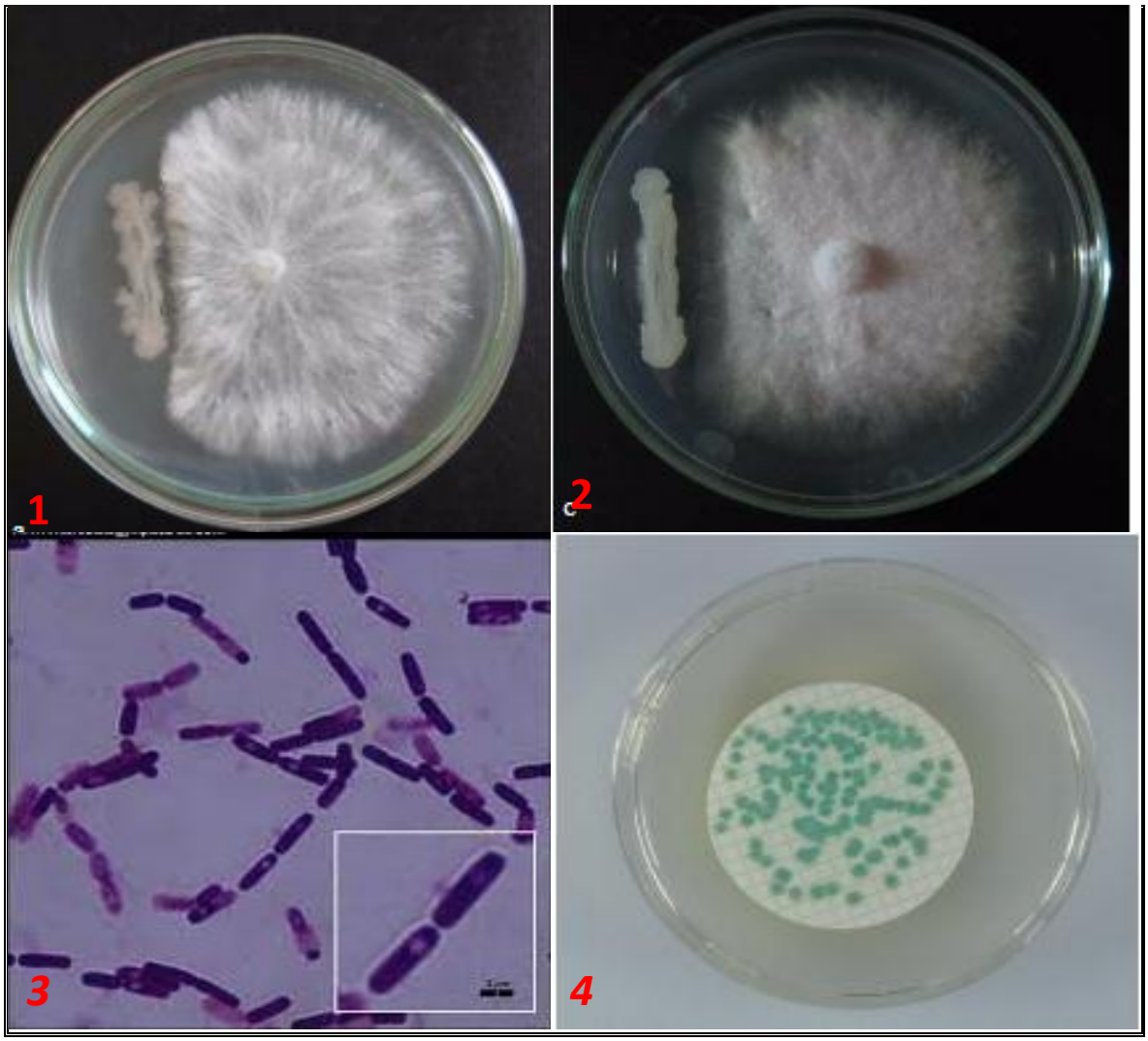

Fig.2: 1.Growth inhibition of soil-borne root pathogen *Sclerotium rolfsii* by *Pseudomonas putida,*

2. Inhibition of *Fusarium oxysporum* by *Pseudomonas putida*,

3. Cells of *Bacillus cereus*; (Gram stained),

4. Cultural characteristics of *Pseudomonas aeruginosa* (Direct isolation from water on PIA

medium)

Despite the extensive research where biological agents have been used to control plant diseases, there have been limited commercial success. An efficient biocontrol agent must meet the requirements of a good colonizer and critical competitor in the rhizosphere besides being viable and non-contaminant along with good shelf life and quality. Root colonization is an active process, which involves the proliferation of microorganisms in/on and around the growing root (Johri et al. 1997). Microorganisms compete with each other for carbon source, mineral nutrients at infection sites on the roots. Competition between the biocontrol agent and pathogen can result in displacement of the latter (Osburn et al. 1989), provided environmental variables are cooperative/favourable.

Pseudomonads have revolutionized the field of biological control of soil-borne plant pathogenic fungi. Most of them fall either in fluorescens or putida group (Fig.2). during the last three decades, they have emerged as the largest potentially most promising group of plant growth promoting rhizobacteria involved in the biocontrol of plant diseases (cook 1993; Pierson and Weller 1994; Barbosa et al, 1995; Gomes et al. 1996; Wei et al. 1996; Compant et al. 2005). Fluorescent pseudomonads have received much attention as they readily colonize roots in nature, besides being common among microorganisms (Weller 1988). The simple nutritional requirement and the ability to use many carbon sources that exude from roots and to compete with indigenous microflora, may explain their ability to colonize the rhizosphere (Mazzola and Cook 1991). Additionally, pseudomonads are amenable to genetic manipulation. These characteristics make them useful vehicle for the delivery of antimicrobial and insecticidal compounds and plant hormones to the rhizosphere. The traits of fluorescent pseudomonads such as production of antibiotics, hydrogen cyanide, siderophore which are involved in suppression of plant root pathogens have been reviewed (O'Sullivan and O'Gara 1992; Kloepper et al 1980a).

There are numerous examples of biocontrol of several devastating fungal plant pathogens of important crops by fluorescent pseudomonads and has been reviewed from time to time (O'Sullivan and O'Gara 1992; Kumar and Dube 1992; Weller and Thomashow 1993; Krishnamurthy and Gnanamanickam 1997; Pierson and Weller 1994; Saxena et al. 2000; Pal et al 2001; Duffy et al. 2004; Compant et al. 2005). Natural disease suppression involving pseudomonads have been reported by many workers. It was noticed that in disease suppressive soils, continued cropping fails to suppress the disease. The results indicate that naturally occurring soil pseudomonads are important elements in these soils to suppress the diseases.

A number of pseudomonas strains have been used as biological control agents in green house and field conditions against an array of plant pathogens. In many cases they not only help in suppressing the pathogens, but also improved the plant yield by acting as plant growth promoters (O'Sullivan and O'Gara 1992; Dowling and O'Gara 1994). *Pseudomonas* spp. have great potential in biological control of plant pathogens (Pal et al. 2001; Manoharachary and Tilak 2012). Few examples of PGPR's as biocontrol agents against plant pathogens are enlisted in Table 1.

TABLE-1. BIOCONTROL OF PATHOGENS BY PGPR

Biocontrol organism	Suppressed pathogen	Crop
Pseudomonas fluorescens	*Erwinia* spp.	Potato
	Erwinia carotovora	Cassava
	Fusarium spp.	Raddish

	Thievaloviopsis basicola	Tobacco
	Rhizoctonia solani	Peanut
	F.oxysporum f. spp. *ciceri*	Chick pea
	Pythium ultimum	Pea
	Xanthomonas malvacearum	Cotton
	Botrytis cinerea	Petunia
	Macrophomina phaseolina	Chickpea
	G.graminis var. *tritici*	Wheat
	Sarocladium oryzae	Rice
Pseudomonas putida	*Fusarium* spp.	Raddish
	Erwinia caratovora	Potato
	F.oxysporum	Flax
	F.oxysporum f. spp *lycopersici*	Tomato
	F.solani	Beans
	Xanthomonas campestris	Potato
P.aureofaciens	*G.graminis* var. *tritici*	Wheat
	Phytophthora megasperma	Asperagus
Pseudomonas (Burkholderia) cepacia	*Fusarium* spp.	Tomato
	F.graminearum	Wheat
	F.moniliforme	Maize
	Rhizoctonia solani	Cotton
	Botrytis cinerea	Apple
	Penicillium expansum	Apple
	Sclerotinia sclerotiorum	Sunflower
	Heterodera glycines	Soybean
	Meloidogyne incognita	Soybean

Pseudomonas spp.	*Fusarium oxysporum*	Carnation
	F.moniliforme	Maize
	Pythium ultimum	Sugarbeet
	Rhizoctonia solani	Cowpea
	Agrobacterium tumefaciens	Grapewine
Bacillus subtilis	*Fusarium roseum*	Corn
Bacillus spp.	*G.graminis* var. *tritici*	Wheat
	Pythium spp.	Wheat
	Rhizoctonia spp.	Wheat
Rhizobim &	*Macrophomina phaseolina*	Soybean
Bradyrhizobium spp.	*Rhizoctonia solani*	Mungbean
	Fusarium solani	Suflower

CONCLUSIONS:

Soil microorganisms are important in the geobiochemical cycles of inorganic and organic nutrients in soil and maintenance of soil health and quality. The rhizosphere of plants is inhabited by complex and dynamic communities of microorganisms, notable among which are plant growth promoting and soil supporting bacteria. Soil-plant-rhizobacteria interactions are complex and there are many ways in which the outcome can influence the plant health and productivity. The PGPR's are also potential biocontrol agents of several soil-borne/root-borne plant pathogens. The rhizosphere provides the frontline defense of roots against attack by pathogens.

PGPR and other beneficial microorganisms including AM fungi and rhizobia have vast potential of their exploitation as beneficial inoculants for crop productivity and establishment of forest seedlings besides their utility in disease suppression and biocontrol agents.

There are problems preventing the commercial use like quality, viability, shelf life and acceptance by the farmer. Improvement of the biocontrol mechanisms of these bacteria by ecological or genetic means is an important approach for enhancing their performance as bioinoculants. However, inconsistency of the bioinoculants demonstrates that there is still a considerable need for extensive studies on rhizobacterial and mycorrhizal populations to

understand the different criteria, which influences the composition of the microflora and their diversity.

Recent advances in our understanding of the ecology and molecular biology of the systems responsible for effective and competitive PGPR bioinoculants are opening the ways for strain improvement the new tools such as recombinant DNA technology, mathematical modeling and computer technology combined with a continuation of the more classical approaches such as crop rotation, various tilling strategies, addition of organic amendments etc. may help to harness the power of PGPR's to improve soil, plant, human health and the environment.

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CANCER STEM CELLS: BENCH TO BEDSIDE

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ABSTRACT:

The molecular basis of carcinogenesis is well established, and existing treatment strategies for several cancers are adequate. However, the major hurdle for ongoing cancer treatments emerges from cancer relapse prompting poor prognosis. Existing literature revealed the therapeutic resistance of cancers due to the occurrence of cancer stem cells (CSCs). The CSC hypothesis provided convincing evidence to understand tumor heterogeneity and therapeutic refractoriness towards cancer treatments. The challenge for targeting CSCs is that they share similar characteristics with normal stem cells. Hence, it is imperative to design novel therapeutic agents that exclusively target CSCs without causing detrimental effects to the normal counterparts improving patient's survival. This review provides insight into CSC origin, its characteristics, and strategies for targeting this subgroup from rest of the tumor.

KEYWORDS: Cancer stem cells; Tumor heterogeneity; CSC origin; Drug resistance; therapeutic strategies

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INTRODUCTION

From the last few decades, there has been remarkable advancement in cancer prevention, diagnosis and treatment régimes for cancer cure. Despite these developments, the lacunas in current cancer therapies are recurrence and metastasis. Existing literature demonstrated that tumors are composed of a heterogeneous group of cells with different phenotypic and functional characteristics. Among these, a minor subgroup of cells that share stem cell properties known as cancer stem cells (CSCs), play a central role in the limitations of cancer therapy and have been related to therapeutic resistance and cancer relapse [1].

Hamburger and Salmon proposed the existence of CSCs in 1977 [2]. Later, Lapidot and colleagues proved CSC hypothesis in 1994 by identifying stem-like cells in acute myeloid leukemia (AML) using cell-surface protein markers [3]. John Dick's group reported the first experimental evidence for CSCs in 1997. They identified $CD34^{+}CD38^{-}$ cells from AML patients which could initiate leukemia upon transplantation in to SCID mice. These cells exhibited stem cell properties such as self – renewal and potency [3, 4]. Al-Hajj in 2003 showed the occurrence of CSCs in solid tumors, where $CD44^{+}CD24^{-}$/low breast cancer cells had higher capability to generate tumors after transplantation into immune-deficient mice [5]. Later, numerous studies have reported the presence of CSCs in solid tumors such as lung [6-9], colon [8], liver [8], prostate [6, 8], head & neck [8], ovarian [10], brain [8, 11], bladder [12-14] as well as melanoma [15-17], hepatocellular carcinoma [18, 19], retinoblastoma [20, 21] and their phenotypic and functional properties have been extensively studied.

TUMOR HETEROGENEITY & ORIGIN OF CANCER STEM CELLS

It is a well-established notion that tumors are composed of a heterogeneous group of cells. Tumor origin and its heterogeneity are well explained by two models. They are called classical or stochastic model, and the hierarchical or CSC model. The classical model is based on the clonal theory of cancer initiation and progression. This model states that each cancer cell has an equivalent potential to generate new homogenous cell mass [22]. As time progresses, the cells undergo genetic & epigenetic alterations and acquire new genetic traits promoting tumor development and enhancing tumor heterogeneity. On the contrary, hierarchical model postulates that within the heterogeneous population of cancer cells, only a small subset of cells generate new tumors with heterogeneous phenotype. These cells are termed as cancer-initiating cells (CICs) or CSCs [5, 7, 23-27]. CSC model was extensively reported in leukemia's [6] and several solid tumors [24, 28-31] except in lymphoma mice models and melanoma [17, 32], where the tumors are homogeneous.

Diverse models have been suggested for the origin of CSCs as shown in figure 1. CSCs may originate from a) transformed adult stem cells or differentiated cells; b) cell fusion of cancer cells and normal stem cells; c) horizontal gene transfer; d) consequence of mutations. These changes initiate cellular transformations, reprogramming these cells to become CSCs and acquire self-renewal capacity [33-35]. These molecular variations are elicited either by activation of oncogenes, inactivation of tumor suppressor genes or as a consequence of the accumulation of additional genetic and epigenetic irregularities [36]. Additional elements that control CSCs maintenance for tumor growth and progression are tumor microenvironment, CSC niche, cytokine loops [1, 4, 37-40] and metabolic swings from glycolytic to oxidative phosphorylation, or vice versa, also prompting cancer stemness [41].

CHARACTERISTICS OF CSCS

SELF-RENEWAL ABILITY & DIFFERENTIATION

CSCs can self-renew and undergo differentiation into multiple cell types that are present in the tumor of origin [42-44]. CSCs share similar self-renewal property as normal stem cells, but its proliferation is influenced by both internal (genetics and epigenetic changes) and external factors (hypoxia, starvation, inflammation) and any disturbance in this leads to abnormal cell proliferation. Furthermore, CSCs undergo rapid symmetric cell divisions producing two daughter stem cells compared to asymmetric divisions producing one stem and one non-stem daughter cells [33].

GENETIC & EPIGENETIC ALTERATIONS

Accumulating data have shown that cancer stemness is directed by genetic changes (oncogene activation and tumor suppressor gene inactivation) and epigenetic changes (chromatin remodeling, altered DNA methylation, and miRNA targeting). The role of chromatin in inducing CSC formation by conferring self-renewal ability is confirmed by sequencing studies. A number of other studies claimed that mutated epigenetic regulators (histone acetyltransferases, methyltransferases, chromatin-remodeling enzymes, etc) stimulate the attainment of self-renewal property of CSCs and initiate the tumorigenic process [45]. The process of CSCs development is also instigated by interactions between non-coding RNAs, specifically microRNA (miRNA) and additional epigenetic mechanism by targeting its components or reciprocally being controlled by epigenetic mechanisms [46].

METABOSTEMNESS

Menendez and Alarcon introduced the term “metabostemness” for explaining the role of cellular metabolic changes responsible for genetic and epigenetic adaptations, required for

reprogramming normal/tumor cells into CSCs [47]. Apart from two crucial epigenetic modifications namely DNA methylation and histone modification, the epigenetic regulation of cell differentiation genes are also crucial for exerting metabolic effects on nuclear reprogramming [48]. These metabolic transformations occur before the changes in stemness happen. The accumulation of by-products of normal metabolism or onco-metabolites causes aberrant signal transduction resulting in initiation and progression of carcinogenesis eventually blocking the acquisition of differentiation markers thereby inducing the expression of stemness genes likely through epigenetic mechanisms [47]. To support this hypothesis, Vermeersch et al found that genes involved in eleven pathways linked with metabolism were differentially expressed between ovarian cancer stem cell and ovarian cancer cells, suggesting that the phenotypic differences between these two cell types are the result of differences in their matabolome [49]. Targeting these metabolic processes specific for CSCs is considered to be one of the new approaches to specifically eliminate this subgroup from the bulk of tumor.

EXPRESSION OF CELL SURFACE MARKERS

CSCs express distinguished cell surface markers such as CD133, CD44, ESA, CXCR4, nestin [50, 51] and functional markers like ALDH1 [52]. The expression of these markers is specific to particular tumor type and tissue of origin. Among these, the most studied marker is CD133 (prominin-1) which is a transmembrane glycoprotein, identified as a primitive hematopoietic and neural stem cell marker overexpressed in both human and mouse tumors [53]. Fu et al demonstrated that the chemoresistance of glioblastoma towards temozolomide is due to expression of $CD133^{+}$ cells in this tumor [54]. $CD133^{+}$ sorted population from ovarian cancer show increased tumorigenic capacity in NOD/SCID mice compared to $CD133^{-}$ cells [55]. In another study, Shmelkov et al proved that both $CD133^{+}$ and $CD133^{-}$ metastatic colon cells could initiate tumors [56]. In retinoblastoma (Rb) primary tumors and Y79 cell line, $CD133^{lo}$ (FSC^{lo}/SSC^{lo}) population sorted by FACS showed CSC properties like increased and larger colony formation, increased invasive potential, more resistance to carboplatin when compared to $CD133^{hi}$ cells [21, 57, 58].

CD44, well documented CSC marker is a transmembrane glycoprotein having a high affinity for hyaluronic acid (HA) ligand. It is involved in cell division, migration, adhesion, and signaling with elevated level in many cancers [59]. The aggressiveness of human non-Hodgkin's lymphomas is attributed to the expression of CD44v6 isoform in these tumors

[60]. The abnormal expression of CD44 and its isoforms CD44v4 and CD44v is also reported in breast, intestinal and colon cancers [59].

ALTERED SIGNALING PATHWAYS

Key signaling pathways disrupted in CSCs are listed in table 1. JAK/STAT, Wnt/β-catenin, notch, NF-kB, Hedgehog, and TGF-β are the common signaling pathways involved in all CSCs. The irregularities in these pathways lead to the acquisition of self-renewal, differentiation, proliferative and survival properties of CSCs through anomalous regulation of target genes, such as transcription factors (Oct-4 Nanog, Sox2), c-Myc, cyclins (cyclin D1) and pro-apoptotic gene (survivin) and others. Notch signaling is the evolutionary conserved and one of the most activated pathways in cancer cells. It has vital role in cell differentiation and cell cycle progression [61]. Wnt signaling pathway includes both, β-catenin-dependent (canonical) and β-catenin-independent (noncanonical) pathways, which regulate asymmetric cell division, cell polarity, migration, survival, and proliferation of cells [62]. The Hedgehog (Hh) signaling pathway plays a critical role in the course of acquiring stemness characteristics through the EMT process [63]. The phosphatidylinositol-3-kinase (PI3K)/Akt signaling pathway has a significant role in angiogenesis, differentiation, and survival of cells and is important for the maintenance of CSCs populations [64]. The JAK/STAT signaling pathway has a critical role in cytokines and growth factor signaling. Aberrant regulation of this pathway leads to the maintenance of stem cell-like cells in various cancers [65]. Understanding the molecular mechanisms responsible for deregulation in these pathways would help to develop new therapeutic approaches that directly target CSCs [9, 10, 28, 66-69].

THERAPEUTIC RESISTANCE OF CSCS

The tumorigenic capacity of CSCs along with therapy resistance property makes them responsible for tumor relapse, metastasis and cancer-related mortality [23, 25, 70-73]. The therapeutic resistance of CSCs is attributed to various intrinsic mechanisms i.e. quiescent state of CSCs during treatment, ABC transporter-mediated drug efflux pumps, ALDH enzyme-mediated cellular detoxification system, loss of immunosurveillance mechanisms due to lack of MHC class 1 molecules, enhanced DNA repair capability, reactive oxygen species (ROS) scavenging, up-regulation of antiapoptotic signaling pathways, higher telomerase activity and extrinsic factors like CSC plasticity through induction of EMT, interaction with its tumor microenvironment, exposure of tumor cells to hypoxic conditions and involvement of extracellular vesicles in cell-cell communication.

TUMORIGENICITY OF CSCS

High tumorigenicity is the most distinct characteristic feature of CSCs. Numerous studies have reported that as low as hundred cells expressing positive markers for CSCs could produce tumors upon serial transplantation, whereas cells expressing alternate phenotype for these markers failed to induce tumors even after transplantation of large number of cells into non-obese/SCID mice [62]. For example, various studies reported that even hundred CSCs, those were cancer and marker and specific like $CD133^+$ brain CSCs, $CD44^+CD24^-$ $ALDH1^+$ breast CSCs, $CD44^+CD117^+$ ovarian CSCs, $CD44^+CD24^+EpCAM^+$ pancreatic CSCs could induce tumors in non-obese/SCID mice [62-64].

STRATEGIES FOR TARGETING CSCS

Several preclinical & clinical studies have documented the possible applications of combining chemotherapeutic drugs with CSC targeting agents. Some drugs are already in use and some are under clinical trials to ascertain their efficacy, safety, and pharmacokinetics. There are diverse range of CSC targeting agents such as ABCG2 inhibitors (GF120918 and tariquidar), cytotoxic lymphocytes against SP cells [74, 75], stem cell inhibitors (cyclopamine), which target signaling pathways (Wnt, Hedgehog, and Notch) which are dysregulated in CSCs [9, 10, 23, 28, 29, 66-69]. A list of CSC targeting agents, their mechanism of action and the tumor types are summarized in Table number 2.

CONCLUSION & FUTURE PROSPECTS

CSCs share normal stem cell-like features and contribute to therapeutic resistance towards systemic chemotherapy resulting in cancer relapse. Even though, the CSC model was well explained in many tumors including AML and other solid tumors, the concept remains controversial. CSCs play a critical role in tumor recurrence after anticancer therapy as they are more resistant than bulk tumor cells. The survival capability of CSCs post cancer therapy is due to their stemness properties such as quiescence, self-renewability, acquisition of immune properties by means of immune evasion, cellular plasticity through EMT along with the manifestation of altered signaling, drug efflux pathways and occurrence of tumor heterogeneity as a result of genetic instability and epigenetic variability protecting them from detrimental effects caused by therapeutic agents. These intrinsic CSC properties along with extrinsic factors such as CSC niche including growth factors and extracellular stimuli play major hurdles in designing effective therapeutic strategies against them. Even though many agents targeting CSCs have been developed, however the shortcomings of these agents can be attributed to non-specifically targeting normal stem cells since, both the

groups share identical surface markers, stem cell niches and common signaling pathways. It is essential to develop novel therapeutic strategies along with efficient drug delivery systems that selectively would target the CSC subgroup and its niche with distinct surface marker without affecting normal stem cells. The development of improved therapeutic strategies may increase the patient's survival rates by preventing cancer relapse.

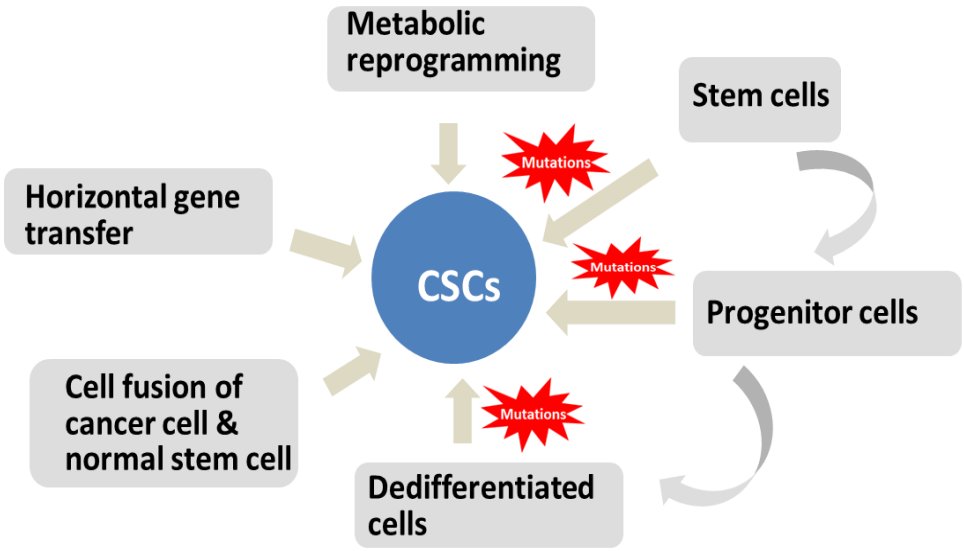

Fig 1 Origin of CSCs - Mutations in adult stem cells, progenitor cells or in dedifferentiated somatic cells can give rise to CSC phenotype. Other factors that prompt CSC origin are horizontal gene transfer, CSC and normal cell fusion and metabolic reprogramming.

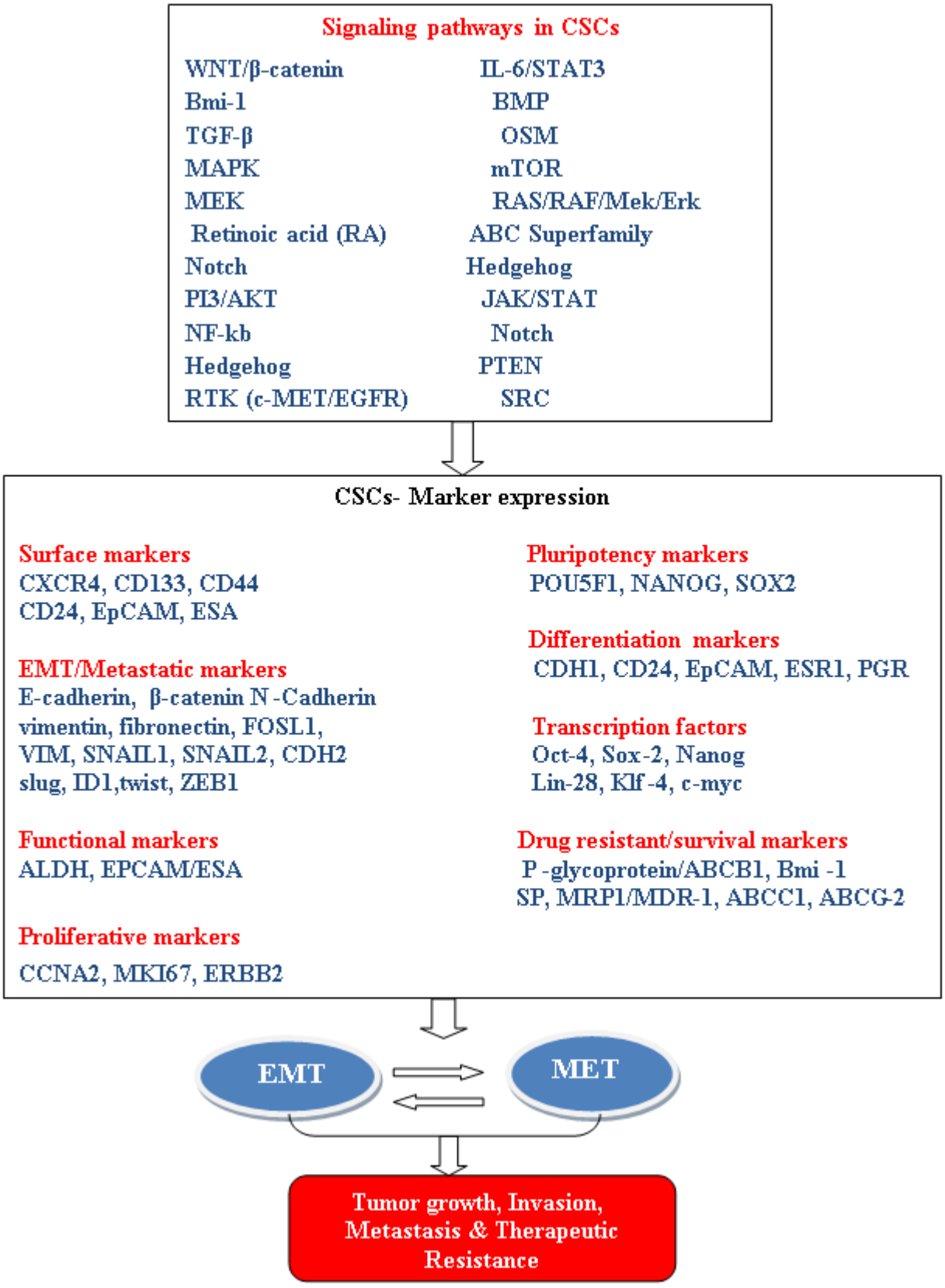

Table 1: List of Signaling pathways involved & markers expressed in CSCs. The cellular signaling crosstalk plays significant roles in self-renewal of CSCs, marker expression, EMT and aggressive tumor phenotype. Different strategies targeting these activated signaling

pathways, markers expressed could abolish CSC population from tumor bulk, which could lead to enhanced treatment outcomes.

Targeting CSCs Property	**Mode of action**	**Drugs name**	**Tumor type**	**References**
Self-renewal & differentiation	Regulation	miR-4319	Breast	[76]
	Induces CSCs to differentiate into a hepatocytic lineage	Oncostatin M	Liver	[77]
	Targets mTOR	Rapamycin	AML	[78]
surface markers	Repression of CD44	miR-34a	Prostate	[79]
	CD133			[77]
	CD33,, IL-3R, CD34, CD90			
	CD133, CD44, CD47, CD123, CLL-1, CD13, CD24		Solid tumors, Hematologic neoplasm	
ABC cassette/ efflux pumps	Inhbits ABCG2/ABC transporters	Anti-ABCG2	Multiple myeloma	[80]
		USP22 block	Lung	[81]
		Cyclosporine A		[82]
	Downregulation of ALDH1	Disulfiram, sorafenib, sulforaphane		
	ALDH1 inhibitors	Tryptostalin		
		Verapamil		
		VX-710, MS-209		
		Tariquidar		
		DS-PLGA/Cu		

Tumor microenvironment a) Hypoxia b) Vasculature niche, c) Endothelial cells d) Invasion e) Migration	Downregulation of HIF-1α CXCL2/CXCR4 VEGEF TAM Adoptive therapy to eliminate ALDH cells	miR-18a-5p PX-478, topotecan & digoxin Sutininb Bevacizumab, anti VEGF-mab Sorafenib ALDH1A1-specific CD8+ T cells	Lung Solid tumors	[83] [77]
Signaling pathways	Inhibits WNT pathway by upregulation of FZD6 transcription Suppression of the WNT/β-catenin pathway Inactivation of BMX–STAT3 NF-kb inhibitor Notch signaling, Hedgehog PI3/AKT, JAK/STAT EGFR, VEGEF BMP-4 pathway	Luteolin Acyclic retinoid Ibrutinib BMS-345541 Dkk1 ICG001 γ-secretase & DLL inhibitors (experimental) Cyclopamine, Cetuximab, Recombinant BMP-4(experimental)	Prostate HCC Glioma Lung	[84] [85] [86] [87] [77] [88]
Gene silencing	Suppression of pro-metastatic genes MDM2 inhibitor	miR-141 AMG232	Prostate Glioblastoma	[89] [90]

Apoptosis/ Autophagy Activation of apoptotic pathways	Induces apoptosis	WYC-209	Melanoma,,lung, and breast	[91]
Cell differentiation	Inhibits CD	ATRA, ATO, vit D3, DMSO)	APL	[92]
Metabolism	Inhibit mTOR, inhibit the mevalonate metbolic			[93]
Metabostemness	Targets OXPHOS & by inhibiting BCL-2 Inhibits mitochondrial biogenesis COX-2 inhibitors Inhibitors of mitochondrial complex I	venetoclax in combination with azacitidine estrogen-related receptor α inhibitor XCT790 Resveratrol, Resveratrol Metformin	Leukemia Ovarian cancer Glioma 293FT cells, A549 cells	[94] [95] [96] [97] [98] [99] [100]
Epigenetic mechanisms	Target self-renewal capacity, Target c-MYC, BET family of chromatin readers: BRD4	BRD4 inhibitors (BETis)	ALL	[101] [102]
Telomerase activity	Inhibitors of telomerase	Imetelstat (Phase III)	breast cancer, non‐small cell lung cancer, multiple myeloma, and chronic lymphocytic leukemia	[103]

Table 2: Strategies targeting CSCs

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ABBREVIATIONS

CSCs - Cancer Stem Cells, AML - Acute Myeloid Leukemia, CD - Cluster of Differentiation, SCID - Severe Combined Immunodeficiency, CICs - Cancer Initiating Cells, DNA - Deoxyribonucleic Acid, miRNA - Micro Ribonucleic Acid, ESA - Epithelial Specific Antigen, CXCR4 - CXC Chemokine Receptors Type 4, ALDH1 - Aldehyde Dehydrogenase 1, NOD - Non-Obese Diabetic, Rb - Retinoblastoma, FSC - Forward Scatter, SSC - Side Scatter, FACS - Fluorescence-Activated Cell Sorting, HA - Hyaluronic Acid, JAK - Janus kinase, FZD6 - Frizzled class receptor 6, STAT - Signal Transducer and Activator of Transcription Proteins, Wnt - Wingless-Related Integration Site, HH - Hedgehog, EMT - Epithelial–mesenchymal transition, NF-κB - Nuclear Factor kappa-light-chain-enhancer of activated B cells, TGF - Transforming Growth Factor

Oct-4 - Octamer-binding transcription factor 4, SOX2 - SRY (sex determining region Y)-box 2, PI3K - Phosphatidylinositol-3-Kinase, AKT - serine/threonine-specific protein kinase, ABC - ATP-Binding Cassette, MHC - Major Histocompatibility Complex, ROS - Reactive Oxygen Species, EpCAM - Epithelial cellular adhesion molecule, mTOR - Mammalian Target Of Rapamycin, miR - Micro RNA, ABCG2 - ATP-binding cassette super-family G member 2, USP22 - Ubiquitin Specific Peptidase 22, DS-PLGA/Cu Poly lactic-co-glycolic acid (PLGA)/copper (Cu)-encapsulated Disulfiram, HIF - Hypoxia-Inducible Factors, VEGF - Vascular Endothelial Growth Factor, TAM - Tumor-Associated Macrophage, BMP-4 - Bone Morphogenetic Protein 4, BMX - bone marrow and X-linked, ICG001 - Inhibits Wnt/β-catenin pathway, Dkk1 - Dickkopf-related protein 1, DLL - Delta-like ligand, HCC - Hepatocellular Carcinoma, MDM2 - Mouse Double Minute 2 Homolog, AMG232 - MDM2 inhibitor, WYC-209 - Synthetic retinoid, ATRA - All-Trans Retinoic Acid, ATO - Arsenic Trioxide, vit D3 - Vitamin D3, DMSO - Dimethyl Sulfoxide, APL - Acute Promyelocytic Leukemia, OXPHOS - Oxidative phosphorylation, Bcl2 - B-Cell Lymphoma 2, COX2 - Cyclooxygenase-2, XCT790 - Potent and specific inverse agonist of Estrogen-related receptor-α (ERRα), BET -

Bromodomain and Extra-Terminal Domain, A549 cells Human Caucasian lung carcinoma, BRD4 - Bromodomain-Containing Protein 4 and ALL - Acute Lymphoblastic Leukemia

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LACTATE, MITOCHONDRIA AND CANCER CELL

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ABSTRACT

Warburg proposed 'aerobic glycolysis' to suggest that cancer cells produce lactate and depend on glycolysis even in the presence of O_2. Lactate is a signal molecule and a symbiotic fuel for cancer growth. The emerging literature suggests that the mitochondria are functional in cancers, and produce the metabolites for the epigenetic modification of histones, and provide the precursor molecules for the biosynthesis of macromolecules. The cell membrane transporters of fatty acids and amino acids regulate the spatiotemporal changes in the gene expression and the metabolic programming in cells, which decide the cell fate. Lactate entry into the cells activates the hypoxia-inducible factor2 (HIF2)/ Aryl hydrocarbon receptor nuclear translocator (ARNT) signalling pathway, which together with MYC mediates glutamine metabolism in the mitochondria. Lactate conversion to pyruvate activates two mitochondrial pyruvate carrier proteins, MPC1 and MPC2, which activate the differentiation of cells and promote the lipogenic and the gluconeogenicprogrammes respectively.

KEYWORDS: Carcinogenesis;Pyruvate; Respiratory chain; Coenzyme ligases, Epigenetics.

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INTRODUCTION

Lactic acid (chemically, the protonated form of lactate) became the focus of attention incancers when Warburg in 1920s observed that proliferative cancer cells produce lactic acid even in the presence of oxygen (O_2). Warburg proposed two hypotheses, 'the Pasteur effect' to suggest that O_2 inhibits fermentation, and 'aerobic glycolysis', to suggest that cancer cell has damaged respiration, and therefore, depends on the fermentation energy of glucose (40,41) The present model of the intermediary metabolism (IM) suggests that glucose is first metabolised to pyruvate in the cytoplasm. If O_2 is available, pyruvate is further oxidised to CO_2 in the mitochondria and produces the reduced cofactors $NADH+H^+$ and FADH2, which areoxidised in the electron transport chain (ETC) to produce ATP. ATP is considered as the energy currency to perform the vital functions of the cell. Meyerhof and his team of scientists working on muscle metabolism developed the model in the first quarter of the 20th century. The investigations on lactic acid production in muscles during the muscle contraction in the 18th century formed the background of the development of the model (Reviewed in 8, 15) The discovery of ATP by Fiske-Subbarow and Lohman strengthened the concept that glucose is the energy fuel and ATP a symbol of 'energy currency'(20, 29). The model became a dogma, with the emergence of the theory of bioenergetics, which proposed that autotrophs utilise sun energy to produce the carbohydrates. Heterotrophs oxidise carbohydrates during respiration to generate ATP for physiological activity (**18**) Warburg adopted glucose energetics to cancer cell growth and introduced two hypotheses, the Pasteur effect and the aerobic glycolysis to suggest that O_2 inhibits the glycolysis and that the mitochondria are dysfunctional in cancers and therefore, depend on the energy of glycolysis for cell growth. Pasteur observed that Yeast can survive in the absence of O_2 (anaerobic conditions) and consumes more sugar during fermentation.Pasteur observed further that lactic acid contamination of alcohol producing units, suspends fermentation and increases the growth of Yeast and that O_2 accelerates the growth of Yeast. The Yeast consumed less sugar (4g as against 60g per 1g of fermenting Yeast) during the growth and transformed the nitrogen in the nutrient medium into albuminoid like material (Protein) and required phosphate as the mineral (2,17). In terms of ATP for the glucose consumed by the Yeast either for growth or fermentation, there is not much of a difference (39). The second half of the 20th century was dominant with studies on growth factors, somatic mutations, and the gain or loss of function of oncogenes, and the tumour suppressors. (42). Aberrant activation of growth factors or their receptors and their downstream signal pathways, Ras-MAP kinase and PI3K-Akt-mTOR signalling remained the focus of attention as the targets for cancer therapy, but with limited success.(33,45) The aggressive relapse of cancers, following the growth factor therapies, led to the resurgence of interest in cancer metabolism over the past two decades (23,31,32).

Sonveaux et al. 2008, demonstrated that lactate fuels the cancer cell respiration and targeting the lactate transporter into the cells, the monocarboxylic acid transporter1 (MCT1), reduces the cancer cell proliferation (34).

Despite glucose considered the fuel for energy, there was a search for alternative fuels supplying energy for cancer growth. It remained a debate for nearly a century of cancer research. (9,24)Recent studies suggest that cancer cells depend on fatty acid oxidation for the energy supply (30).

FATTY ACID TRANSPORT AND SECOND MESSENGERS

G-protein coupled receptors (GPCR) modulate the entry of the fatty acids into the cells. Several hundreds of these receptors are known, which span across the cell surface as well as the intracellular membranes. Some of them exhibit polymorphism (12). The NC-IUPHAR Subcommittee on free fatty acid receptors, classified them into four categories (FFA1-4), based on the carbon chain length.(19) In brief,a wide range of saturated and unsaturated fatty acids containing from 6 to 22 carbons activate the FFA1 and FFA4, the short chain fatty acids of 2-4 carbon chain length activate the FFA2 and FFA3 receptors, are activated by, which include acetic, propanoic, butyric, trans-2-methylcrotonic, 1-methylcyclopropanecarboxylic acids. These receptors are proteins with seven transmembrane helical domains interconnected with three external, and three internal loops. The N-terminal end of the protein is the ligand binding site, which is towards outer face of the membrane, while the internal cytoplasmic c-terminal end is attached to three proteins (a heterotrimeric complex), the Gα, the Gβ, and the Gγ. There is polymorphism in each of these trimeric proteins; in humans, there are four Gα subunits, $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$, $G\alpha_{12/13}$, each with distinct functions. There are about5 and 12 distinct subunits Gβ and the Gγ units. In the unstimulated state, the three proteins form a cohesive unit and remain bound to GDP in an inactive state. The ligand binding induces a conformational change in the receptor, which results in the exchange of GDP with GTP at Gα subunit and the heterotrimeric proteins segregate into Gα and the dimeric Gβγ subunits. The activated Gα subunits activate specific lipases and transmit the messages to the targets inside the cells, through the second messengers, the diacylglycerol (DAG), the inositol triphosphate (IP_3), the cyclic AMP (cAMP) and the Rho effectors (reviewed by Priyadarshini et al. (28).

LACTIC ACID REGULATES THE cAMP, CELL PROLIFERATION AND DIFFERENTIATION

A distinct family of the GPCR, the hydroxycarboxylic acid receptors (HCA1-3), formerly known as the nicotinic acid receptor family (GPR81, GPR109A, and GPR109B), act as negative regulators of fatty acid oxidation in adipocytes by binding to the receptors with $G\alpha_{i/o}$ subunit. Activated $G\alpha_{i/o}$ subunit inhibits adenylate cyclase (AC) and the cAMP production and reduces the protein kinase A (PKA) signalling pathway. While HCA1 is the receptor for the lactic acid, HCA2 is the receptor for the β-hydroxybutyric acid, and the HCA3 is the target of the β-hydroxy-octanoic acid, which is an intermediate of the fatty acid β-oxidation (1,4). They have immune and anti-inflammatory functions (5,25); a recent study suggests that the β- hydroxy-octanoic acid binding to HCA3 activate the EGFR dependent ERK1/2 MAP kinase pathway (47). Given the limitations of the space, the article presents only the highlights of the significant developments in cancer metabolism. The references cited will provide adequate information for further reading.

EMERGING DEVELOPMENTS IN CANCER METABOLISM

Three main lines of developments in the past few years threw new light on the role of mitochondria in cancer growth.1) Epithelial cells lose their anchorage and transform to tumour initiating mesenchymal cells (EMT) or cancer stem cell (CSC) phenotype (11,22). They acquire the mitochondrial genome from the host genome for stepwise assembly of respiratory supercomplexes to acquire the respiratory capacity to increase the potential for proliferation and increase the tumour mass (36,43). Besides, the mitochondria produce metabolites for epigenetic modification and the precursor molecules for macromolecular synthesis, which promote tumour growth (7,26).

2) Long recognised as the genome guardian and activated in response to DNA damage, p53 has metabolic functions and activates mitochondrial respiration and oxidative phosphorylation through transcription of synthesis of cytochrome C oxidase 2 (SCO2), required for cytochrome oxidase assembly (21,44). P53 remains in the inactive deacetylated state in stem cells (46), and the wild type of p53 is acetylated to facilitate its translocation into mitochondria (37,48). The p53 can negatively regulate the PDK2 and activates the PDC (6), which migrates into the nucleus to acetylate histones (35). The fatty acid oxidation in mitochondria generates the bulk of acetyl-CoA, a substrate for trans acetylation reactions affecting epigenetic modifications and ketone body production. The p53 dependent respiration is also linked to pyrimidine biosynthesis and in maintaining the balance between life and death. The acetylation and deacetylation dynamics by the CREBBP (cAMP response element binding(CREB) binding protein) and sirtuins regulate the p53 dependent ATP regenerating system (14,38). 3) Lactate conversion to pyruvate in cells is catalysed by LDH1, which is constitutive and occurs even in unfertilized eggs. Pyruvate activates the two mitochondrial pyruvate carrier proteins (MPC1& 2). They are inactive in stem

cells, and their activation transforms stem cells to a differentiated state. Pyruvate metabolism integrates with amino acid metabolism in mitochondria in transforming cells. Under low nutrient conditions, pyruvate entry through MPC1 activates the glutamine and BCAA metabolism, which generates 2-OG for reductive carboxylation and α- ketoacids, which generate succinate and acetoacetate from BCAA. Succinate oxidation by CII suppresses NADH oxidation and ATP production in ETC and increases the acidity in the matrix. The transfer of NADH to NADPH promotes reductive carboxylation and export of citrate out of mitochondria into cytoplasm, which activates de novo lipogenesis. The oncometabolite 2-HG reduces the function of 2-OG and increases ROS production. ROS shifts mitochondrial metabolism from glutamine metabolism to asparagine metabolism, which activates MPC2 mediated pyruvate carboxylase, and the biosynthesis of pyrimidines, gluconeogenesis and glutamine synthesis. Reduced mitochondrial glutamine metabolism activates HIF1 transcription factors and activation of glycolysis. Citrate export into cytoplasm activates lipogenic pathways. Unlike the classical theory, which suggests that mitochondrial metabolism takes place in the oxygenated environment, the bulk of metabolism in mitochondria and cytoplasm takes place only under hypoxic conditions. Elevated levels of O_2 utility is only during respiration and ATP production, which is tightly regulated. Hammarlund et al. recently suggested that the stemness of life maintains the global biodiversity, which exists under hypoxia (<1-3%), while O_2 promotes the adult organogenesis; and the reproductive potential to increase the population size of the diversified lineages (10). At the organism level, the stem cell/ progenitor population maintain tissue diversity. There is a self-regulatory mechanism, which facilitates these transformations to be bi-directional. In times of need, like in tissue damage or severe injury, somatic cells transform to mesenchymal state (EMT) to support the demand for increased cell number for tissue repair and wound healing. Cancer cells exploited these mechanisms successfully.Hypoxia-inducible factors play a crucial role in the maintenance of stemness as well as in EMT. While HIF1 activates glucose-dependent glycolysis in somatic cells and promotes EMT during cell transition to carcinogenesis (4,13,16), HIF2 maintains stem cell population (27). Respiration increases the oxidative phosphorylation and triggers the transformation from stem cell to adult cells. Nitric oxide imposes a limitation on the respiration, and limits the ATP production, for harvesting hydrogen for biosynthesis.

CONCLUSIONS

Lactate entry into tumour initiating cells and its conversion to pyruvate by LDH1 activates the cell transformation by activation of p53 and the mitochondrial carrier proteins MPC1 and MPC2. The mitochondrial metabolism has three compartments, the FAO, the ETC and the metabolism of the amino acids. Oxygen regulates the hydrogen transfers between the nutrient oxidations and the biosynthesis of molecules through the ETC. The proliferative cells limit the hydrogen

expenditure for ATP production to conserve it for the reduction reactions during biosynthesis. The p53 dependent mitochondrial respiration promotes ATP production and generates acetyl-CoA. MPC1 activates the GPT and links pyruvate metabolism to glutamine and the BCAA metabolism, which regulates NADH oxidation in ETC and activates NADPH dependent reductive carboxylation, citrate synthesis, which supplies acetyl-CoA for epigenetic programmes and de novo lipogenesis. MPC 2 activates the pyruvate carboxylase, which integrates metabolism of multiple amino acids like serine, glycine, asparagine, aspartate, arginine, methionine threonine for activation of pyrimidine, polyamine, glutamine and gluconeogenesis related biosynthetic programmes. Bothp53 and the MPC are inactive in stem cells, but proliferative progenitor cells have active p53, and the arginine metabolic pathways, which activate the nitric oxide synthesis, and ribosome biogenesis. Mitochondrial respiration and the proliferative potential is active in metastatic tumours, and activation of the MPC proteins initiates differentiation and cell growth by activating the lipogenic and gluconeogenicprogrammes transform to senescence state. The HIF1 pathway is activated in somatic cells under hypoxic state, and after the suppression of glutamine metabolism. More than the O_2 levels, the relative ratios of 2-OG /succinate decide whether the HIF1 is activated or not. The falling levels of glutamine metabolites activate the reductive carboxylation by activating HIF1, which simultaneously activates the oxidation of 2-OG by the oxoglutarate dehydrogenase (OGDH) to increase the succinate levels at the same time, providing the GTP for cytoskeletal reorganisation and mTORC1 dependent biosynthetic activity. There are three mechanisms by which the glucose is available for cell functions; 1) GLUT 4 mediated glucose uptake downstream of AMPK and mTORC2, which activates the hexosamine pathway and 4F2HC dependent nutrient uptake; 2) gluconeogenesis, which is activated by pyruvate carboxylation; and 3) the HIF1 mediated GLUT1 dependent glucose uptake, which supports the fatty acid biosynthesis.

Contrary to the bioenergetic theories and Warburg's 'aerobic glycolysis', which promoted ATP as the principal driver of cell growth, it is the chromatin remodelling, which promotes the cell transformations. Mitochondria are biosynthetic hubs fixing hydrides, NH3 and CO2 into biomass and not the catabolic hubs of glucose catabolism to CO_2. Both ATP and acetyl-CoA contribute to epigenetic modification of chromatin in reprogramming the metabolism during cell transformation.

In short, it is suggested that mitochondria are the centres of oxidation of fatty acids and recycle amino acids to fix CO_2, NH_3 and hydrides generated from the oxidation of fatty acids and amino acids for de novo synthesis of metabolites required for epigenetic modifications and precursor molecules for biosynthesis of macromolecules and supramolecular assemblies and organelles in cytoplasm. The model suggests that the mitochondria have three metabolic compartments: 1) the fatty acid oxidation (FAO), which supplies the reduced cofactors NADH and FADH2; b) the ETC. The dehydrogenases transfer hydrogens from the nutrients to reduce the cofactors,

NADHand FADH2. 2) The ETC oxidises the reduced cofactors to generate ATP and reduce O_2 to H_2O.3) The metabolism amino acid is divided into i) arginine glutamine metabolism, which produces the nitric oxide, urea, polyamines, and the ribosomes, ii) MPC1 mediated metabolism of glutamine and BCAA (lipogenic amino acids), which activate the reductive carboxylation and lipogenicprogrammes and iii) MPC2 mediated metabolism of asparagine and gluconeogenesis, which inhibits glutaminolysis in favour of asparagine metabolism and glutamine synthesis.

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FIG. 1

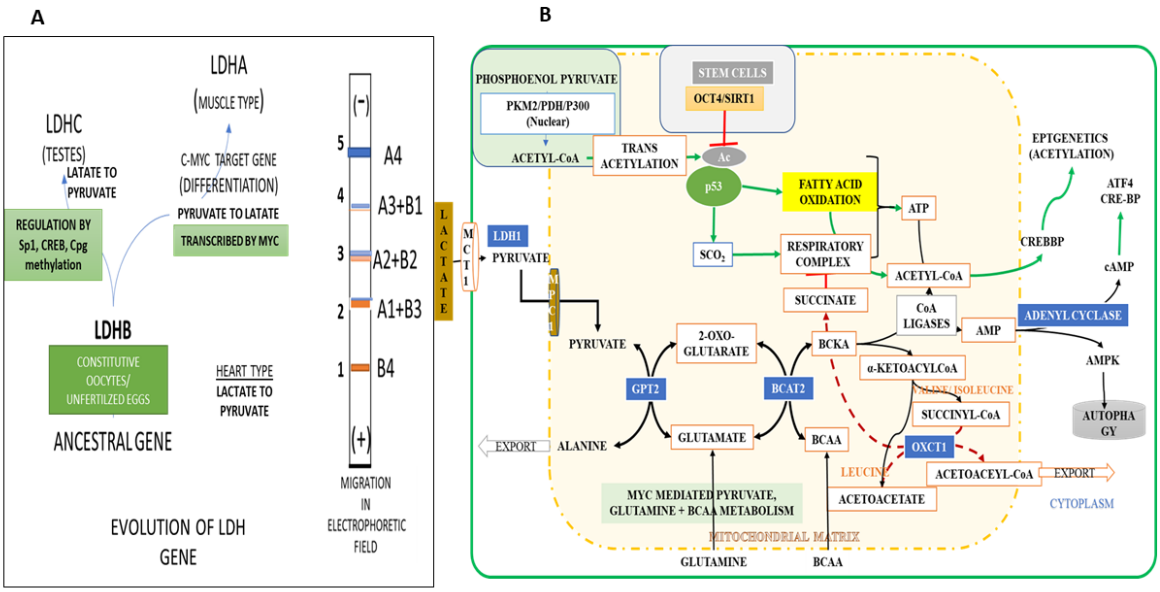
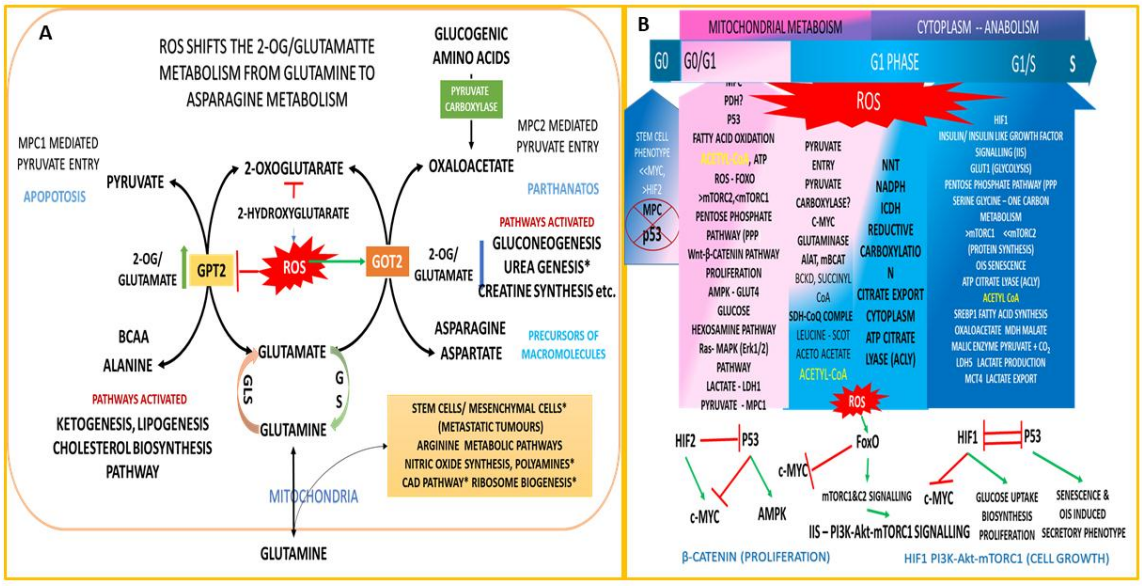


FIG. 2



LEGENDS FOR FIGURES:

FIG. 1 A Lactate dehydrogenase isoforms:

The model is depicting the evolution of LDH isozymes in the vertebrates. LDH B is constitutive, present in unfertilized eggs. MYC activates LDHA during differentiation. LDHC is present mainly in testes and transcribed during development.The transcription factors Sp1 and CRE-BP promote the transcription of LDHB/C under the hypomethylated state;the methylation status of its promoter region regulates the LDHB transcription. Right side: Graphic representation of the electrophoretic mobility of the LDH isoforms. The A and B genes generate homo and heterotetramers, which produce five isoforms differently expressed and in different proportions in adult tissues.

FIG. 1 B Lactate induced metabolism in cancer cells:

Lactate enters the cell through MCT1 (HCA1). LDH1 converts it into pyruvate. Pyruvate activates pyruvate dehydrogenase complex (PDC), which translocates into the nucleus generates acetyl-CoA and activates the transacetylase p300, which acetylates histones and activates the genotoxic response elements. The p53 remains inactivated in stem cells by deacetylation by OCT4 and SIRT1. PDC activation in the nucleus activates P300, which trans acetylates p53 and activates it. Activated p53 translocates into mitochondria and activates the respiratory complex and the fatty acid oxidation (FAO). Pyruvate entry through MPC1 into the mitochondria

activates glutamine and BCAA metabolism, which activates simultaneous transamination of glutamate to 2-OG, and BCAA to BCKA, which maintains 2-OG/ glutamate cycle (see the text).

FIG. 2. A. Pyruvate activates two different routes of metabolism in mitochondria

Glutamine in mitochondria is deaminated to glutamate by GLS2 in mitochondria. Glutamate in tumour initiating mesenchymal cells activates the glutamine-aspartate-arginine metabolic pathways, which activate the nitric oxide, polyamine, urea, and nucleotide biosynthetic pathways. It results in metastatic tumours (not part of the essay). Pyruvate entry through MPC1 activates the GPT2 which converts pyruvate to alanine and glutamate to 2-OG. The 2-OG produced regenerates glutamate by activating the transamination of the BCAA to BCKA which produce succinate to shift NADH oxidation in ETC to produce NADPH in the matrix. Reductive carboxylation leads to citrate synthesis and lipogenicprogrammes. Pyruvate entry through MPC2 activates to OXA, which integrates the metabolism of asparagine and aspartate metabolism, which modulate several amino acid metabolic pathways that culminate in gluconeogenesis, pyrimidine synthesis, ureagenesis and methylation pathways. OXA is reduced to malate and activates aspartate-malate shuttle for gluconeogenesis. Deprivation of glutamine activates apoptosis, while long term deprivation of glucose/ nucleotide production activates PARP-1 dependent parthanatos.

2. B. Cell transformation is a balance between catabolism and the anabolism

Cell transformation is a dynamic process, which has the temporal programming of the metabolism interconnected to gene regulatory networks. Much of the anabolic programming in cancer cells takes place in a hypoxic environment. During the proliferative state, mitochondria increase the respiration and activate the metabolite production from the nutrients, which are utilised for epigenetic modification and as precursors for biosynthesis. Pink colour depicts the transitional oxidative state. The bottom of the figure gives the interactions between the transcription factors.

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EVOLVING GENOME TECHNOOGY AND HUMAN HEALTH

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ABSTRACT

The international collaborative Human Genome Project (HGP) where the whole human genome was sequenced led to the development of Genomic medicine as an emerging medical discipline. The exponential technological innovations in Next generation sequencing has led to the "Lab on chip era", and has also enabled the sequencing of the genome, transcriptome and the epigenome at the single cell resolution. The impact of genome sequencing on clinical care is observed in the fields of oncology, pharmacology, rare and undiagnosed diseases, and infectious diseases. A new field termed 'precision medicine,' is emerging where genomics, epigenomics, environmental exposure, would be used to accurately guide individual diagnosis and treatment. A newly discovered gene editing tool CRISPR-Cas9 is revolutionizing the treatment of genetic diseases at the DNA level.

Keywords : DNA,Human Genome, NGS,WGS,WES, Single Cell Omics.

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INTRODUCTION

The DNA of a living organism is the Genome which contains information to build and maintain the organism throughout its life. A gene is a segment of DNA that provides the cell with instructions for making a specific protein, which then carries out a particular function in the body. DNA is made up of molecules called nucleotides. Each nucleotide contains a phosphate group, a sugar group and a nitrogen base. The four types of nitrogen bases are adenine (A), thymine (T), guanine (G) and cytosine (C). The order of these bases is what determines DNA's instructions, or genetic code. The human genome is the complete set of nucleic acid sequences, which is encoded as DNA within the 23 chromosome pairs in cell nuclei the nuclear genome and a small DNA molecule found within individual mitochondria called as the mitochondrial genome. The haploid human genome (23 chromosomes) is about 3 billion base pairs long and contains approximately 20,000 genes. The whole genome is about 750 megabytes of data. Human genomes include both protein-coding DNA genes and noncoding DNA which is controls biochemical activities of the cell, including regulation of gene expression, organization of chromosome architecture, and signals controlling epigenetic inheritance. Allmost all humans have the same genes arranged in roughly the same order and more than 99.9% of an individuals DNA sequence is identical to any other human. A small number of individual traits are mainly controlled by a single gene, however most traits are influenced by multiple genes. The external environment influences how the genome works. The number of genes in the human genome is not entirely clear because the function of numerous transcripts remains unclear. Although the number of protein coding genes are approximately estimated, there are still about 1,400 questionable genes which may or may not encode functional proteins, usually encoded by short open reading frames. The sequential arrangement of the four base pairs A,T,G,C in a human gene will differ from person to person and will decide the structure and function of a protein, how much protein is made, when it's made, or where it's made. For example the DNA sequence affects the color of a person’s eyes, hair, and skin. More importantly, this variations in the genome also influences human health by increasing the risk of developing diseases and also individual responses to medications and pathogens.

Changes in the DNA sequence are called genetic variants. Generally genetic variants have no phenotypic effect but, sometimes, the effect of one missing or changed nucleotide may result

in a defective protein, extra protein, or no protein at all, with serious consequences on health. Additionally, the passing of genetic variants from one generation to the next helps to explain why many diseases are inherited, to name a few such as in sickle cell disease, cystic fibrosis, and Tay-Sachs disease. Some diseases are caused by mutations that are inherited from parents are called germline mutations and those acquired during a persons lifetime are called somatic mutations(for example in cancer). The understanding of our Genome has come through several discoveries over time and culminated in the sequencing of the human genome. The genetic discoveries from chromosomal basis of disease to gene to DNA variants have revolutionized the way diseases are diagnosed and treated. From one gene one protein to one SNP variant and the combination of several SNPs causing disease we have come a long way in understanding our genome. Additionally most of the vast quantities of noncoding DNA within the genome have associated biochemical activities, including regulation of gene expression, organization of chromosome architecture, and signals controlling epigenetic inheritance.

HUMAN GENOME PROJECT

The human genome project was an international collaborative effort launched in 1990 and the first complete genomic sequence was completed in april 2003 which cost many billions of dollars. A major part was the time and effort to build a “framework” where the organization of chromosome could be deciphered by known markers so that the 3 billion, bases of the genome sequence could be placed in relative position. The extraordinary research effort of Human Genome Project (HGP) was to generate knowledge that could advance the understanding of biology and disease and to improve human health. According to the National Genome research institute Genomic medicine is an emerging medical discipline that involves using genomic information about an individual as part of their clinical care (e.g. for diagnostic or therapeutic decision-making) and the health outcomes.(https://www.genome.gov/health/Genomics-and-Medicine). Post genomic era the advent of modern massively parallel sequencers are able to do sequencing of 3.3 billion bases at very high speed and at low cost. This is further simplified by sequencing the protein coding part of the genome that is whole exome sequencing (WES) which constitutes about 1.5-2% of the genome. This rapid development of WES now enables researchers to interrogate all known protein-coding genes in a single experiment. (1,2).

TECHNOLOGY ADVANCES IN GENOME TECHNOLOGY

While discussing the evolving role of genome in human health the earlier discoveries in identification of chromosomes and DNA as genetic material responsible for inheritance should not be forgotten because these discoveries are responsible for the recent advances in genomic research. For many years scientists believed that proteins were the molecules which held all our genetic material. The story of the discovery of the DNA "The molecule of life" began in 18th century.

There is a misconception that James Watson and Francis Crick discovered DNA in 1950s. In reality, DNA was discovered decades before. It was by following the work of the pioneers before them that James and Francis were able to come to their ground-breaking conclusion about the structure of DNA in 1953. However the molecule now known as DNA was first identified in the 1860s by a Swiss chemist called Johann Friedrich Miescher. While trying to identify the key components of white blood cells which were obtained from pus-coated bandages collected from a medical clinic, Johann identified a substance which separated from the solution which dissolved again when an alkali was added. This substance had unexpected properties different to those of the other proteins. This substance was named 'nuclein', because he believed it had come from the cell nucleus. Unknowingly the molecular basis of all life – DNA was discovered. However according to the scientific understanding of that time the newly discovered substance called nuclein present in the nucleus was not complex enough to contain all the information required to make up a genome. In the early 1900s, the work of Gregor Mendel who identified the genetic basis of inheritance was rediscovered and his ideas about inheritance were being accepted in the scientific community. In the middle of the nineteenth century, Walther Flemming, an anatomist from Germany, discovered a fibrous structure within the nucleus of cells 'chromatin' what are now known as chromosomes and he also discovered the process of cell division and mitosis.

Walter Sutton and Theodor Boveri first presented the idea that the genetic material was passed down from parent to child is within the chromosomes. Their work helped explain the inheritance patterns that Gregor Mendel had observed over a century before. Walter Suttons studies could distinguish individual chromosomes undergoing meiosis in the testes of the grasshopper and, through this, he correctly identified the sex chromosome. In 1902 (3) he proposed the chromosomal theory of inheritance based around these principles: Chromosomes contain the genetic material, chromosomes are passed along from parent to offspring and

chromosomes are found in pairs in the nucleus of most cells (during meiosis these pairs separate to form daughter cells). During the formation of sperm and eggs cells in the male and female, respectively, chromosomes separate and each parent contributes one set of chromosomes to its offspring. Most of these discoveries were done in other organisms however parallel studies started emerging on the genetic basis of inheritance and human disease. The one gene-one enzyme hypothesis was first proposed by the English physician Archibald Garrod in 1909, suggests that each gene codes for a single enzyme whereby each gene would be responsible to facilitate a single step in the metabolic process. This theory was experimentally further validated by Tatum and Beadle in 1940s.

A significant discovery in the field of cytogenetics was the use of "hypotonic shock" by Hsu and Pomerat to prepare a metaphase spread (4) followed by the correct determination of the human diploid chromosome number as 46, by J-H Tjio and A Levan(5).With the discovery of human cytogenetics started the association of the human genome to disease.

ADVANCES IN KARYTYPING TECHNOLOGY AND WITH HUMAN DISEASE

A chromosome is a DNA molecule with part or all of the genetic material (genome) of an organism. Chromosomes are normally visible under a light microscope only when the cell is undergoing cell division during the metaphase stage (where all chromosomes are aligned in the center of the cell in their condensed form. Karyotyping which is the ordered arrangement of chromosomes was termed as karyotype by Levitsky (6). Chromosomal aberrations are disruptions in the normal chromosomal content of a cell and are a major cause of genetic conditions in humans, such as Down syndrome. Chromosome abnormalities can be numerical, as in the presence of extra or missing chromosomes, or structural, as in derivative chromosome, translocations, inversions, large-scale deletions or duplications. Numerical abnormalities, also known as aneuploidy, often occur as a result of nondisjunction during meiosis at the time of gamete formation. Common numerical abnormalities are trisomies, in which three copies of a chromosome are present instead of the usual two, and monosomy where one copy of a chromosome is present instead of the usual two. Chromosomal abnormalities that lead to disease in humans include Turner syndrome results from a single X chromosome (45,X or 45,X0), Klinefelter syndrome, the most common male chromosomal disease, otherwise known as 47,XXY,: Edwards syndrome is caused by trisomy of chromosome 18, Down syndrome, a common chromosomal disease, is caused by

trisomy of chromosome 21, Patau syndrome is caused by trisomy of chromosome 13. Trisomy 9, believed to be the 4th most common trisomy, has many long lived affected individuals but only in a form other than a full trisomy, such as trisomy 9p(where a part of the chromosome 9 is in three copies) syndrome or mosaic trisomy 9. They often function quite well, but tend to have trouble with speech. Also documented are trisomy 8 and trisomy 16, although they generally do not survive to birth.

Then came the discovery of banding of chromosomes where a banded pattern is observed when treated with some stains. Bands are alternating light and dark stripes that appear along the length of chromosomes. Unique banding patterns are used to identify individual chromosomes which in turn can identify structural chromosomal aberrations and to diagnose chromosomal aberrations, including chromosome breakage, loss, duplication, translocation or inverted segments. Some disorders arise from loss of just a piece of one chromosome for example Cri du chat syndrome, arises from a truncated short arm on chromosome 5. The name comes from where the babies have a distinctive cat like cry, caused by abnormal formation of the larynx. 1p36 Deletion syndrome, is due to the loss of part of the short arm of chromosome1. Angelman syndrome where 50% of cases have a segment of the long arm of chromosome 15 missing; a deletion of the maternal genes, example of imprinting disorder. Prader-Willi syndrome where 50% of cases have a segment of the long arm of chromosome 15 missing; a deletion of the paternal genes, which is also an example of imprinting disorder. Chromosomal abnormalities also occur in cancerous cells of an otherwise genetically normal individual; one well-documented example is the Philadelphia chromosome, a translocation commonly associated with chronic myelogenous leukemia and less often with acute lymphoblastic leukemia. More than half of all leukemias have detectable chromosomal abnormalities by karyotypic analysis. Many chromosomal abnormalities are associated with particular subtypes of leukemia, and appear to have prognostic significance. Different chromosomal abnormalities are associated with different characteristic features. For example, Auer rods are often associated with translocation t(8;21) and t(15;17). Eosinophils have been associated with abnormalities of 16q22, and this is a good prognostic indicator, inv(16), t(8,21) respond better to high dose chemotherapy cytarabine, whereas patients with del(5q), del(7q) trisomy 8, and abnormal IIq show poor response to chemotherapy. These are a few examples of medical conditions with cytogenetic abnormalities which can be detected by karyotyping.

FLURESECEMCE IN SITU HYBRIDIZATION (FISH)

With the advent of fluorescent microscopy advanced cytogenetic tests to localize small fragments of DNA/gene were developed. FISH is a molecular cytogenetic technique that uses fluorescent probes that bind to only those parts of a nucleic acid sequence with a high degree of sequence complementarity. It was developed to detect and localize the presence or absence of specific DNA sequences/gene on chromosomes. Fluorescence microscopy can be used to find out where the fluorescent probe is bound to the chromosomes. FISH was initially utilized to localize the position of genes on chromosomes. FISH can also be used to detect and localize specific RNA targets (mRNA, lncRNA and miRNA) in cells, circulating tumor cells, and tissue samples. Multicolor FISH is a further enhancement of the technique used to identify structural chromosome aberrations in cancer cells and other disease conditions when light microscopy karyotyping, Giemsa banding or other techniques are not accurate enough. In this method, a single-stranded fluorescent-labeled nucleic acid sequence (probe) complementary to a target genomic sequence is hybridized to detect the presence or absence of a given abnormality. FISH is a method of choice for diagnosis, prognosis, and treatment response in hematopoietic neoplasms (leukemia, lymphomas, multiple myeloma, and myelodysplasia) and solid tumors (breast cancer, non-small cell lung cancer, colorectal cancer, and cervical cancer).Testing of oncology specimens, whether the sample is blood, bone marrow, fresh tissue,
or paraffin block, is available. FISH testing can also be done for prenatal diagnosis on uncultured amniotic cells for detecting chromososmal numerical as well as structural abnormalities in the fetus. You can also have for example a diagnostic Lung Cancer Panel which consists of EGFR, ALK and ROS1 FISH. It can also help define the spatial-temporal patterns of gene expression within cells and tissues. FISH is often used for finding specific features in DNA for use in genetic counseling, medicine. Fluorescently labeled probes for each chromosome are made by labeling chromosome-specific DNA with different fluorophores. *Digital karyotyping* is a technique used to quantify the DNA copy number on a genomic scale. Short sequences of DNA from specific loci all over the genome are isolated and enumerated. This method is also known as virtual karyotyping and analysis of human cancer cells by using this method identified gross chromosomal changes as well as amplifications and deletions, including regions not previously known to be altered at the whole genome level(7).

POLYMERASE CHAIN REACTION (PCR)

PCR is a method widely used in molecular biology and genomic research to make many copies of a specific DNA segment. Using PCR, copies of DNA sequences are exponentially amplified to generate thousands to millions of more copies of that particular DNA segment. Most of the modern genomic technologies are based on PCR technology. The invention made by Mullis allowed PCR to become a central technique in biochemistry and molecular biology, described by *The New York Times* as "highly original and significant, virtually dividing biology into the two epochs of before PCR and after PCR"(8). Because of its extensive application in every aspect of genomic research he was awarded the Nobel Prize in Chemistry in 1993 (along with Michael Smith) for his work in developing the method. Some of the applications of PCR include DNA sequencing to determine unknown PCR-amplified sequences in which one of the amplification primers may be used in Sanger sequencing, isolation of a DNA sequence to expedite recombinant DNA technologies involving the insertion of a DNA sequence into a plasmid, phage, or cosmid (depending on size) or the genetic material of another organism. Bacterial colonies *(such as E. coli*) can be rapidly screened by PCR for correct DNA vector constructs. PCR can also be used for genetic/DNA fingerprinting; a forensic technique used to identify a genetic blue print of an individual or organism by comparing DNAs through different PCR-based methods STRs.

SANGER SEQUENCING

Sanger sequencing is a method of DNA sequencing developed by Frederick Sanger in 1977 which is based on the selective incorporation chain-terminating dideoxynucleotides by DNA polymerase during invitro DNA replication. It was the widely used sequencing technology for 40 years. Sequencing is generally utilized to determine the order of nucleotides in small targeted genomic regions or entire genomes. Automated DNA-sequencing instruments (DNA sequencers) can sequence up to 384 DNA samples in a single batch. DNA sequencers separate strands by size (or length) using capillary electrophoresis, they detect and record dye fluorescence, and output data as fluorescent peak trace chromatograms. Current methods can directly sequence only relatively short (300-1000 nucleotides long) DNA fragments in a single reaction. Developed by Frederick Sanger and colleagues in 1977(9) was a breakthrough that helped scientists determine the human genetic code, but it is time-consuming and expensive. The Sanger method has been automated to make it faster and is still used in laboratories today

to sequence short pieces of DNA, but it would take years to sequence complete genome of an individual. Next-generation sequencing has sped up the process (taking only days to weeks to sequence a human genome) while reducing the cost. However, the Sanger method remains in wide use, for smaller-scale projects, and for validation of Next-Gen results. It still has the advantage over short-read sequencing technologies (like Illumina) that it can produce DNA sequence reads of > 500 nucleotides.

MICROARRAY TECHNOLOGY

The human genome sequencing led to the development of high throughput technology known as the microarray technology. A DNA microarray (also commonly known as DNA chip or biochip) is a collection of microscopic DNA spots attached to a solid surface. DNA microarrays are utilized to measure the expression levels of large numbers of genes simultaneously or to genotype multiple regions of a genome. The most extended microarray platforms are Affymetrix and Illumina. Thousands of spotted samples known as probes (with known identity) are immobilized on a solid support (a microscope glass slides or silicon chips or nylon membrane). The spots can be DNA, cDNA, or oligonucleotides. These are used to determine complementary binding of the unknown sequences (DNA or RNA of the disease or normal sample) thus allowing parallel analysis for gene expression and gene discovery. An experiment with a single DNA chip can provide information on thousands of genes simultaneously. An orderly arrangement of the probes on the support is important as the location of each spot on the array which is used for the identification of a gene. Using this technology Microarray Expression Analysis can be done where, the cDNA derived from the mRNA of known genes is immobilized. Probe-target hybridization is usually detected and quantified by detection of fluorophore, labeled targets to determine relative abundance of nucleic acid sequences in the target. Spots with more/less intensity are obtained for normal as well as diseased tissue, and over/under expressed data obtained in diseased condition is compared to the expression of the normal tissue. For Mutation Analysis using Microarray the genomic DNA of the sample is hybridized to the chip where the genes might differ from each other by a single nucleotide base or otherwise known as SNP(Single nucleotide polymorphism).Whereas Comparative Genomic Hybridization is used for the identification of increase or decrease of chromosomal fragments harboring genes involved in a disease. The main application of microarray technology has been a genome-

wide test to provide molecular diagnosis to infer copy number variation. Microarray has the capacity to interrogate chromosomal rearrangements at the submicroscopic level in the range of size (50 bp−5 Mb). These chromosomal structural variation (SV) is usually a rearrangement of a genomic region with can cause Mendelian disease and contribute to complex diseases (10, 11). These include, genomic imbalances (duplications and deletions), the latter commonly known as copy number variants (CNVs) (12). The advent of chromosomal microarrays can detect large genomic de novo structural variants and also recurrent de novo (or inherited) CNV in both parents and child (e.g., 1q21.1, 16p11.2, or 22q11.21) or ultra-rare or unique de novo CNVs. These discoveries provided an additional aspect of disease etiology to identify and bring rare variants with unusual phenotypes to the forefront (13).

“NEXT-GEN" SEQUENCING

More recently, higher volume Sanger sequencing has been replaced by "Next-Gen" sequencing methods, especially for large-scale, automated genome analyses. Next generation sequencing (NGS), can be performed on Illumina, Applied Biosystems, SOLiD and Roche Life Science systems. NGS can be utilized to determine the order of nucleotides not only in small targeted genomic regions but also entire genomes. Using Next generation sequencing (NGS) an entire human genome can be sequenced and analyzed within a month. The genome of the test sample is sequenced and computationally compared to a reference genome (which is the complete genome sequence of a normal individual) to identify many types of genetic variation. In contrast, the previous Sanger sequencing technology, used to decipher the human genome, required over a decade to deliver the final draft. Although in genome research NGS has mostly superseded conventional Sanger sequencing, it has not yet translated into routine clinical practice. NGS or massively parallel or deep sequencing are related terms that describe a DNA sequencing technology of the whole genome which has revolutionized genomic research. High-throughput genome sequencing involves fragmenting the genome into small single-stranded pieces, followed by amplification of the fragments by PCR. All NGS platforms perform sequencing of millions of these small fragments of DNA in parallel. Bioinformatics analyses are used to piece together these fragments by mapping the individual reads to the human reference genome. Each of the three billion bases in the human genome is sequenced multiple times, providing high depth to deliver accurate data and an insight into unexpected DNA variation. The advent of NGS technologies has created a paradigm shift in our approach to both the discovery of new disease genes and the timely diagnosis of previously unknown genetic

disease. DNA variation in a human genome comprises small base changes substitutions, insertions and deletions of DNA, large genomic deletions of exons or whole genes and rearrangements such as inversions and translocations. Traditional Sanger sequencing can discover substitutions and small insertions and deletions. For the remaining genetic alterations dedicated assays are frequently performed, such as fluorescence in situ hybridisation (FISH) for small deletions and duplications, or comparative genomic hybridisation (CGH) microarrays to detect submicroscopic chromosomal copy number changes such as microdeletions. However, all these data can be derived from NGS sequencing data directly, in a single experiment. The only limitations reside in regions which sequence poorly or map erroneously due to extreme guanine/cytosine (GC) content or repeat architecture, for example, the repeat expansions underlying Fragile X syndrome, or Huntington's disease. NGS can be used to sequence entire genomes or constrained to specific areas of interest, including all 22,000 coding genes (a whole exome) or small numbers of individual genes called as clinical genome(14). Sanger sequencing depends on preknowledge of the gene or locus under investigation. However, NGS is completely unselective and used to interrogate full genomes or exomes to discover entirely novel mutations and disease causing genes.

WHOLE GENOME SEQUENCING (WGS):

This term implies the determination of the sequence of the entire human genome of an individual simultaneously. However, there may be components of the genome that are not included in a present-day “whole genome sequence.” It includes the coding as well as the noncoding part of the genome.The assembly of the genome is computationally laborious and most of the noncoding sequence is difficult to interpret. WGS is not routinely done on clinical sampes but only for research purposes where no genetic aberration could be identified in the protein coding region of the genome.

WHOLE EXOME SEQUENCING (WES):

The “exome” is the component of the genome that encodes protein; these DNA segments are referred to as “exons” and can include noncoding exons. The exome comprises about 1.5-2% of the genome comprising 20,000 genes and is, so far, the component most likely to include interpretable mutations that result in clinical phenotypes/disease. Whole exome sequencing involves determination of the DNA sequence of most of these protein-coding genes and may

also include some DNA regions that encode RNA molecules that are not involved in protein synthesis. WES offers lower cost analysis than WGS. In some cases, exome testing or analysis may be targeted to particular genes of clinical interest and are called clinical genome for a given application. These clinical panels include a set of genes associated with for example neurology panel, a microcephaly gene panel, nephrotic syndrome gene panel, cardiomyopathy gene panel to name a few. These gene panels include genes which have been discovered in the particular set of disorders. Diseases that were previously intractable to gene identification given their rarity, clinical and genetic heterogeneity, and paucity of multiplex families have shown tremendous discovery success using WES. The pace of novel disease gene discoveries has increased dramatically with this technology over the last few years (15).

WES has become a diagnostic approach for the identification of molecular defects in patients with suspected genetic disorders. WES as a powerful tool for effective discovery and diagnostics of monogenic genetic diseases and has clinical usefulness, and allows timely medical interventions, informed reproductive choices, and avoidance of additional testing. It also helps in phenotype expansion and identification of new candidate disease genes that would have been impossible to diagnose by other targeted testing methods.

RNA SEQUENCING

The NGS technology takes advantage of a broad range of techniques, from targeted RNA to whole-transcriptome sequencing by RNA-Seq a recently developed deep-sequencing technology. Following RNA sequencing, the resulting reads are either aligned to a reference genome or reference transcripts, or assembled *de novo* without the genomic sequence to produce a genome-scale transcription map that consists of both the transcriptional structure and/or level of expression for each gene(17). To investigate associations of nucleotide polymorphisms to complex diseases and traits, several studies have shown that gene expression analyses provide unbiased way to investigate complex traits and pathogenesis of common disorders'. Whole-transcriptome analysis therefore acquiring a key role in understanding the mechanisms responsible for complex diseases. Hybridization- and tag-based technologies have elucidated the involvement of multiple genes and pathways in pathological conditions, providing insights into the expression of thousand of coding and noncoding RNAs, such as microRNAs. RNA-Seq, can identify, in a single experiment, potentially novel

genes/exons and splice isoforms, RNA editing, fusion transcripts and allele-specific expression in normal and disease tissues. RNA-Seq has been fruitfully applied to study cancer, host-pathogens interactions, neurodegenerative diseases (ND) as well as neuropsychiatric diseases and cancer.

METHYLATION SEQUENCING

A growing body of evidence has suggested that DNA methylation patterns can be modulated by environmental factors (18,19). Both genome-wide analysis and targeted approaches can provide insight into methylation patterns at a single nucleotide level. Until a decade ago, DNA methylation studies only focused on small regions of the genome because of technical limitations. Recent advances in DNA sequencing technology has made it possible to construct single-base resolution maps of 5-methylcytosine (5mC) at the genome-wide scale (20). The technology is collectively called whole-genome bisulfite sequencing (WGBS) or methylome analysis. DNA methylation involves the addition of a methyl or hydroxymethyl group to bases in the DNA sequence. The most commonly studied modification is the methylation of the C5 position on cysteine bases, or m5c. DNA methylation can be measured using chromatin immunoprecipitation (ChIP) or bisulfite-based methods.

ChIP-based methods use methylation-specific antibodies to purify methylated regions of the genome. The DNA is then analysed by microarray or NGS to identify these regions (21). Bisulfite-based methods involve bisulfite treatment of the DNA sample, which converts unmethylated cytosine bases to a uracil base, while leaving methylated residues as a cytosine. The treated DNA is then analysed by microarray or NGS. This method can only detect types of methylation that are susceptible to bisulfite-induced changes. For NGS, the treated and untreated samples are sequenced and compared to identify specific methylation sites within the genome. Bisulfite sequencing requires a well annotated genome. Aberrant DNA methylation can cause a number of human diseases such as developmental diseases (ICF syndrome, Prader-Willi and Angelman syndromes etc), aging related diseases (i.e. Alzheimer's disease), heart disease, diabetes, and autoimmune diseases and cancer(22,23,24,25,26,27) . Therefore, it is very important to understand how methylation patterns are established and maintained during normal development and under pathological conditions.

WGBS-seq has been successfully used to map the complete methylomes of several human embryonic stem (ES) cell lines(28,29), human peripheral mononuclear cells(30) and hematopoietic progenitor cells several cancer samples have been completed(32,33,34) at the single methylcytosine resolution(31). Genome-wide DNA methylation profiles using high-throughput platforms, such as methylation arrays, MeDIP, MIRA, and functional epigenomic approaches have been used for epigenetic profiling of many common cancers, including lung, breast, and colorectal cancer. WGBS is increasingly important in biology and medicine and the International Human Epigenome Consortium (IHEC) recommends WGBS as the standard method for DNA methylation analysis (http://ihec-epigenomes.org/.

SINGLE-CELL OMICS

Single-cell omics is the profiling/sequencing of single cells sampled from heterogeneous population of cells from different cellular states, where the normal development and disease processes can be studied and dissected at a single-cell resolution. The fast-evolving next-generation sequencing technologies have enabled high-throughput multidimensional analyses of individual cells that will produce detailed knowledge of the cell lineage trees of higher organisms, including humans (35). The molecular term omics implies a comprehensive, or global, assessment of a set of molecules (http://omics.org/) (36). The majority of functional genomics studies are currently based on the analysis of multiomics layers, which are termed genomics, transcriptomics, epigenomics, and proteomics. A cell's state is determined by the complex interplay of these multiomics layers (35). Sequencing of genome, transcriptome, epigenome and the proteome has revolutionized genomic studies by speeding up the discovery of and simultaneously recording the identity as well as the function of a cell. Multiomics investigation of single cells has advanced our understanding of normal developmental processes as well as disease processes. Single-cell RNA sequencing has become an indispensable tool in biological discovery and its downstream applications in medicine to design tailored therapies. A comprehensive cellular atlas utilizing single-cell omics has already been created for breast epithelium, paving the way for similar blueprints of other tissues in the future. This comprehensive atlas will form the foundation for understanding the cellular level perturbations that lead to breast cancer process as well as other cell types in future.

CRISPR-CAS TECHNOLOGY

CRISPR-Cas9 was first is a gene editing tool developed in 2012 (37). using the CRISPR technology scientists are able to edit any genetic mutation at will, curing the disease it causes. The first

CRISPR trial in Europe and the US, enrolled its first patient in February this year, aims to treat beta-thalassemia and sickle cell disease, two blood disorders that affect oxygen transport in the blood with FDA approval. In another study Hangzhou Cancer Hospital starts with the extraction of immune T cells from the patient. Using CRISPR, the cells are modified to remove the gene that encodes for a protein called PD-1 some tumors are able to bind to this protein on the surface of immune cell and instruct them not to attack. The modified cells are then re-infused into the patient with a higher capacity to attack cancer cells. CRISPR therapy is also being used for Leber congenital amaurosis, which is the most common cause of inherited childhood blindness, for which there is no treatment. The CRISPR technology is used to target the most frequent mutation behind the disease, to restore the function of light-sensitive cells before the children lose sight completely. In another experiment scientists are treating Huntington's disease with a newly developed KamiCas9, a version of CRISPR-Cas9 that includes a "self-destruct button" with an enzyme called nickase to make the gene editing more precise. In practice, we are just at the beginning of the development of CRISPR as a therapy and there are still many unknowns.

CONCLUSIONS

The impact of genome sequencing on clinical care is observed in the fields of oncology, pharmacology, rare and undiagnosed diseases, and infectious disease. New discoveries are very rapidly translated into patient care especially in the field of oncology. Genomics is incorporated into diagnostics for identifying genetic and genomic markers which are included in cancer screening, and to guide tailored treatment for cancer patients. **Predictive testing** is for those who have a family member with a genetic disorder. The results help to determine a person's risk of developing the specific disorder and can be done before any symptoms present themselves. **Diagnostic genetic testing** is used to confirm or rule out a suspected genetic disorder. The results of a diagnostic test may help you make choices about how to treat a specific disorder based on the genetic profile of the individual. Some of the examples of targeted therapies developed in cancer by these new technologies are imatinib mesylate targets the BCR-ABL fusion protein, larotrectinib *NTRK* fusion for metastatic cancers, herceptin forHER2 positive for breast cancer, epidermal growth factor receptor (EGFR) colon cancer, crizotinib for alk-eml4 translocation in lung cancer etc. Patients with *KRAS* mutation do not respond to targeted therapies such as cetuximab (Erbitux) and panitumumab (Vectibix) and genetic testing in these patients will help in avoiding unnecessary treatment.

Pharmacogenomic testing is an emerging field which provides information about how an individual responds to a particular medication, and adjust the dosage as well as avoid subsequent side effects. According to the National Academy of Sciences post HGP physicians are able to provide personalized medical care based on the genome of the individual patient. A new field termed 'precision medicine,' is emerging where genomics, epigenomics, environmental exposure, and other data would be used to more accurately guide individual diagnosis and treatment.

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CRISPR/CAS9 – A VERSATILE TOOL FOR GENOME ENGINEERING

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ABSTRACT

The CRISPR/Cas (clustered regularly interspaced short palindromic repeats/CRISPR-associated proteins) system, first identified in bacteria and archaea, can degrade exogenous DNA. It was developed as a gene editing technology in 2013.Over the subsequent years; it has received extensive attention owing to its easy manipulation, high efficiency, and wide application. The process of CRISPR/Cas is optimized constantly and its application has also expanded dramatically.Compared with previously developed gene editing tools zinc finger nucleases (ZFNs) and transcription activator–like effector nucleases (TALENs) CRISPR/Cas is more efficient and it can edit multiple target genes simultaneously. Therefore, CRISPR/Cas is considered a revolutionary technology in biology. and has rapidly developed and successfully applied to alter metabolic pathways and improve crop quality and drug development via gene mutation, gene silencing, and transcriptional regulation. The applications of type II CRISPR have had a tremendous impact on bioengineering and molecular biology; however, scientists are still searching for more flexible and applicable CRISPR-derived systems to apply to molecular biology research. CRISPR/Cas9 system can also be used as programmable antibiotics to kill the bacteria sequence specifically and therefore can bypass multidrug resistance. Furthermore, CRISPR/Cas9 based gene drive may also hold the potential to limit the spread of vector borne diseases. What the future holds with CRSIPR/Cas9 is both fascinating and intriguing, however much further research is necessary to overcome the shortcomings at hand, to tackle any possible adverse effects on humans, and the ethical aspects of such experiments must not be overlooked.

KEYWORDS:CRISPR/Cas9,ToolforGenomeEngineering,applicationsgenetics,human, diseaese,

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INTRODUCTION

Clustered regularly interspersed short palindromic repeats (CRISPR) were first discovered in *E. coli* in 1987 (Ishino et al. 1987) and later in many other bacterial species (Kampmann 2018). For several years, the role of the short repeat sequences remained obscure. In 2005, the CRISPR spacer sequence was found to be highly homologous with exogenous sequences from bacterial plasmids and phages(Pourcel et al. 2005;Mojica et al. 2005; Bolotin et al. 2005) proposed the hypothesis that these sequences are part of an adaptive immune system in bacteria. As a result of this homology between host and exogenous substances, CRISPR is able to cleave foreign DNA. Notably, the vital site-specific gene editing tool called the CRISPR/Cas system was developed in 2013. CRISPR/Cas only requires a short guideRNA sequence to recognize the target loci according to Watson–Crick base pairing. These studies have been to extended to experimentally demonstrate that CRISPR and its CRISPR-associated proteins (Cas) are linked to the adaptive immunity targeting foreign viral DNA (Barrangou et al 2007). The discovery that a single-guide RNA (sgRNA) could take the place of the crRNA and the tracrRNA further simplified the use of the CRISPRCas9 system such that only one protein and one RNA molecule are needed to achieve RNA- programmed DNA cleavage (Jinek et al. 2012).In 2013 the type II Cas protein from *Streptococcus pyogenes* (SpCas9) was used for RNA guided DNA cleavage in mammalian cells for the first time, laying the basis for using CRISPR/Cas9 as a widely applicable genome-editing tool (Cong et al. 2013; Mali et al. 2013) (Table 1).

Table 1 Comparison of CRISPR/Cas9, ZFNs, TALENs, and RNAi

	CRISPR/Cas9	**ZFNs**	**TALENs**	**RNAi**
Target site	19-22 bp	18-36 bp	24-40 bp	Target site should be located 50–100 nt from ATG
Retargeting possibility	Easily retargeted without any complexity	Yes, but requires complex molecular cloning	Yes, but requires protein engineering	Yes
Nuclease	Cas9	FokI	FokI	Dicer and Argonaute proteins
Recognition mechanism	RNA-DNA	Protein-DNA	Protein-DNA	RNA

Targeting restrictions	Protospacer adjacent motif (PAM) must be present	Non-G rich sequences are difficult to target	T in the start and A at the end	Only targets mRNA
Efficiency	High	High	High	High
Limitations	Off targets	Both expensive and time consuming to construct	Takes long to construct	Off targets
Cytotoxicity	Low	Low	Variable to high	Variable to high
Multiplexing ease	High	Low	Low	High
Cost	Low	High	Moderate	low

GENE EDITING TOOLS

Genomes of eukaryotic organisms are composed of billions of DNA bases. The ability to change these DNA bases at precisely predetermined locations holds tremendous value not only for molecular biology, but also for medicine and biotechnology. Therefore, introducing desired changes into genomes, i.e., "genome editing", has been a long sought-after goal in molecular biology. Genome editing is not a new concept to the scientific community and has been around for decades. However, directing precise sequence changes at desired sites has remained a difficult and tedious challenge for researchers. Limited successes have been achieved with oligonucleotides. The ability to manipulate the genome in a precise and targeted fashion is crucial for understanding genetic contributions to biology and disease. Genome engineering of cell lines or animal models has traditionally been accomplished through random mutagenesis or low-efficiency gene targeting. To facilitate genome editing, programmable sequence-specific DNA nuclease technologies have enabled targeted modification of endogenous genomic sequences with high efficiency, particularly in species that have proven traditionally genetically intractable (Carlson et al. 2012; Geurts et al. 2009; Takasu et al.2010; Watanabe et al. 2012).

Before the establishment of RNA-guided engineered nucleases such as the CRISPR/Cas9, programmable DNA-binding nucleases such as zinc finger nucleases (ZNFs) and transcription activator-like effector nucleases (TALENs) were used to edit DNA. However, engineering such sequence-specific DNA-binding proteins was time-consuming and challenging, which significantly hampered the widespread usage of these techniques. Compared to these tools, CRISPR/Cas is more efficient and it can edit multiple target genes simultaneously. Based on these advantages, applications of CRISPR/Cas are rapidly developing. The ZFN and TALEN gene editing tools search valid sequences with proteins, while CRISPR/Cas depends on guideRNA (gRNA) (Table 2). Recently, a new genome editing technology was developed called NgAgo,

which is applicable for editing genes in human cells with the DNA-mediated NgAgo endonuclease (Gao et al. 2016). To date, only one study using NgAgo has been published by Qi et al (2016). The authors reported that gDNA/NgAgo led to a gene knockdown that resulted in an abnormal phenotype in zebra fish, but no gene mutation could be detected. Unfortunately, other groups have not successfully repeated the utilization of NgAgo for genome editing, and therefore NgAgo is still a topic of discussion in the field. Another technology is RNA interference (RNAi), which has also been used to some extent for gene expression modification. But, this technique has certain limitations. The effects of RNAi are generally non-specific, temporary and the technique is restricted to the knocking down of only transcribed genes (Zhang et al. 2013). Gene editing technologies are developing rapidly, including those using the CRISPR/Cas system. Foreseeably, gene editing technologies will have an impact on the progress of medicine, agriculture, and other scientific fields because it will allow for direct and fast genetic modifications of model systems used in these fields (Liu et al. 2017)

Table 2: Milestones in the history of CRISPR research

S. No	Year	Research outcome
1	1987	Ishino *et al*. first describe a pattern of short, palindromic repeats of DNA interspersed with short, non-repetitive "spacers" of DNA in *E.coli* bacteria.
2	2002	Jansen *et al*. name the pattern CRISPR, short for "clustered regularly interspaced short palindromic repeats".
3	2005	Spacer sequences were found to be originated from phage genomes
3	2007	Barrangou *et al*. show that CRISPR, mediated by Cas proteins, provides bacterial immunity against viruses by matching DNA in spacer sequences with DNA from viruses.
4	2010	Garneau *et al*. show that the CRISPR/Cas system can acquire new spacers from foreign DNA
5	2011	TracrRNA and crRNA were combined into a single guide RNA (gRNA)
6	2012	Jinek, Doudna, Charpentier *et al*. develop CRISPR/Cas9, which can be programmed to recognize and target any DNA sequence.
7	2013	• Cong, Zhang *et al*. show that CRISPR/Cas9 can precisely edit DNA in human & mouse cells, and that a single CRISPR/Cas9 array can be programmed to edit several sites at once. • Tan *et al*. use CRISPR/Cas9 in pig, goat and cattle cells. • Ran, Zhang *et al*. report that a technique called "double nicking" which breaks both strands of DNA, can reduce CRISPR/Cas9 off-targeting by 50-to 1,500-fold. • Scientists use CRISPR/Cas9 to modify the genome of silkworm and frog embryos.

8	2014	• Fu, Sander *et al*. report that using truncated guide RNAs can reduce CRISPR/Cas9 off-targeting by 5,000-fold or more. • Shalem, Zhang *et al*. use CRISPR/Cas9 for genome-scale screening of cancer-related genes in human cells. • Niu *et al*. report the birth of twin monkeys that have been genetically engineered with CRISPR/Cas9. • Hu, Khalili *et al*. use CRISPR/Cas9 to eradicate HIV from human immune cell lines.
9	2015	• Wu *et al*. use CRISPR/Cas9 to correct genetic disease in mice germ cells. • Scientists publish editorials in Nature and Science calling for a pause on researching clinical applications of CRISPR/Cas9 in human reproductive cells. • Hilton *et al*. create a CRISPR/Cas9-based system that can edit the epigenome, a set of chemical "switches" that can turn genes on and off. • Liang et al. report that they have used CRISPR/Cas9 to gene-edit non-viable human embryos with limited success.
10	2016	• CRISPR-associated DNA-cleaving enzyme Cpf1 also processes precursor CRISPR RNA (Fontara *et al*. 2016). • Cas9 variants with altered with PAM sequence specificity (Hirano *et al*. 2016). • Targeted gene manipulation in plants using the CRISPR/Cas9 technology (Zhang *et al*. 2016). • In vitro gene editing in dystrophic mouse muscle and muscle cells (Tabebordbar *et al*. 2016)
11	2017	Optimization of the production of knock-in alleles by CRISPR/Cas9 microinjection into the mouse zygote (Raveux *et al*. 2017)
12	2018	RNA dependent RNA targeting by CRISPR/Cas9 (Strutt *et al*. 2018)
13	2019	Chinese scientist He Jiankui and his colleagues claimed HIV resistant twin babies borne through manipulation of embryo by CRISPR technology

BIOLOGICAL FUNCTIONS OF THE CRISPR-CAS SYSTEM

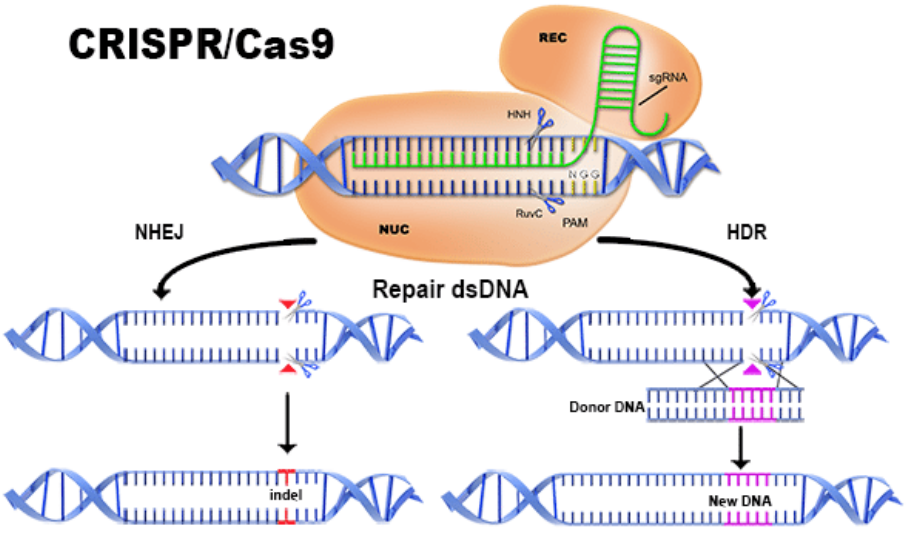
Immunity function and endogenous regulation: The combination of CRISPR sequences and Cas proteins provides bacteria with their own adaptive immune system which can resist interference from external nucleic acid. Further, the CRISPR-Cas system plays a crucial role in endogenous regulation of gene expression. A previous study suggested that the CRISPR-Cas system was associated with lysogenic modifications of *Pseudomonas aeruginosa*, including reduced biofilm formation. Any reference

Genome evolution and DNA repair: The CRISPR-Cas system also has positive effects on genome evolution by self-targeting. CRISPR-Cas-mediated DNA cleavage can potentially lead to large-scale genome rearrangements. Previous research has shown that the repetitive CRISPR sequences play active roles in the process of genome rearrangements. In other words, the

CRISPR system is related to evolution. Furthermore, this system not only defends against exogenous elements and viral infections, but also has DNA repair functions.

CRISPR TECHNOLOGY

CRISPRs belong to a class of repeated DNA sequences that work together with CRISPR-associated (Cas) genes to protect bacteria and archaea from invading foreign nucleotides, such as those from phages and plasmid DNA. Upon injection of phage-DNA into the bacteria, the phage-DNA is cut into small pieces and incorporated into the CRISPR locus. This locus, now containing information about the foreign phage-DNA is transcribed; this CRISPR-RNA (crRNA) along with a second non-coding RNA, trans-activating CRISPR RNA (tracrRNA), then forms a ribonucleoprotein complex with the Cas9 endonuclease and cuts the foreign DNA from this specific phage. So, a bacteria carrying information about a specific phage-DNA can have immunity towards attack from that phage. In this way, bacteria and archaea have evolved an adaptive immune response against invading DNA and can protect themselves in future attacks by the same phage. This system that provides adaptive immunity against foreign elements in bacteria and archaea has been modified today to be enable to edit genes in various cell types (mammalian, plant, insect, fungi, etc). Researchers combined the crRNA and tracrRNA and generated a single guide RNA (sgRNA), which recruits the Cas9 nuclease to specific genomic locations with guidance from the sgRNA. sgRNA then pairs with the complimentary genomic sequences via Watson-Crick pairing and the Cas9 nuclease induces double strand breaks (DSB). The DSB can be corrected by an error-prone mechanism called, non-homologous end joining (NHEJ), which causes insertions or deletions. If a synthetic template is present, the DSB can also be corrected by homology-directed repair (HDR), allowing the introduction of desired base-changes into the genome (Fig.1).



TERMINOLOGY, DEFINITIONS OF COMPONENTS OF CRISPR COMPLEX

This is being provided to enable the reader to understabd and comprehend the subjcet

CRISPR array: The genetic locus of the CRISPR/Cas systems is called "CRISPR array"; the locus contains CRISPRs separated by variable short DNA sequences termed as "spacers". These spacers are preceded by leader sequence rich in AT. The sequence of DNA in the invading microbe possesses a sequence identical to the spacers; this foreign sequence is termed as "protospacer".

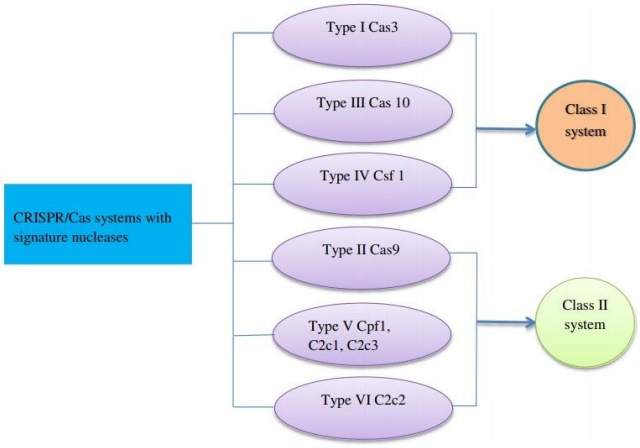
CRISPR: Clustered regularly interspersed short palindromic repeats (CRISPR) (~ 20–50 bp) first discovered in *E. coli* in 1987 and later on in many other bacterial species. These sequences interspersed by spacer sequences were found to be highly homologous with exogenous sequences from bacterial plasmids and phages.

Cas genes: These are conserved genes regularly present adjacent to the CRISPR regions. The genes were designated CRISPR-associated genes 1 through 4 (*cas1* to *cas4*).

Cas proteins: Coded by *Cas* genes. These are DNA endonuclease that can be derived from different bacteria.

dCas9: Nuclease-deficient Cas9

CRISPR/Cas system: prior to DSB, the gRNA makes a complex with specific Cas protein known as CRISPR/Cas system. There exists a great diversity among CRISPR/Cas systems given the range of foreign genetic elements. CRISPR/ Cas system has been classified into six main types and two main classes shown in Fig. 2. Type I-III is better understood, whereas types IV-VI have been identified recently. In the type I system the Cas-3 nuclease-helicase is involved, the type II system has the nuclease Cas9, while the type III systems possess the least understood Cas10. Type IV system possesses an uncharacterized protein Csf1. Type V systems contain either Cpf1, C2c1or C2c3, which are very much similar to Cas9. Type V1 contains a large protein C2c2. Class 1 system comprises of type I, III and IV and the class 2 system comprises of II, V and VI (Fig. 2).



Among different CRISPR/Cas systems, CRISPR/Cas9 most commonly used variant, with 1,368-residues from *Streptococcus pyogenes* (SpCas9) is the best understood and has wide applications due to its versatility.

crRNA: The CRISPR targeting (crRNA). Processed product of pre-crRNA, contains a single spacer

Pre-crRNA: A non-coding RNA transcribed from CRISPR array.

tracrRNA: The trans-activating RNA (tracrRNA), a non-coding RNA transcribed from a gene present upstream of CRISPR array and Cas loci.

gRNA: The sgRNA is a synthetic RNA with a length of about 100nt. Jinek et al. demonstrated that a single guideRNA (sgRNA) formed by fusing crRNA to tracrRNA plays the same role as a crRNA-tracrRNA hybrid. Its 50-end has a 20-nt sequence that acts as a guide sequence to identify the target sequence accompanied by a protospacer adjacent motif (PAM) sequence, which is often the consensus NGG(N, any nucleotide; G, guanine).The design of sgRNA is one of the key factors in editing target genes successfully using CRISPR/Cas9. Until now, dozens of online tools and stand-alone software have been developed to devise efficient and specific sgRNA.

PAM: Protospacer-adjacent motif present adjacent to protospacer

PI: PAM-interacting domain of the Cas endonuclease required for PAM interrogation

Spacer, Protospacer: The variable short DNA sequences in a CRISPR array are termed as spacers. These spacers are preceded by leader sequence rich in AT. The sequence of DNA in the invading microbe possesses a sequence identical to the spacers; this foreign sequence is termed asProtospacer.

NHEJ: Non-homologous end joining (NHEJ), a type of DNA repairing mechanism after double strand breaks by CASPR/Cas system.

HDR: Homology-directed repair (HDR), another type of DNA repairing mechanism after double strand breaks by CASPR/Cas system

INDELs: Insertions and deletions together simply known as INDELs

DSB: Double stranded breaks in DNA

HNH, RuvC: Domains of NUCCas9.HNH domain for cleaves the target DNA strand whereas the RuvC cleaves the complimentary strand.

RGN: RNA-guided nuclease

DIVERSITY AND CLASSIFICATION OF CRISPR-CAS

It is striking to note that closely related strains can vary considerably in their CRISPR content and distribution. For example, in the *Mycobacterium* genus, CRISPR exists in M. tuberculosis but not in *M. leprae*. On the other hand, phylogenetically distant *E. coli* and *Mycobacterium avium* as well as *Methanothermobacter thermautotrophicus* and *Archaeoglobus fulgidus* carry nearly identical CRISPR sequences (Jansen et al. 2002). The number of CRISPR arrays in one genome varies from 1 to 18, and the number of repeat units in one CRISPR array varies from 2 to 374 (Marraffini et al. 2010).Based on the CRISPR database (http://crispr.i2bc.paris-saclay.fr), as of May 2017, CRISPRs were identified in the whole-genome sequences of 202 (87%) out of 232 analyzed archaeal species and 3,059 (45%) out of 6,782 bacterial species. Interestingly, a survey of 1,724 draft genomes suggested that CRISPR-Cas systems are much less prevalent in environmental microbial communities (10.4% in bacteria and 10.1% in archaea). This large difference between the prevalence estimated from complete genomes of cultivated microbes compared to that of the uncultivated ones was attributed to the lack of CRISPR-Cas systems across major bacterial lineages that have no cultivated representatives (Burstein et al. 2016).

As shown in Fig. 2, the latest classification of CRISPR-Cas systems includes two classes, 1 and 2, based on the encoded effector proteins (Shmakov et al. 2017). Class 1 CRISPR-Cas systems work

with multisubunit effector complexes consisting of 4 to 7 Cas proteins present in an uneven stoichiometry. This system is widespread in bacteria and archaea, including in all hyperthermophiles, comprising 90% of all identified CRISPR-cas loci. The remaining 10% belongs to class 2, which use a single multidomain effector protein and are found almost exclusively in bacteria (Burstein et al. 2017).

Each class currently includes three types, namely, types I, III, and IV in class 1 and types II, V, and VI in class 2. Types I, II, and III are readily distinguishable by virtue of the presence of unique signature proteins: Cas3 for type I, Cas9 for type II, and Cas10 for type III. The multimeric effector complexes of type I and type III systems, known as the CRISPR-associated complex for antiviral defense (Cascade) and the Csm/Cmr complexes, respectively, are architecturally similar and evolutionarily related (Rouillon et al.2013;Jackson and Wiedenheft 2015; Makarova et al. 2011, 2015;Koonin and Makarova 2013;Koonin et al 2017; Venclovas 2016). Unlike all other known CRISPR-Cas systems, the functionally uncharacterized type IV systems do not contain the adaptation module consisting of nucleases Cas1 and Cas2 (Rouillon et al. 2013; Makarova et al. 2015). Notably, the effector modules of subtype III-B systems are known to utilize spacers produced by type I systems, testifying to the modularity of the CRISPR-Cas systems (Garrett et al 2011). Although many of the genomes encoding type IV systems do not carry identifiable CRISPR loci, it is not excluded that type IV systems, similar to subtype III-B systems, use crRNAs from different CRISPR arrays once these become available (Makarova et al. 2015). Finally, each type is classified into multiple subtypes (I-A to F and U and III-A to D in class 1; II-A to C, V-A to E and U, and VI-A to C in class 2) based on additional signature genes and characteristic gene arrangements (Shmakov et al. 2017; Koonin et al. 2017). While all CRISPR types have in common the use of RNA-guided ribonucleoprotein complexes that recognize foreign invaders to trigger host defense, recent work has uncovered additional features for some of these types like, (i) collateral, nonspecific, cleavage of host nucleic acids; (ii) secondary messengers that amplify the immune response; and (iii) immunosuppression of CRISPR targeting by phage-encoded inhibitors (Varble and Marraffini 2019)

CLASS 2 SYSTEMS ARE SUITABLE FOR GENOME EDITING TECHNOLOGY

The simple architecture of the effector complexes has made class 2 CRISPR-Cas systems an attractive choice for developing a new generation of genome editing technologies. Several distinct class 2 effectors have been reported, includingCas9 in type II, Cas12a (formerly Cpf1), Cas12b (C2c1) in type V, and Cas13a (C2c2) and Cas13b (C2c3) in type VI. The most common and best studied multidomain effector protein is Cas9, a crRNA-dependent endonuclease, consisting of two unrelated nuclease domains, RuvC and HNH, which are responsible for cleavage of the displaced (nontarget) and target DNA strands, respectively, in the crRNA-target DNA complex. Type II CRISPR-cas loci also encode a trans-activating crRNA (tracrRNA) which might have evolved from the corresponding CRISPR. The tracrRNA molecule is also essential for pre-crRNA processing and target recognition in the type II systems. The molecular mechanism

of the target DNA cleavage by the Cas9-crRNA complex has been elucidated at the atomic level by the crystal structure analysis of the DNA-Cas9-crRNA complex (Nishimasu et al. 2014).

THE CAS9 NUCLEASE: THE STRUCTURE AND THE WORKING MECHANISM

The CRISPR/Cas9 system achieves its sequence specificity by the special structure and conformation of the Cas9 protein. The Cas9 protein contains a conserved core and a bi-lobed architecture including adjacent active sites and two nucleic acid binding grooves: a large recognition (REC) lobe and a small nuclease (NUC) lobe that are connected by a helix bridge (Anders et al. 2014; Jinek et al.2014; Nishimasu et al. 2014). REC determines the Cas9-specific function, whereas the NUC incorporates two nuclease domains, RuvC and HNH, and a protospacer-adjacent motif (PAM)-interacting domain (PI). Under natural conditions, Cas9 is inactive. It is activated when combined with the sgRNA at its REC lobe. The Cas9-sgRNA complex scans a DNA double strand for rigorous PAMs (the trinucleotide NGG) using Watson–Crick pairing between sgRNAand targeted DNA. Once anchored at the proper PAMs, the HNH nuclease domain cleaves the RNA–DNA hybrid, while RuvC cleaves the other strand to form a double-strand break (DSB).

DSBs can be repaired by non-homologous end joining (NHEJ) and homology-directed repair (HDR) mechanisms that are endogenous to both prokaryotes and eukaryotes (Puchta 2005). NHEJ employs DNA ligase IV to re-join the broken ends, an operation that can introduce insertion or deletion mutations (indels), whereas HDR repairs the DSBs based on a homologous complementary template and often results in a perfect repair. NHEJ is, therefore, characterized as error-prone. It can occur at any phase of the cell cycle and is the primary cellular DSB repair mechanism. In contrast, HDR uses a homologous repair template to precisely repair the DSB. HDR typically occurs in late S- or G2-phase, when a sister chromatid can serve as the repair template. In general, the incidence of HDR for DSB repair is extremely low compared with NHEJ, at least in instances where both pathways are equally available to an organism. Given the significant gene editing enabled by HDR, development of methods to increase the incidence/efficiency of HDR for gene editing with site-specific nucleases is an active field of research. The error-prone NHEJ has advantages for gene knock-out.HDR is used for gene replacement and gene knock-in in plants (Shukla et al. 2009; Schiml et al. 2014).

The use of site-specific nucleases and NHEJ or HDR generally results in one of four gene editing products. These include gene knockout, deletion, correction, or addition. The error-prone character of NHEJ can be exploited to introduce indels and frame shifts into the coding regions of a gene. This knocks the gene out via nonsense-mediated decay of the mRNA transcript. In gene deletion, paired nucleases excise regions of the coding gene, resulting in premature truncation and knockout of the protein in a manner more generally efficient than introducing frameshifts. Both gene correction and gene addition require an exogenous DNA template that can be introduced as either single-stranded (Radecke et al., 2010; Chen et al., 2011; Soldner et al., 2011) or double-stranded DNA (Rouet et al. 1994). The DNA template contains homologous sequence arms that flank the region containing the desired mutation or gene cassette.

SOFTWARE AND EXPERIMENTAL TOOLS FOR SGRNA DESIGN

The use of CRISPR/Cas9 as a genome editing tool rapidly spread across the scientific community. This distribution of knowledge was supported by the development of open bioinformatics resources for the design and analysis of CRISPR-associated experiments. Hundreds of online methods are available for CRISPR/Cas9 gene editing, construct designing with double stranded breaks, single stranded breaks, functional knockouts, plasmid with active gene expression, repress gene expression, tagging protein, finding target sequences and many others(Table 3). The algorithms that determine sgRNA on-target efficiency are derived from large screening data and integrate both the nucleotide composition of the binding site as well as its relative position within the gene model. Furthermore, for the design of custom sgRNA libraries and the analysis of pooled CRISPR/Cas9 screens, specific web-based and local bioinformatics solutions have been developed. In addition, miscellaneous online resources are available for the designing of sgRNAs which provide information about OTs without limiting the PAM or number of mismatch bases, for finding potential off targets in any genome, identification and ranking all sgRNA targets sites according to off target quality, help in inquiry of guide sequences (Sanjana et al. 2014).

One problem with the CRISPR/Cas9 system is that many sgRNAs have low efficiency. This leads to evaluation of an excessive number of sgRNAs for a particular experiment thus slowing the progress. Development of software to design potent and specific sgRNAs has therefore become an important area of research for the success story of CRISPR/Cas9 technology. However, it became clear that even for a single species, there is no single tool that can satisfy all the requirements of users because these tools are highly multidimensional in their features, as arethe experimental purposes and technical requirements of their users (Prykhozhij et al. 2016)

Table 3 Software for the design and analysis of CRISPR experiments.

Software for the design of single sgRNAs			
Software	**Platform**	**Organism**	**URL**
E-CRISP	Web-based	Multiple	http://www.e-crisp.org/
CRISP Design Tool	Web-based	Multiple	http://crispr.mit.edu/
CHOPCHOP v2	Web-based	Multiple	http://chopchop.cbu.uib.no/
Protospacer Workbench	Offline / graphical interface	Multiple	www.protospacer.com
CRISPRscan	Web-based	Multiple	http://www.crisprscan.org/
sgRNA Scorer 1.0	Web-based	Human	http://crispr.med.harvard.edu/sgRNAScorer

Software for design of sgRNA libraries			
Software	Platform	Organism	URL
caRpools	R/Bioconductor	/	http://github.com/boutroslab/caRpools
MAGeCK-VISPR	Offline/graphical interface	/	https://bitbucket.org/liulab/mageck-vispr/
ScreenBEAM	Offline/graphical interface	/	https://github.com/jyyu/ScreenBEAM
CRISPRanalyzer (CAR)	Web-based and Offline/graphical interface	/	http://www.crispr-analyzer.org http://www.github.com/boutroslab/CRISPRAnalyzeR

CRISPR/CAS9 RESOURCES

A large number of resources have been developed which are being used for application in genome engineering, for identification of CRISPR target site and for selection of gRNA. Multiple online resources are available for commercially available kits/ plasmids and CRISPR/Cas9 construction, as few are mentioned in Table 4.

Numerous vectors are used for Cas9 according to the desired gene modification to be performed. The desired modifications include single strand break (SSB), double strand break (DSB), activation of gene expression, repression of gene expression and tagging of proteins knockout genes, these tools working so that any user can design construct with selectable marker, and different gene to be inserted according to their own demands. Many of them are freely accesible, but some are paid as well.

Table 4 Commercial available kits/plasmids and services for CRISPR/Cas9 construction

S No	Company Name	Web link
1	Addgene	www.addgene.org/crispr/guide/
2	Thermo Fisher Scientific	www.thermofisher.com/pk/en/home/life-science/genome-editing/geneart-crispr/crispr-libraries/lentiarray-crispr-libraries/lentiarray-cas9-lentivirus.html
3	ATUM	www.atum.bio/products/expression-vectors/mammalian?exp=5
4	Synthego	www.synthego.com/products/synthetic-sgrna/
5	GeneCopoeia	www.genecopoeia.com/product/transgenic-mouse/
6	Origene	http://www.origene.com/CRISPR-CAS9/

7	Clonetech	http://www.clontech.com/US/Products/Genome_Editing/CRISPR_Cas9/Resources/About_Guide-it_Kits
8	Sigma-Aldrich	https://www.sigmaaldrich.com/webapp/wcs/stores/servlet/LogonForm?storeId=11001
9	CHOPCHOP	https://chopchop.rc.fas.harvard.edu/
10	Active Motif	http://www.activemotif.com/catalog/1172/enchip

CRISPR: BEYOND GENE EDITING

In addition to site-specific gene editing, the DNA-binding properties of CRISPR/Cas9 may prove useful in other important applications. Qu et al. repurposed CRISPR into an RNA-guided platform for controlling gene expression by developing a catalytically-dead Cas9 enzyme (dCas9) that retained its capability to recognize and bind a target DNA sequence (Qi et al. 2013). Instead of cleaving the bound DNA, the dCas9 enzyme remained bound to the target DNA sequence, disrupting RNA polymerase or transcription factor binding. They showed that this system, termed CRISPR interference (CRISPRi), could repress expression of multiple genes simultaneously without altering the genome. They demonstrated this gene repression in both Escherichia coli and human cells.

The CRISPR system may also serve as a powerful tool for epigenetic studies, allowing for targeted manipulation of epigenetic markers to interrogate epigenetic and transcriptional control relationships. A fusion protein of dCas9 and acetyl transferase was developed by Hilton et al. (2015), catalyzing acetylation of histone H3 lysine 27 at target sites. They showed highly specific gene activation across the genome. Other epigenetic markers (e.g. methyl groups) may be modulated using this approach.

Inducible CRISPR systems were also developed. A photoactivated Cas9 was generated from a split of Cas9 fragments and photoinducible dimerization domains. In response to blue light, the CRISPR/Cas9 system performed gene sequence modification (Nihongaki et al. 2015). Editing activity was extinguished by removing the light source. A similar blue light activating system was developed for the epigenetic gene activator CRISPR system of Hilton et al. discussed above (Polstein and Gersbach 2015). Chemically induced CRISPRs have also been created.

The specific DNA binding function of CRISPR has also been repurposed to detect the location of genes within undisturbed nuclei of fixed cells (Deng et al., 2015) and living human cells (Ma et al. 2015). Termed CASFISH, dCas9 proteins were labelled with differently colored fluorophores and coordinated to a specific sgRNA. This allowed for multicolour detection of specific genomic loci with high spatial resolution and the assessment of DNA compaction.

Some recent cutting-edge work suggests that RNA with no PAM site can also be an active substrate for Cas9. Strutt and colleagues demonstrated that Cas9 subtypes II-A and II-C can recognize and cleave RNA in a directed manner utilizing RNA–RNA interactions independent of

the presence of a PAM site in the target RNA molecule (Strutt et al. 2018). This cleavage protected *E. coli* cells from infection with bacteriophage MS2 particles, suggesting that Cas9 can provide cellular defence against both DNA and RNA viruses. This exciting work allows for the possibility of direct RNA targeting via CRISPR/Cas9, further expanding the scope of the system for practical applications.

FACTORS AFFECTING EFFICACY OF THE CRISPR/CAS9 SYSTEM

While the CRISPR/Cas9 system has demonstrated great promise for site-specific gene editing and other applications, there are several factors that influence its efficacy which must be addressed. These factors include target DNA site selection, sgRNA design, off-target cutting, incidence/efficiency of HDR vs. NHEJ, Cas9 activity, and the method of delivery.

TARGET DNA SITE SELECTION AND SGRNA DESIGN

A great advantage of the CRISPR/Cas9 system is the ability to target any 23-bp sequence that contains a PAM motif on either strand of DNA. Cas9 proteins from other species are being characterized and found to have differing PAM sequences. This provides a great flexibility in target sequence selection, and this flexibility will increase as new Cas9 proteins with differing PAMs are identified. Additionally, directed evolution and structure-guided rational design has allowed for engineering of Cas9 variants with altered PAM sequence specificity (Kleinstiver et al. 2015; Anders et al. 2016; Hirano et al. 2016).

Nevertheless, reports from several groups have shown that target site selection and sgRNA design are not as simple as perhaps originally assumed. As mentioned previously, single and multiple-base mismatches can be tolerated, with mismatches more tolerated at greater distances from the PAM. One report suggests that CRISPR/Cas9 may be less specific than ZFNs or TALENs due to the relatively shorter targeting sequence (Cradick et al. 2013). This contrasts with many reports that show no detectable off-target cleavage from CRISPR/Cas9 editing, with off-target effects being guide-RNA-specific (Cho et al. 2014). Rational design of the sgRNA has, therefore, been the subject of a significant body of work resulting in many criteria and no simple rules. There are now many computational tools and software packages available that facilitate sgRNA design. However, caution is still needed, as shown in a recent study by Haeussler et al. (2016) that compared predictions from several sgRNA design tools with experimental results. The authors showed evidence of algorithmic over fitting and the importance of using a model trained on data from the same guide RNA expression system.

OFF-TARGET CUTTING

One of the drawbacks of the CASPR/Cas9 system is its off-target cutting. Efforts to improve specificity and reduce off-target cutting have resulted in the design of mutant Cas9 systems. One mutant system disrupts the Cas9 protein so that it introduces only single-stranded DNA nicks. The nickase CRISPR/Cas9 is then used as a pair with one Cas9 binding to the forward DNA sequence and another Cas9 binding to the reverse DNA sequence flanking the target site. Only when binding in this configuration is a DSB formed through cooperative nicks. Off-target cutting

results in only a single stranded nick that is repaired with simple DNA ligases. The use of this system in mammalian cells reduced off-target cutting by three orders of magnitude with little to no reduction in on-target cutting efficacy (Cho et al. 2014).

Another mutant Cas9 system, a fusion protein of inactive dCas9 and a FokI nuclease dimer was developed to increase specificity and reduce off-target cutting (Tsai et al 2014). This system also significantly increased the size of the gene editing tool and provided greater constraints on in vivo delivery approaches. Another reported mutant Cas9 systems designed to reduce off-target effects include fusions of Cas9 with ZFPs or TALEs that can target nearly any genomic locus with improved precision (Bolukbasi et al. 2015). Cas9 mutants have also been designed to reduce non-specific DNA contacts by weakening binding of the target DNA strand (Kleinstiver et al. 2016) or the non-target DNA strand (Slaymaker et al., 2016) to Cas9 while maintaining robust on-target cleavage.

INCIDENCE/EFFICIENCY OF HDR

The incidence of HDR-mediated DNA repair from DSBs is typically extremely low in mammalian cells. Even in the presence of donor template DNA, NHEJ is the more frequent repair mechanism observed from CRISPR/Cas9 editing (Maruyama et al. 2015). Several approaches have emerged to increase HDR efficiency and suppress NHEJ, including use of small molecular inhibitors of NHEJ, gene silencing, cell cycle synchronization, and use of cell lines deficient in NHEJ components (Lino et al). One of the most commonly used inhibitors, Scr7, targets the NHEJ component DNA ligase IV, and has been reported to increase efficiency of HDR from Cas9 editing by up to 19-fold (Vartakand Raghavan, 2015). While the use of Scr7 and other inhibitors have resulted in increased HDR-mediated gene editing efficiency, these inhibitors may have toxic effects on the host cells. Recent work to synchronize cells into late S and G2 phase, where HDR can occur,along with direct nucleofection of Cas9 ribonuclease complex, may prove a viable alternative to chemical suppressionof NHEJ (Lin et al. 2014).

CAS9 ACTIVITY

Several Cas9 proteins from different species have been identified and used for gene editing. Each has differing PAM sequences and variable activity. Thus, selection of a specific Cas9 ortholog may provide improved gene editing efficiency for a given target sequence and should be considered as part of gene editing system design. In addition to the inherent activity of a given Cas9 protein, other factors have been shown to influence activity. For gene editing in eukaryotic cells, Cas9 must translocate into the nucleus. In these systems, the nuclear location signal (NLS) is connected to the Cas9 protein. Increasing access to the NLS by adding a 32 amino acid spacer between the NLS and Cas9 was shown to increase DNA cleavage activity (Shen et al. 2013). Increasing the relative concentration of sgRNA to Cas9 protein was also shown in increase on-target cutting activity, presumably by ensuring all Cas9 proteins formed the active ribonucleoprotein complex (Kim et al. 2014).

Finally, in comparison with other enzymes, the activity of Cas9 is quite low, with a single turnover rate of 0.3–1.0 min_1 (Jinek et al. 2012). Once bound to the target DNA sequence,

displacement of Cas9 from the DNA strand, even after DSB formation is challenging. Thus, Cas9 is less like a catalytic enzyme and more like a single-shot actuator. While this characteristic may be useful in some instances, such as gene activation/inhibition or short-lived activity for gene editing with lower off-target effects, it may be undesirable for other applications where catalytic activity is useful.

CRISPR DELIVERY SYSTEMS

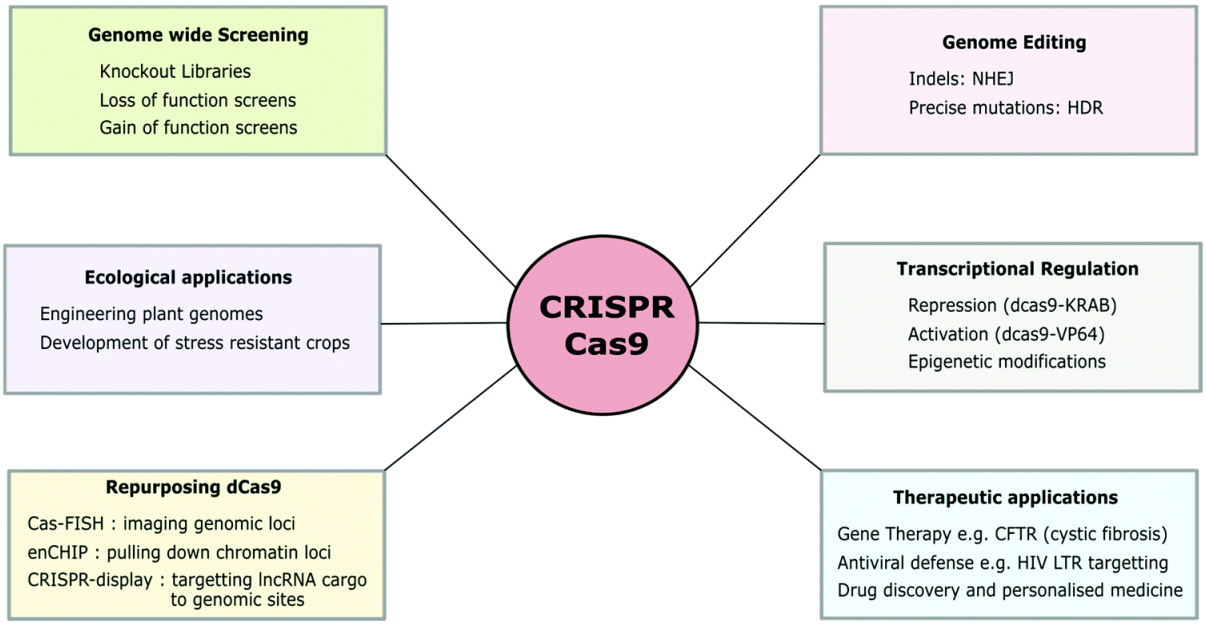
Delivery can be classified, into two categories: cargo and delivery vehicle. Regarding CRISPR/Cas9 cargoes, there are three approaches that are commonly reported: (i) DNA plasmid encoding both the Cas9 protein and the guide RNA, (ii) mRNA for Cas9 translation alongside a separate guide RNA, and (iii) Cas9 protein with guide RNA (ribonucleoprotein complex). The delivery vehicle used will often dictate which of these three cargos can be packaged, and whether the system is usable in vitro and/or in vivo. Vehicles used to deliver the gene editing system cargo can be classified into three general groups: physical delivery, viral vectors, and non-viral vectors. The most common physical delivery methods are microinjection and electroporation, while methods such as hydrodynamic delivery are currently under investigation. Viral delivery vectors include specifically engineered adeno-associated virus (AAV), and full-sized adenovirus and lentivirus vehicles. Especially for in vivo work, viral vectors have found favor by reserachers and are the most common CRISPR/Cas9 delivery vectors. Non-viral vector delivery is not as prominent as viral-based delivery; however, non-viral vectors possess several advantages over viral vectors and are a bourgeoning area of research. Non-viral vector systems include systems such as lipid nanoparticles, cell-penetrating peptides (CPPs), DNA 'nanoclews', and gold nanoparticles. There are additionally many delivery technologies that have not been demonstrated in the literature as suitable to CRISPR/ Cas9 delivery, though they appear to naturally lend themselves to the application. Four such technologies are streptolysin O, multifunctional envelope-type nanodevices (MENDs), lipid-coated mesoporous silica particles, and other inorganic nanoparticles.

ADVANTAGES OF CRISPR TECHNOLOGY

- The target can be designed simply and quickly, without having to construct endonuclease repeatedly.
- RNA-guided genomic DNA specific recognition needs no consideration of DNA methylation.
- Targeting operation can be highly efficient and flexible. Multiple genes can be edited at the same time and genetic modification can be genetic.
- CRISPR has the same or highly efficiency of gene editing comparing ZFN and TALEN.
- Knock-out and knock-in cell lines can be established.
- CRISPR/Cas9 lentivirus system can infect division and non-division cells.
- CRISPR can be applied to any species.

APPLICATIONS OF CRISPR TECHNOLOGY

The applications of type II CRISPR have had a tremendous impact on basic research, bioengineering and molecular biology, medicine and agriculture (Fig.3).



Scientists around the globe are working to find out the cure of various diseases such as heart disease, cancer, mental illness, HIV infection etc. Recently, FDA has approved the launch of a clinical trial of a drug made using the CRISPR/Cas9 technology named Edit-100, which is supposed to cure the common childhood blindness 'Leber Congenital Amaurosis type 10' as per press release of the Editas Medicine

Rapid generation of cellular and animal models: Cas9-mediated genome editing has enabled accelerated generation of transgenic models and expands biological research beyond traditional, genetically tractable animal model organisms.

Functional genomic screens: The efficiency of genome editing with Cas9 makes it possible to alter many targets in parallel, thereby enabling unbiased genome-wide functional screens to identify genes that play an important role in a phenotype of interest.

Transcriptional modulation: dCas9 binding alone to DNA elements may repress transcription by sterically hindering RNA polymerase machinery (Qi et al. 2013), likely by stalling transcriptional elongation. This CRISPR-based interference, or CRISPRi, works efficiently in prokaryotic genomes but is less effective in eukaryotic cells.

Epigenetic control: Cas9 epigenetic effectors (epiCas9s) that can artificially install or remove specific epigenetic marks at specific loci would serve as a more flexible platform to probe the causal effects of epigenetic modifications in shaping the regulatory networks of the genome.

Live imaging of the cellular genome: Fluorescently tagged Cas9 labelling of specific DNA loci was recently developed as a powerful live-cell-imaging alternative to DNA-FISH (Chen et al., 2013). Advances in orthogonal Cas9 proteins or modified sgRNAs will build out multi-color and

multi-locus capabilities to enhance the utility of CRISPR-based imaging for studying complex chromosomal architecture and nuclear organization.

Inducible regulation of Cas9 activity: By exploiting the bilobed structure of Cas9, it may be possible to split the protein into two units and control their reassembly via small-molecule or light-inducible heterodimeric domains.

Cas9 as a therapeutic molecule for treating genetic disorders: Although Cas9 has already been widely used as a research tool, a particularly exciting future direction is the development of Cas9 as a therapeutic technology for treating genetic disorders. Regarding the various genetic diseases, CRISPR/Cas9 technology can be used with ease to treat monogenic diseases; where a correction in the culprit gene could reverse the genetic disease. On the other hand, polygenic diseases are not so straightforward, having multiple mutations in the genome; they possess a far strenuous challenge to treat in comparison to monogenic diseases.A description about the use of CRISPR/Cas9 in the correction of genetic diseases is given in Table 5.

Table 5 Overview of gene correction of genetic diseases using CRISPR/Cas9

Genetic disease	Method of CRISPR/Cas9 delivery	Conclusion/outcome
Tyrosinemia	Tail vein hydrodynamic injection into adult mice	Correction of Fah gene mutation (1 nt substitution)
Hemophillia A	Transfection based delivery into iPSCs	Inversion based correction of the blood coagulation factor VIII (F8) gene
Hemophillia B	Tail vein hydrodynamic injection into Fah mice	Correction of mutation in F9 gene
Cataract	Injection into Oocyte of mouse	Correction in mutation of CRYGC gene (1 nt insertion)
Sickle cell anemia	Adenovirus based transduction into human IPSCs	Correction in sixth codon of beta globin gene
Beta Thalassemia	Transfection and piggyback removal in IPSCs from patients	HBB mutations corrected (1 nt substitution 4 nt insertion)
Cystic fibrosis	Transfection into intestinal stem cells from patients	Correction of CFTR gene mutation (3 substitution)

CRISPR/CAS9 IN DIRECT CANCER GENE THERAPY

Previous research has suggested the potential of CRISPR/ Cas9 in the treatment of cancer. The ability of cancer cells to develop resistance to chemotherapy drugs is a primary cause of failure of chemotherapy. The application of the CRISPR/Cas9 system to inactivate drug resistance genes in a given cancer is a potential therapeutic strategy to increase the efficacy of

chemotherapy. For cancer, until now, the role of CRISPR/Cas9 has been predominantly about the generation of cancer models in animals and cell lines (Table 6). These cancer models are and will be highly advantageous in understanding oncogenic pathways, new markers of cancer progression, identifying novel tumor suppressor genes, and will definitely provide an improved and efficient repertoire of strategies for cancer therapies. For instance, transcriptomic studies using CRISPR/Cas9 revealed a novel TSG "FOXA2" in pancreatic cancer, which was previously not known to function as a TSG (Vorvis et al. 2016).

Table 6: Role of CRISPR/Cas9 in Cancer modeling

Type of cancer	Method of CRISPR/Cas9 delivery	Conclusion
Pancreatic cancer	Transfection based multiplexed delivery into mice	Editing of multiple gene sets in pancreatic cells of mice
Acute myeloid cancer (AML)	Lentiviral based delivery into Hematopoirtic stem cells	Loss of function in nine targeted genes analogous to AML
Liver cancer	Hydrodynamic injection into wild type mice	Mutation in the Pten and p53 genes leading to liver cancer in mice
Breast cancer	Plasmid transfection into JygMC (mouse cell line)	The stem cell marker Cripto-1 was shown to be as a breast target
Pancreatic cancer	Lentivirus/Adenovirus based delivery into somatic pancreatic cells of mice	Knockout of gene Lkb1
Lung cancer	Plasmid transfection into human cell line (HEK 293)	Chromosomal rearrangement among EML4 and ALK genes
Lung cancer	Lentivirus/Adenovirus mediated	Gain of function of KRS and loss of function of p53 and Lkb1
Colon cancer	Plasmid transfection into DLD1 and HCT116 cell lines (human)	Loss of function in protein kinase c subgroups
Colorectal cancer	Electropolation into organoids intestinal epithelium (human)	Loss of function and directed mutation in APC, SMAD4, TP 53 and KRAS genes
Gliobastoma Medulloblastoma	Postnatal PEI-mediated transfection and in utero electroporation into mice	Deletion of TSGs (Ptch 1, Trp 53, Pten and Nf1)
Renal cancer	Renca (mouse cell line)	Knockout of TSG VHL to induce cancer

Radiotherapy has also been used in the treatment of cancer for a while. However, poor radiation sensitivity has been reported in tumors having mutations in the p53 and p21 genes. Correction of these mutations in the cancer cells and interruption of the cellular radiation injury repair pathway may be a potential alternative way to augment radio-sensitivity. A combination of radiotherapy and CRISPR/Cas9-mediated gene therapy with synergistic anticancer effects may become a promising therapeutic strategy for cancer therapy (Yi and Li, 2016). Another aspect of CRISPR/ Cas9 in cancer therapy is to enhance the host cells immune response to cancer. This could be possible through CRISPR/Cas9 mediated modification of T-cells. The reinfusion of genetically modified T-cells into cancer patients has shown promising results in clinical trials (Siggs 2014) and could be a way forward for anti-cancer therapies.

Another potential way to used CRISPR/Cas9 in cancer therapy could be the development of genetically engineered oncolytic viruses (OVs). These OVs have antitumor properties and can kill the cancer cells without causing any harm to the normal cells (Yuan et al 2016). The killing of the cancer cells takes place via virus-mediated cytotoxicity or by an increased anticancer immune response. CRISPR/ Cas9 can play an important role in oncolytic viral therapy by addition of cancer-specific promoter to genes that are indispensable for viral replication, and inducing mutations in viral genomes (Xiao-Jie et al. 2015). In both pre-clinical models and clinical trials promising results have been reported about the use of OVs in cancer therapy (Yuan et al. 2016). Recently, a research group has conducted clinical trials using CRISPR/Cas9 in a patient suffering from lung cancer. In this clinical trial, immune cells from the patient were removed and the Programmed death (PD-1) gene, which encodes for the protein PD-1 was disabled. This protein PD-1 is used by the cancerous cells to keep the host immune response in check. This is the first report of human trials using the CRISPR/Cas9 in clinical trials on human patients (Cyranoski 2016).

VIRAL DISEASES AND CRISPR/CAS9

The therapeutic challenge of viruses is captivating these obligate parasites rely on host metabolic machinery to replicate. It is a much arduous task to treat viruses as compare to bacteria due to their unique nature and machinery. Antiviral therapy targeting various viral proteins showed promising results, but anti-viral drug failure is becoming common, however, scientists have recently used the CRISPR/Cas9 phenomenon against a congregation of pathogenic viruses (Table 7).

Table 7. Overview of CRISPR/Cas9 in virus genome modification

Virus	Method of delivery of CRISPR/Cas9	Conclusion/outcome
HSV-1	Transfection into HEK293 cells	Modification of ICP0 gene in different locations of genome

EBV	Nucleofaction into Burkhitt's lymphoma cell line	Complete virus clearance in 25% cells, partial in 50%
EBV	Transfection into HEK 293-BX1 and C666–1 cells	Loss of BART Micro RNA expression
HPV	Lentiviral transduction into HELA and SiHA cell lines	Indel mutations in the E6 and E7 genes
HBV	Transfection in to Huh cells	Cleavage of the HBV genome-expressing template
HBV	Hydrodynamic injection into C57BL/6 mice	Cleavage of the HBV genome-expressing template
HBV	Transfection into HepG2 cell line	Fragmentation of viral genome
HBV	Lentiviral transduction into HepAD cell line (Chronic HBV infection)	Inhibition of viral DNA production
HIV	Lentiviral transduction into SupT1 CD4+ T cell line	Inactivation of virus and acceleration of virus escape
HIV	Lentiviral transduction into T-cells	Inhibition of early phase viral infection, but anti-HIV potency was not consistent in multiple rounds
HIV	Retro-orbital injection into transgenic mice	Decrease of viral gene expression in T-cells
Polyomavirus (JCV)	Transfection into TC 620 cell line	Inactivation of T-antigen gene

ALLERGY AND IMMUNOLOGICAL DISEASES

Scientists are using methodologies, which provide a critical analysis of the use of CRISPR/Cas9 as a treatment for allergic and immunological diseases. Single nucleotide polymorphisms (SNPs) are known to contribute to allergic diseases such as asthma and allergic rhinitis. The main emphasis of CRISPR/Cas9 in relation to allergic diseases has been about the investigation of the potential role of particular genes. Using the technology, certain gene knockout models can be created, which will provide an evaluation of the role of certain genes in allergic diseases and

immunological disorders. Moreover, CRISPR/Cas9 is rapidly becoming the primary tool to create mutant mouse models of diseases.

CRISPR/Cas9 possesses potential against allergic and Mendelian disorders of the immune system. Janus Kinase 3 (JAK 3) deficiency in humans is characterized by normal but poor functioning B-lymphocytes, and the absence of natural killer cells (NKs) and T-lymphocytes. For correction of this immunological disorder, CRISPR/Cas9 was used in induced pleuripotent stem cells. Correction of the JAK 3 mutation was made, resulting in restoration of normal T-lymphocyte development and number (Chang et al. 2015).

POTENTIAL OF CRISPR/CAS9 AS ANTIMICROBIALS

Another manner of using CRISPR/Cas9 could be putting it to use as an antimicrobial entity.Using CRISPR/Cas9 as an antimicrobial tool, Bikard et al. (2014) reported promising results that used a phagemid-based delivery of programmable, sequence-specific antimicrobials using the RNA-guided nuclease Cas9. The reprogrammed Cas9 only targeted the virulence genes of *Staphylococcus aureus* killing virulent strains, and did not kill avirulent strains. In a mouse skin model, the CRISPR/Cas9 antimicrobials showed extreme potential in killing of *Staph. aureus*. This technology creates opportunities to manipulate complex bacterial populations in a sequence-specific manner (Bikard et al. 2014).The true capability of CRISPR/Cas9 as an antimicrobial can be further exploited by developing delivery systems using phages that can help in the injection of cargo into diverse bacterial strains.

APPLICATIONS OF CRISPR/CAS9 IN PLANT GENOME EDITING

In plants, the application of CRISPR/Cas9 is just emerging. In Arabidopsis, a model plant, several genes including AtPDS3, AtFLS2, AtADH, AtFT, AtSPL4, and AtBRI1 are targeted with varying mutational efficiencies, from 1.1% to as high as 84.8%, in the first generation. These mutations are stably heritable across multiple generations with high percentages (up to 79.4%) (Feng et al.2014). A single CRISPR/Cas9 with two sgRNA expression cassettes has been developed to modify two genes (CHLOROPHYLL A OXYGENASE1 and LAZY1) simultaneously (Mao et al. 2013). In tobacco (Nicotiana benthamiana), CRISPR/ Cas9 has been coupled with VIGS (virus-induced gene silencing) technology, a transient expression system (Ali et al. 2015; Gao et al. 2015; Nekrasov et al. 2013), while in tomato, the knockout of ARGONAUTE 7 causes clear morphological changes in compound leaves [30]. Several cases have been successful in rice (Jiang et al 2013; Song et al. 2016). Successful examples have also been reported for several other crops with more complex genomes, such as sorghum (*Sorghum bicolor*), maize (*Zea mays*), citrus (*Citrus sinensis*), poplar (*Populustricocarpa*), tomato (*Solanum esculentum*) and wheat (*Triticum aestivum*) (Jiang et al. 2013; Liang et al. 2014; Svitashevet al. 2015; Jia and Wang 2014). Thus, the potential of CRISPR/Cas9 in crop genome editing and its applications are certain to be further developed over time.

Although the CRISPR/Cas9 can be applied to plant genome editing, there are still certain challenges, such as minimizing off- target rates, elucidating the precise mechanism for this minimization, and how to optimize Cas9 function. Further study is needed to improve the

experimental application of CRISPR/Cas9 to promote the development of its basic and applied abilities in the future.

GENE DRIVE AND CRISPR

A gene drive is a process by which an altered gene is introduced inside an animal population. The aim of gene drive is to get desired traits a population through natural reproduction alone. The use of novel gene drives resides in the use of CRISPR, the CRISPR technique has great potential in genome engineering. By using it scientists edit genes with precision, quickness, and economy, in addition it also has the potential of generating genetic alterations in wild animals that may persist in nature. So far, gene drives have been tested and evaluated only in laboratories, and the main emphasis of research has been on mosquitoes that transmit infectious diseases, as well as lab animals such as mice. The objectives are numerous however, some of the pivotal ones include control of the size of the population, or to suppress it completely, the last but not least is its use to combat against infectious diseases. Gene drives therefore have the potential to reduce the occurrence of, and possibly eradicate various infectious diseases by upsetting their transmission chains.

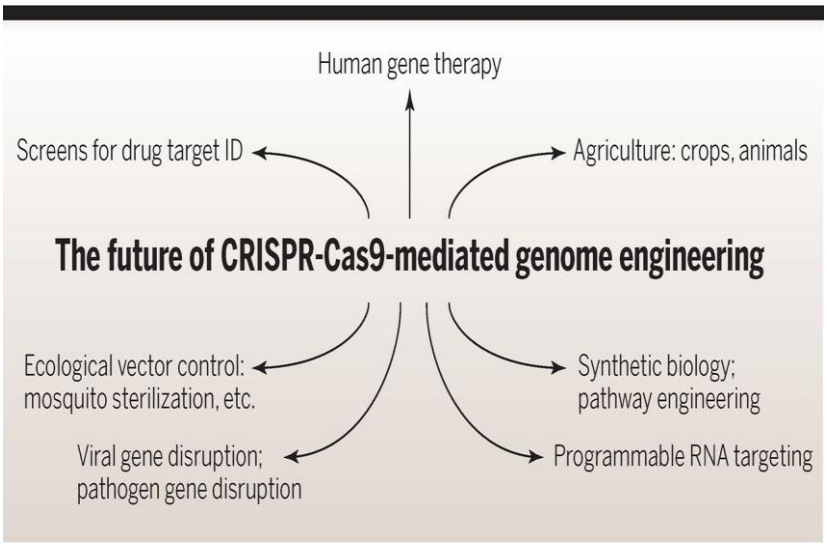
ETHICAL ISSUES- CRISPR TWINS

Although the CRISPR/Cas9 has brought increasing convenience and efficiency, several concerns regarding CRISPR/Cas9 ethical and biosafety have emerged. Recently, a paper reporting gene editing in human embryos was published in the journal Protein & Cell, which raised concerns about the ethics of employing the CRISPR/Cas9system (Liang et al. 2015). Thereafter, both the editorial team of Nature and Science announced that although the CRISPR/Cas9 system shows huge potential for genome editing, its use for modifying human germ line cells should be considered very seriously, and progressive policy on this issue should be developed (Baltimore et al., 2015).Thus, embryo editing or engineering of human foetuses is becoming increasingly controversial among scientists. Especially, some countries have already restricted CRISPR/Cas9 technology, with some completely banning its use in humans. To address such a complicated debate, positive and negative aspects of germline editing should be weighed by an authoritative national agency, and both the scientific and social ethical concerns should be taken into consideration simultaneously (Baltimore et al.2015).Due to the ethical concerns, many scientific journals declined to publish their work. An international summit was organized in response to these concerns. As part of the National Academy of Sciences and the National Academy of Medicine's Human Gene-Editing Initiative, a summit was co-hosted by the Chinese academy of Sciences and the U.K.'s Royal Society, taking place in Washington, D.C. on December 1–3, 2015.Experts from all around the world gathered to discuss the ethical, scientific, and regulatory issues associated withhuman genome-editing research and practice. Various countries banned the gene-editing in the human while few countries have do not taken any steps so far. However, recently, Chinese scientist from Southern University of Science and Technology in Shenzhen, China claimed that his team has successfully edited the gene in twin baby born and this inheritable gene-editing using CRISPR/Cas9 technology will prevent them

from HIV infection and also impact their coming generations too. There has been a huge hue and cry all over the world.

PROSPECTS OF CRISPR/CAS9

The CRISPR-Cas9 system can edit genomes accurately; it has been rapidly and widely applied to molecular biology and cell research (Fig.4).



However, due to the fact that the specificity of CRISPRCas9is determined by base pairing, a low targeting specificity can result in uncertain research results and the waste of manpower and material resources. Therefore, the biggest problem with the system is off-target effect. It affects the efficiency of the system, and can lead to a series of adverse consequences such as oncogene activation in the genome or other important mutations. For clinical applications and gene therapy in particular, off-target effects can cause great harm.55In addition, the plasmids needed for CRISPR-Cas9 editing are large, and there are difficulties with the transfection process. Furthermore, the gene deletion rate is low, and even if the vectors are successfully imported into the cells, a lack of effective screening methods is also a problem. In spite of the successful generation of gene-knockout cell lines, the biological functions of cell lines formed by different monoclonal cells may not be exactly the same. This may lead to poor reproducibility in subsequent experiments with such cell lines. Despite these problems, CRISPR-Cas9 technology has many advantages compared with other gene editing tools, such as simple operation, high targeting efficacy and robust sequence specificity. It has a broad range of applications in site-directed genome modification. The discovery and characterization of CRISPR systems have transformed genome editing and the life sciences. These technologies have armed researchers with powerful new tools to study living systems and human disease. The rapid progress in developing Cas9 into a set of tools for cell and molecular biology research has been remarkable, likely due to the simplicity, high efficiency and versatility of the system. Of the designer nuclease systems currently available for precision genome engineering, the CRISPR/Cas system is by far the most user-friendly. It is now also clear that Cas9's potential reaches beyond DNA

cleavage, and its usefulness for genome locus-specific recruitment of proteins will likely only be limited by our imagination. What the future holds with CRSIPR/Cas9 is both fascinating and intriguing, however much further research is necessary to overcome the shortcomings at hand, to tackle any possible adverse effects on humans, and the ethical aspects of such experiments must not be overlooked.

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Helicase-primase interactions in eubacteria: diverse sequences and unified structures

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Abstract

DNA replication is a highly dynamic process involving multiproteins complexes. Many replication proteins are either contain unstructured regions or highly flexible, making to be challenging for the structural studies of these proteins by X-ray crystallography or cryo-EM for their mechanistic details. The dynamics of the replisome complex are facilitated and controlled through the web of protein-protein interactions of diverse strengths. Here, we explored the structural insights into the two essential proteins involved in DNA replication; DnaG primase and DnaB helicase. The N-terminal domain of DnaB-helicase interacts to the C-terminal domain of DnaG-primase, and these interacting domains are highly diverse in sequence. However, the structures of these domains and the mode of complex formation are very similar and retain the essential interaction albeit with different affinities.

Keywords: DNA replication initiation, DnaG-primase, DnaB-helicase, crystal structures, helicase-primase interaction

Introduction

The process of transfer the genetic material to the next generation is the essential process for persistent life (Dawkins, 1978). In all the living organisms except some RNA viruses, genetic information is present in the form of chromosomal DNA (Griffiths et al., 2005). DNA replication is the fundamental process that needs a high grade of accuracy to sustain faithfulness in the genetic material while

transferring to the new life (Kornberg and Baker, 1992). DNA replication is firmly regulated in various steps and uses multi-protein complex molecular machines called the replisome (Baker and Bell, 1998). In the bacterial replicon model, the initiation of DNA replication involves the *trans*-acting proteins, and it is controlled from specific chromosomal regions, known as oriC (Fuller et al., 1984). The initiators bind to the oriC that starts the recruitment and assemble the replisome to generate the replication fork (Sekimizu et al., 1987). A series of mechanisms involved ensuring the timely onset and accurate completion of DNA replication initiation (Kornberg and Baker, 1992). DNA replication process is divided into three main stages: initiation, elongation, and termination. Each step involves a specific set of the multi-protein complex with highly coordinated actions. Though the general functions of replisome from diverse forms of life are similar, the constituents and mechanistic details can be distinctive (Mott and Berger, 2007). In bacterial DNA replication, one of the most prominent events is the formation of the replisome.

Replisomal machines are highly mobile and flexible. The dynamics of the replisome complex are facilitated and controlled through the web of protein-protein interactions of diverse strengths (Baker et al., 2003). Many replication proteins are either contain unstructured regions or highly

flexible, making to be challenging for the structural studies of these proteins by X-ray crystallography (Xu and Dixon, 2018). Though, over decades of struggles, structures of replication proteins of *E.coli* or homologs from other bacteria have been solved as domains, whole proteins, or protein complexes (Xu and Dixon, 2018). Here, the review is restricted to the DNA replication initiation in eubacteria and exploring the helicase-primase interaction during DNA replication initiation. The *E.coli* model has highly studied DNA replicon system. Developing scientific information for the replicon model on other eubacteria has been showing differences with *E.coli* model (Mott and Berger, 2007; Nitharwal et al., 2007). Therefore, the replicon model of *E.coli* cannot be generalized for all the eubacterial organisms. Although there are differences in the execution of DNA replication initiation in different organisms of eubacteria, the general biochemistry of DNA replication is the same. Moreover, the eubacterial replicon model is facilitating to explore the corresponding proteins in archaebacteria and eukaryotes. The structural information of proteins involved in DNA replication initiation would have contributed immensely to understand the replication in eubacteria. Here, we explored the structural insights into the two essential proteins involved in DNA replication; DnaG primase and DnaB replicative helicase. The interacting region of the helicase-primase complex does not share the sequence homology among the eubacteria (Rehman et al., 2013; Sharma et al., 2018). The structural studies of these two proteins from the different eubacterial organisms will contribute comprehensive information that may support us for the better understanding of the mechanisms of the DNA replication at the species level as well as its modifications during the evolution.

Initiation of bacterial DNA replication

DNA replication initiation in a living cell is firmly organized to confirm that the chromosome is replicated only once in each cell division. Bacterial chromosomes are generally present in the circular double-stranded form of DNA with a single replication initiation locus called the replication origin, oriC (Fuller et al., 1984). In Bacteria, the *E.coli* DNA replication initiation model has been highly explored (Fig.1). The size of *E. coli* chromosomal DNA is 4.6 Mbp with a specific 250-bp long fragment of oriC locus (Bergthorsson and Ochman, 1995). The sequences of initiation locus are highly conserved and contain five conserved 9-bp regions having the consensus sequences 5'-TTATCCACA-3' known as DnaA boxes (Schaper and Messer, 1995). The upstream of DnaA boxes contains three tandemly repeated 13 base-pairs AT-rich consensus DNA sequences (5'-GATCTNTTNTTTT-3') are called DNA unwinding element (DUE) (Bramhill and Kornberg, 1988; Kowalski and Eddy, 1989). The oriC are recognized by the initiator protein DnaA (Schaper and Messer, 1995). A series of repeating trinucleotides (5'-TAG/A), termed DnaA-trio, was recognized as a key component in the DNA unwinding region (Richardson et al., 2016). The hexameric DnaB helicase is recruited at the DNA bubble and loaded onto separated ssDNA strand by helicase loader DnaC. The DnaC helicase loader inhibits the helicase activities, and ATPase of DnaB helicase and keeps the hexameric ring to an open right-handed state that is organized to bind onto single-stranded DNA (Arias-Palomo et al., 2013; Chodavarapu et al., 2015). The AAA+ domain of DnaC interacts to DnaA filament with the AAA+ domain of DnaA and assist in loading hexameric DnaB onto the ssDNA bubble (Mott et al., 2008). Helicase loader is not found in *Mycobacterium tuberculosis* and *Helicobacter pylori* (Soni et al., 2005;

Zhang et al., 2014). In *Helicobacter pylori*, DnaB assembles as a double hexamer (dodecameric state), which separate into two hexameric helicases during replication initiation (Bazin et al., 2015).

The DnaG primase interacts with the hexameric DnaB and dissociates the DnaC from replication initiation complex (Makowska-Grzyska and Kaguni, 2010). The two hexameric DnaB move towards the heads of the DNA bubble and generate two replication forks that move in opposite directions (Kim et al., 1996). DnaG primase recognizes the particular priming sites (favorably 5'-CTG) on leading-strand of unwound DNA, and the bound form of DnaG to ssDNA synthesise an RNA primer for DNA polymerase to elongate the DNA (Hiasa et al., 1990). At the lagging strand, repeated Okazaki-fragment are synthesized. RNA primer offers the 3'-OH required by DNA polymerase to carry out the elongation phase of DNA replication (Baltimore and Smoler, 1971).

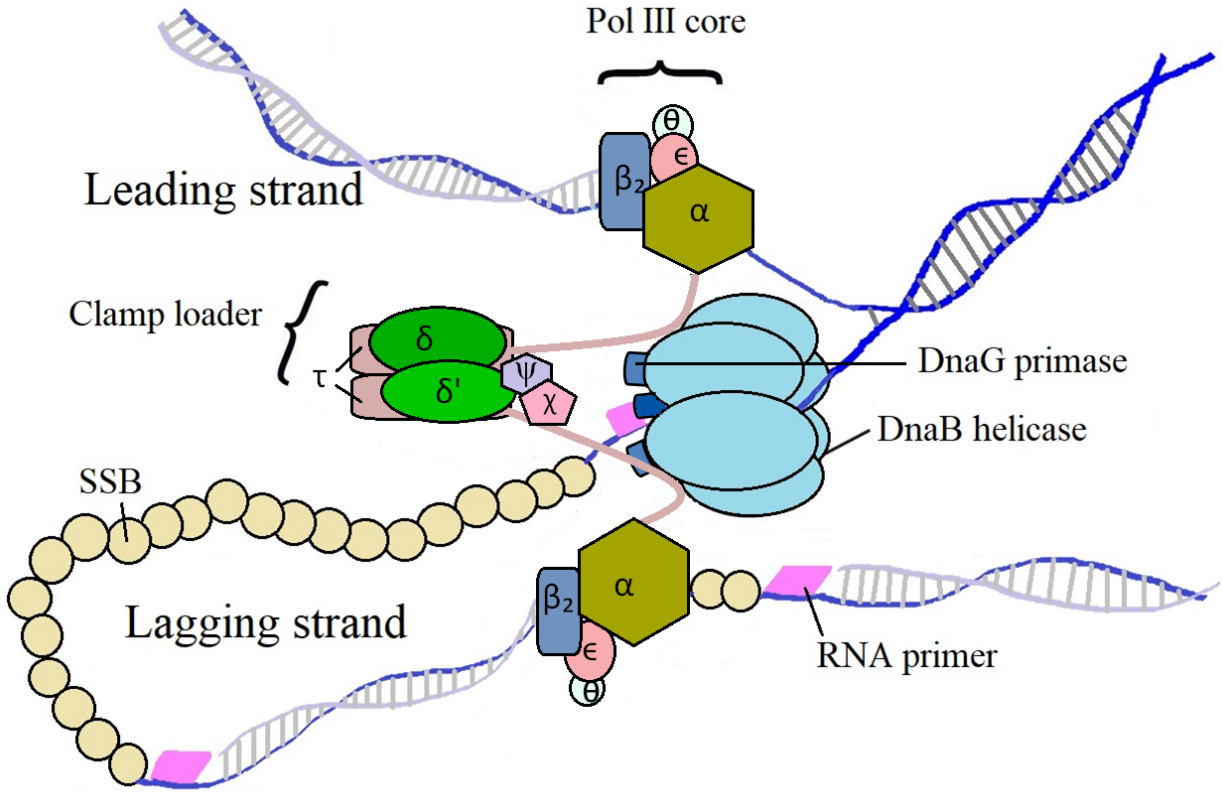

Figure.1: Diagram representing the DNA replisome machine in *E. coli***.** [Adapted from (Lewis et al., 2016)].

DnaB helicase

DNA helicases are nucleotide hydrolysis enzymes that unwind the duplex DNA (Arai, Yasuda et al. 1981). During the unwinding process, these enzymes also show the translocation on DNA strand. There are six superfamilies of helicases, and DnaB helicase belongs to the fourth superfamily, having a RecA-type domain (O'Shea and Berger, 2014). The functional form of DnaB exists as a hexameric state with all the same protomers. DnaB protomer has two domain; highly variable N-terminal domain and the conserved C-terminal helicase motor domain connected by a linker (Fig.2a). The sequence of N-

terminal domain is diverse among the different organisms in eubacteria (Fig.2b). The N-terminal domain interacts to the C-terminal domain of DnaG-primase (Kashav et al., 2009; Rehman et al., 2013; Sharma et al., 2018). The C-terminal domain of DnaB hydrolysis the nucleoside triphosphate and unwind the duplex DNA with translocating in the 5′-to-3′ direction regarding the enzyme bounded single-strand of DNA (Galletto et al., 2004). The structures analysis of *E. coli* DnaB and thermophilic bacterial DnaB revealed the doughnut form of DnaB (Arias-Palomo et al., 2019; Bailey et al., 2007). Fluorescence energy transfer experiments with DnaB that was attached with the artificial replication fork indicated the orientation of the larger C-terminal domain towards the fork junction (Jezewska et al., 1998). The single-stranded DNA attached to DnaB passes into the inner void and interact with precise amino acids residues present at the inner voids of DnaB (Lyubimov et al., 2011).

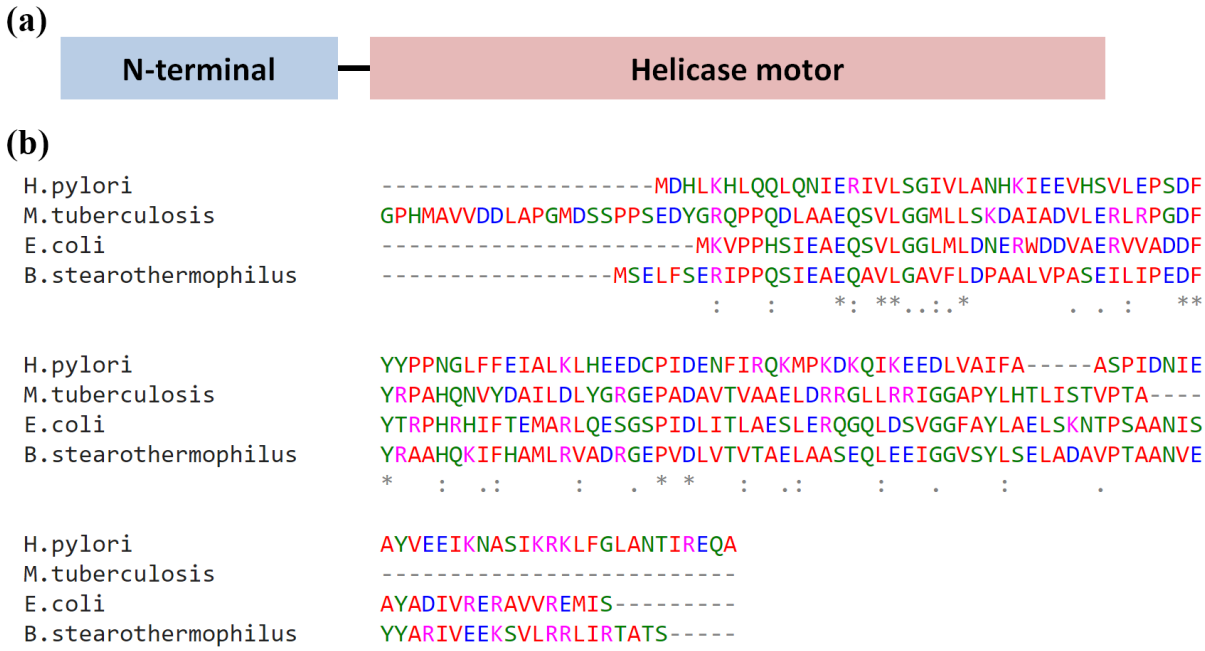

Figure.2: DnaB-helicase: (a) Domain organization of DnaB. (b) Multiple sequence alignment of DnaB-NTDs or primase binding domain of different bacterial species shows insignificant sequence homology.

Afterward melting of double-stranded DNA at the oriC site, the DnaB–DnaC complex binds at this site of replication initiation and establishes an intermediator complex known as the pre-priming complex. In *E. coli*, DnaA showed the direct interaction with DnaB but not with DnaC, therefore

DnaA is thought to be lead the recruiting of the DnaB–DnaC complex on both the melted strand (Sutton et al., 1998). Two regions of DnaA (domain I and domain III) are recognized that interact with DnaB (Keyamura et al., 2009). However, the DnaB form a hexameric closed ring structure, the complex of the hexameric ring of DnaB with DnaC binds to the single-stranded DNA at oriC. The mechanism of opening of the closed ring is still not understood. The biochemical studies, the structure of the DnaC-DnaB complex, electron microscopic images of DnaB and its 3D reconstruction showed the opening of DnaB hexameric ring by binding the DnaC to DnaB (Arias-Palomo et al., 2013). Though in the other experiment based on hydrogen-deuterium exchange showed the spontaneous opening and closing conformation of hexameric DnaB, and only the open conformation of hexameric DnaB ring traped the DnaC (Chodavarapu et al., 2015).

DnaB helicase activation

Unwound DNA of oriC occupies the interior canal of the helical DnaC filament during loading of the DnaB-DnaC complex (Arias-Palomo et al., 2013). After loading the DnaB-DnaC complex, DnaC dissociates from DnaB, and after that, DnaB starts its helicase activity. Dissociation of DnaC from DnaB has been occurring with ATP hydrolysis after the interaction of DnaG to the N-terminal domain of DnaB that causes the changes in the conformation of C-terminal domain of DnaB or DnaC binding region (Makowska-Grzyska and Kaguni, 2010). The DnaC is a member of the AAA+ superfamily and also have a characteristics arginine finger that involves conformational changes with ATP hydrolysis (Makowska-Grzyska and Kaguni, 2010).

In *E. coli,* the DnaC shows the fundamental role in loading the DnaB at oriC. However, all bacteria do not have a dnaC gene. *H. pylori* and *M. tuberculosis* do not encode dnaC. The DnaB helicase of *H. pylori* is showing unique characteristics and form a dodecameric structure by two hexamers interacting through N-terminal domains (Bazin et al., 2015; Stelter et al., 2012). A plasmid encoding *H. pylori* dnaB was transformed into the temperature-sensitive *E. coli* strain with dnaC mutant to study complement of dnaC (Soni et al., 2003; Soni et al., 2005). This complement assay showed that DnaB helicase of *H. pylori* can be loaded at the oriC for replisome formation in the absence of the DnaC and the DnaC is not required in all bacteria. In *H. pylori*, the DnaB require ATP to bind with single-stranded DNA. Additionally this DNA binding and ATP hydrolysis are not adequate to break the DnaB dodecamer into the hexameric DnaB (Bazin et al., 2015). The structural analysis of *H. pylori* DnaB suggested that the primase interaction region of DnaB are partially covered by the interface joining the two hexamers (Bazin et al., 2015). Once, the primase makes contact with this interface, cause the breakdown of the dodecamer into two hexamers. A few structures of DnaB-NTD are available from different bacterial species. Though the N-terminal domains of DnaB from different bacterial species show insignificant sequence homology, the structures of different DnaB-NTDs are very similar (Fig.3a). The DnaB-NTD comprises only helices with one extended hairpin-like structure. The hairpins from two molecules form 4 helical bundles interacting with each other and form a dimer like A and B (Fig.3b). Various dimers also interact with each other and form a hexameric structure as a trimer of dimers (Kashav et al., 2009) (Fig.3b). The hexameric arrangement of N-terminal

domain is conserved in all bacterial species.

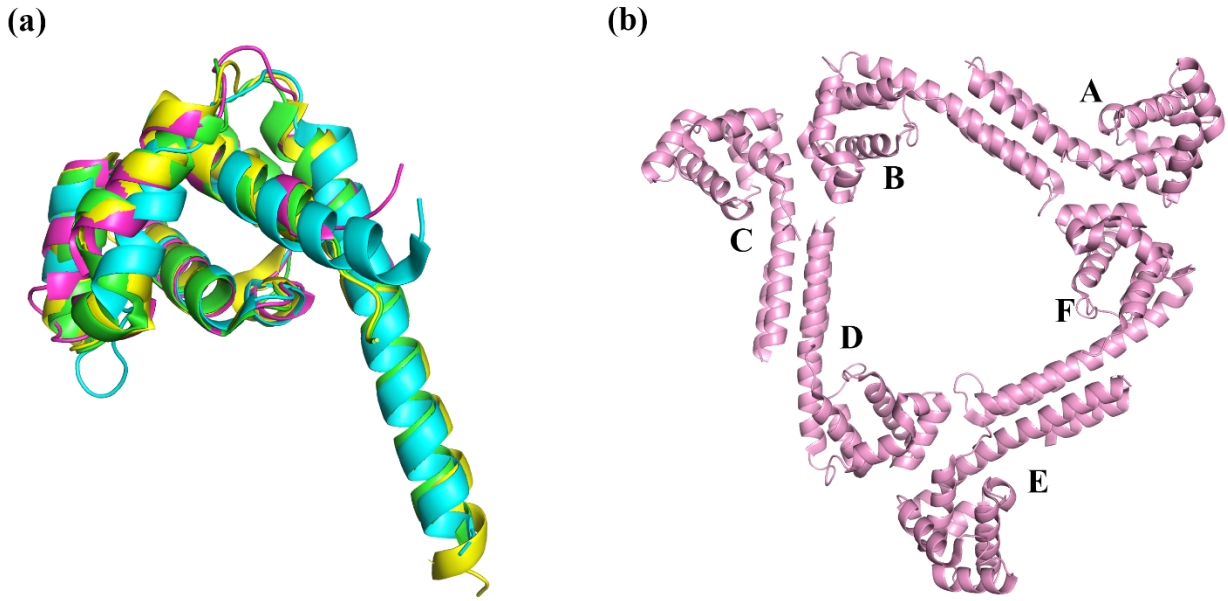

Figure.3: Structure of DnaB-NTD. (a) Structural superimposition of DnaB-NTD from *H.pylori* (cyan) (PDB:3GXV), *M.tuberculosis* (green) (PDB:2R5U), *B.stearothermophilus* (yellow) (PDB:2R6A) and *E.coli* (pink) (PDB:1B79) shows structural similarity. (b) Structure of hexameric DnaB-NTD in *M.tuberculosis* (PDB:2R5U). N-terminal region of DnaB with linker form the hexamer of DnaB. AB, CD, and EF were forming a trimer of dimer.

DnaB modulates the replisome

The C-terminal domain of DnaG primase mediates the binding with DnaB helicase through the N-terminal domain (Bird et al., 2000; Tougu and Marians, 1996). The bacterial DnaB helicase-DnaG primase interaction is essential for the synthesis of primers to initiate the DNA replication and also shows the highly dynamic interaction in synthesis of lagging strand at the replication fork (Frick and Richardson, 2001). At the lagging strand, DnaB-DnaG complex regulates the constantly synthesizing primers. DnaB-helicase supports in reaching the DnaG-primase to its recognition region at oriC, increase the flexibility in the specificity of recognition site of the primase, control the primase activity, and retain the same length in synthesis of new primers (Frick and Richardson, 2001). The DnaG to DnaB ratio is also significant for optimum activity of primase. Since very less level of DnaB compares to DnaG results in suboptimal primase activity, while very much DnaB cause the inhibition in primase activity (Thirlway and Soultanas, 2006). The helicase bounded primase molecules are thought to regulate the primase activity through “trans interactions” of the primer synthesizing domain of a primase with the zinc-binding domain of another primase (Corn et al., 2005). Thus, DnaG shows two types of primase activity modes, firstly

"trans mode" occurs in the presence of the DnaB, where smaller primers are synthesized and secondly "cis mode" are found in the absence of the DnaB, where longer and variable primers are synthesized (Thirlway and Soultanas, 2006). The "trans mode" is dominant when there is number of DnaG molecules that bind to the DnaB.

Therefore, DnaB-helicase modifies the activity of DnaG-primase in several manners.

(a)

(b)

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B.stearothermophilus      ------------------------KLLPAFQNAERLLLAHMMRSRDVALVVQERIGGRFN
M.tuberculosis            ----------------------PTLWPQREALK-----------SALQYPALAGPVFD
H.pylori                  ERVSFQPFYPKTEKPNRPQRFAHVSSAPSLEFLEKLVIRYLLEDRSLLDLAVGYIHS---
E.coli                    -----------AAESGVSRPVPQLKRTTM-----RILIGLLVQNPELATLVPPLENL---
V.cholerae                ---------------NRPAPHKAIKRTPM-----RDVIALLIQNPSYAELVPDLASV---
                                                .

B.stearothermophilus      IEEHRALAAYIYAFYEEG---HEADPGALISRIPGE--------------LQPLASELSL
M.tuberculosis            ALTVEGFTHPEYAAVRAA---IDTA-GGTSAGLSGAQWLDMVRQQTTSTVTSALISELGV
H.pylori                  -----GVFLHKKQEFDALCQEKLDDPKL-VA---------------------LLLDANLP
E.coli                    ----DENKLPGLGLFRELVNTCLSQPGL-TT----GQLLEHYRGTNNAATLEKLSMWDDI
V.cholerae                ----RHLMIPGLDTFSEVLEKCRQYPHI-TT----GQLLEHWRDSKNETLLSRLASWEIP
                                                  :                    *

B.stearothermophilus      LLIADDVSEQELEDYIRHVLNRP--KWLMLKVKEQEK------------TEAERRKDFLT
M.tuberculosis            --EAIQVDDDKLPRYIAGVLARLQEVWLGRQIAEVKSK-------LQRMSPIEQGDEYHA
H.pylori                  --LKK----GGFEKELRLLILRY----FERQLKEIPKSSLPFSEKMICLKKA-RQAIMKL
E.coli                    --ADKNIAEQTFTDSLNHMFDSL----LELRQEELI-----ARERTHGLSNEERLELWTL
V.cholerae                --LVEDNQEELFLDSLDKILAQC----VEKQIENLQ-----AKERSVGLSTEEKRELQDL
                                :   :  ::       .  :  :              .   :

B.stearothermophilus      AARIAKEMIEMKKMLSS----S-----
M.tuberculosis            ---LFGDLVAMEAYRRSLLEQASGDDL
H.pylori                  ---KQGELVAI-------LE-------
E.coli                    ---NQELA-KK----------------
V.cholerae                ---ILKGL-KA-------LE-------
```

Figure.4:DnaG-primase: (a) Domain organization of DnaG and (b) multiple sequence alignment of DnaG-CTDs or helicase binding domain of different bacterial species shows insignificant sequence homology.

Prokaryotic primases

Genome sequencing uncovered the various proteins that have significant resemblance with primases. Mostly primases are divided into two major groups. The first group comprises the bacterial primases and primases from bacterial phages while the second group includes primases from eukaryotes. Although all the primases exhibit the synthesis of primers, the primases can also be categorized into two groups depends on structural as well as its interacting partner during the replication process. Generally, the prokaryotic primases are the individual protein that binds to the helicase in replisome complex, while the eukaryotic primase exists as an integral part of DNA polymerase α (Pol α). Though there are some primases that cannot be classified in these two groups (Frick and Richardson, 2001). Both bacterial primases and phage primases are included in the prokaryotic group (Ilyina et al., 1992). Though they are distantly related, still showing many common features, such as consensus signature sequences and association with replicative helicase (Iyer et al., 2005; Versalovic and Lupski, 1993). *In vitro* complementation assay with the purified 60-kDa *E. coli* DnaG protein showed the formation of small RNA primers (Bouché et al., 1975). The *E. coli* DnaG primase needs either the oriC with single-stranded DNA binding protein or DnaB helicase to efficiently synthesize oligo-ribonucleotides on ssDNA. Therefore, the activity of DnaG is explored in the framework of two diverse methods. In the first method, DNA from G4 phage is used as a single-stranded DNA template, where the primer synthesis depends on the presence of SSB proteins and synthesis starts from oriC (Hourcade and Dressler, 1978). The length of the synthesized primer is up to 29 nucleotides (Bouché et al., 1978). In the second method, the effect of DnaB helicase on the DnaG activity is determined. Here, the length of the synthesized primer is mostly 11 nucleotides long, and this method shows the general primase activity during the DNA replication (Wu et al., 1992). The DnaG primase is divided into three domains (Fig.4a). The Zn binding domain and RNA polymerase domain show conservation in sequence homology among different organisms in eubacteria, whereas the multiple sequence alignment of DnaG-CTDs of different bacterial species shows insignificant homology (Fig.4b). Interestingly despite this insignificant sequence homology, all the DnaG-CTDs comprises two subdomains, a globular region of six to eight helices and a helical hairpin region interacting with the DnaB-helicase (Rehman et al., 2013; Sharma et al., 2018). The helixhairpin regions from the different sources adopted different orientations relative to the globular region (Fig.5) and helixhairpin region also showed much greater structural differences than the globular regions (Sharma et al., 2018).

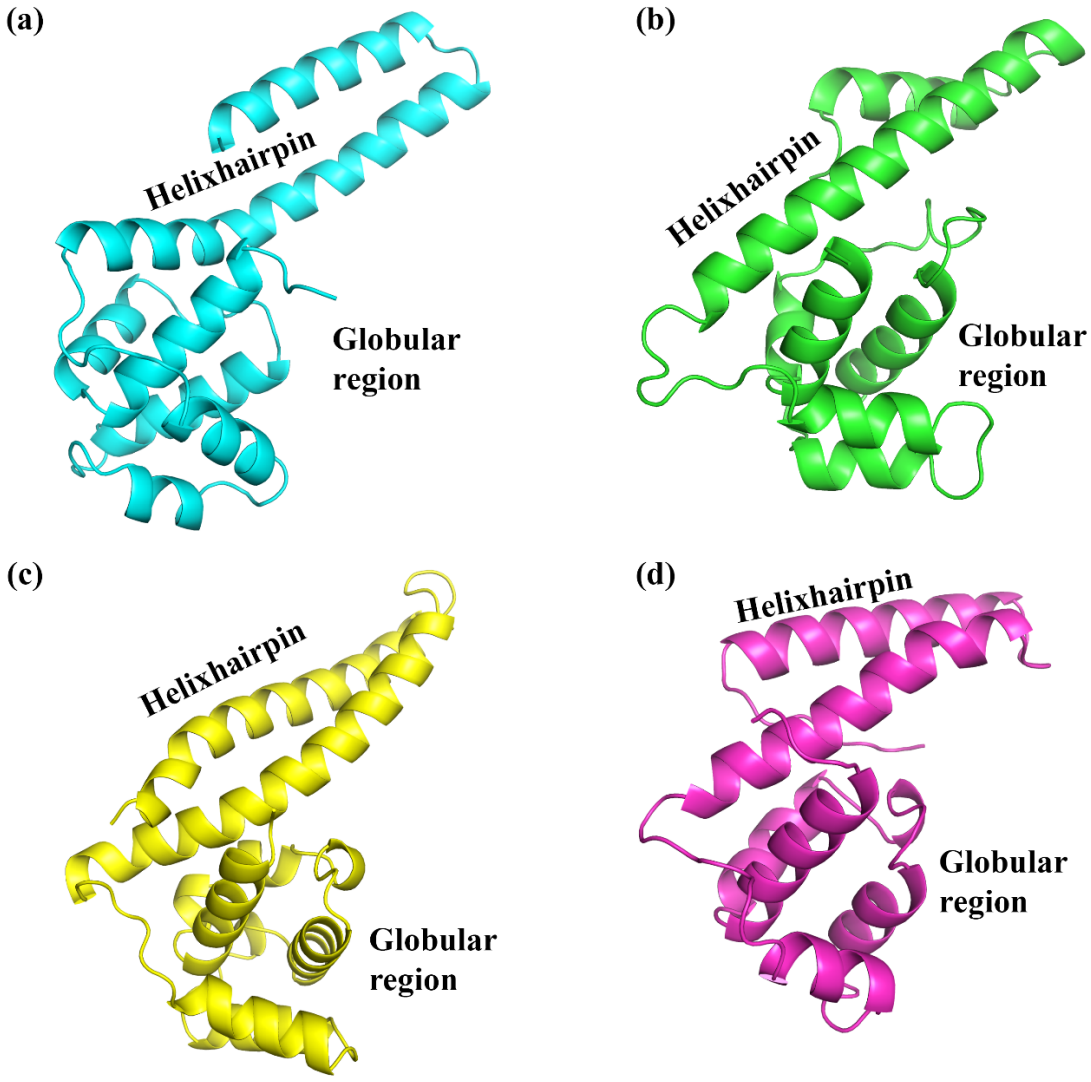

Figure.5: Crystal structures of helicase binding domain of DnaG from (a) *B. stearothermophilus* (PDB:2R6A), (b) *V.cholerae* (PDB:4IM9), (c) *M.tuberculosis* (5Z51), and (d) *H.pylori* (4EHS) showing the different shape and orientation of helixhairpin region.

Evolution in primases

The sequence of a large number of primases from different organisms was analyzed for the study of evolutionary relations amongst the primases (Iyer et al., 2005). Several discrete primase families are classified without sequence relationship, supported the hypothesis of the parallel evolution of primases. Structurally, primases are classified based on a number of subunits

required for its activity and the presence of the toprim structure in phage/bacterium primases or archaeal/eukaryotic fold. Primases from eubacteria and several phages that play a role in plasmid duplication are single subunit primases (Frick and Richardson, 2001). The rate of

primase activity of these enzymes are slow (Sheaff and Kuchta, 1993). However, the presence of helicase stimulates the primase activity. The archaeal and eukaryotic primases are mysterious to their relationship. Both the primases exhibit two subunits and also comprise a Fe-S cluster (Weiner et al., 2007). Sequence alignments of both the large and small subunit from these enzymes show some homology (Iyer et al., 2005).

Based on the structural fold, the primases are classified into two groups. Similar to the structure of topoisomerases, the structure of polymerase domain of primases from *E. coli* and *Bacteriophage T7* consist a toprim fold structure (Corn et al., 2008; Kato et al., 2003; Keck et al., 2000). Berger group determined the complex structure of single-stranded DNA bounded with *E. coli* primase in two distinct conformations (Corn et al., 2008). Generally, primases have a Zn^{+2}-binding domain that binds to DNA and the primer synthesizing domain (Biswas and Weller, 1999). The large subunits of primases from the eukaryotic and archaeal have an uncharacterized Fe:S group that has an essential role in primase activity (Weiner et al., 2007).

The interaction of primase-helicase complex in eubacteria

The DnaB helicase interacts with many proteins such as single-stranded DNA binding proteins, DnaA, DnaC-helicase loader, DnaG-primase, τ-subunit of DNA polymerase III, and replication termination proteins (Xu and Dixon, 2018). DNA polymerase III, DnaG primase, and DnaB helicase establish replication machinery known as replisome (Yao and O'Donnell, 2010). The binding of DnaG primase and DnaB helicase remains into the central action in the dynamics of replication machines and also the foundation of complex replication machine formation at the replication fork (Yao and O'Donnell, 2010). The DnaB helicase regulates the length of newly synthesized primer to around 10-14 nucleotide long and stimulates the DnaG to increases the rate of primer synthesis (Corn and Berger, 2006). Binding of the DnaB to DnaG increases the single-stranded DNA binding affinity of the DnaG at the primer synthesizing sites (Johnson et al., 2000; Mitkova et al., 2003). On the other side, DnaG stimulates the helicase and ATPase activity of DnaB helicase (Syson et al., 2005).

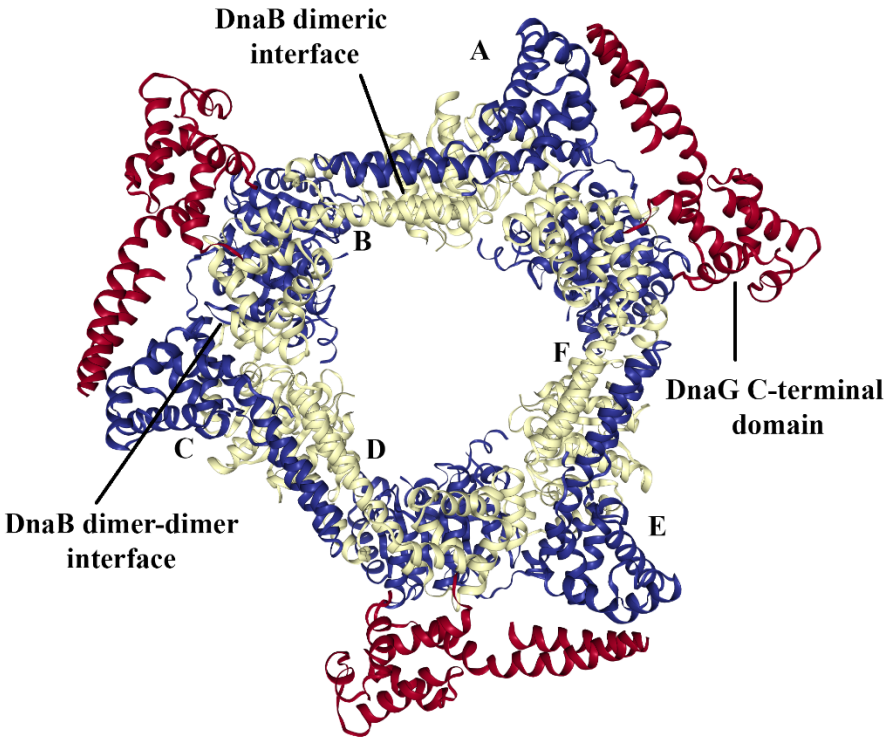

Figure. 6: Crystal structure of DnaB/DnaG-CTD complex in *B. stearothermophilus*. [PDB: 2R6A]. AB, CD, and EF formed hexameric DnaB as a trimer of dimer and DnaG-CTD bind to the dimer-dimer interface of DnaB.

The strength of the interaction between DnaB and DnaG varies in different bacteria. In *E.coli*, DnaB and DnaG show weak interaction contrast to the *Bacillus stearothermophilus* where the DnaB-DnaG interaction shows very high binding affinity that the DnaB-DnaG complex can be purified in gel filtration chromatography (Bailey et al., 2007; Thirlway et al., 2004). Earlier in our lab, we found the moderate binding affinity in DnaB-DnaG complex of *H. pylori* (Kashav et al., 2009; Nitharwal et al., 2007; Rehman et al., 2013). Fluorescence anisotropy showed that three molecules of DnaG bind with hexamer of DnaB, thus the binding stoichiometry of DnaB: DnaG is 2:1 that was also confirmed by the complex structure of DnaB/DnaG-CTD (Fig.6) (Bailey et al., 2007; Mitkova et al., 2003). DnaG stabilizes the hexameric arrangement of DnaB. In *B. stearothermophilus*, Tyr88, Ile119, or Ile125 residues in DnaB are found to be crucial for the complex formation with DnaG (Bailey et al., 2007). Tyr 88 is essential for stabilizing the helical bundle of DnaG binding domain, (NTD) of DnaB, while Ile119 and Ile125 involve in the dimerization of DnaB (Bailey et al., 2007). Mutation at any of these residues of DnaB inhibit the interaction with DnaG due to disruption of the trimers of dimers form of the DnaB and the corresponding amino acid residues in *S. typhimurium,* and *E.coli* also causes the same effect (Bailey et al., 2007; Chang and Marians, 2000; Stordal and Maurer, 1996). A non-conserved residue on DnaG –CTD (Phe534) (Fig.7a) was identified as a critical residue for DnaB-DnaG complex in *H. pylori* (Rehman et al., 2013). In *M.tuberculosis*,

Ile605 (Fig.7b) was found to be crucial hydrophobic residue present at the loop of HHR region of the *Mt*DnaG-CTD and interact with Val82 and Ala85 of *Mt*DnaB-NTD (Sharma et al., 2018). The amino acid residues present at the helical hairpin loop of DnaG-CTDs suggested the presence of non-conserve hydrophobic residues that have a crucial role in the stability of DnaB-DnaG complex (Sharma et al., 2018). The flexibility of loop allows maximizing the access of its hydrophobic residue for contacts with the DnaB-NTD (Sharma et al., 2018).

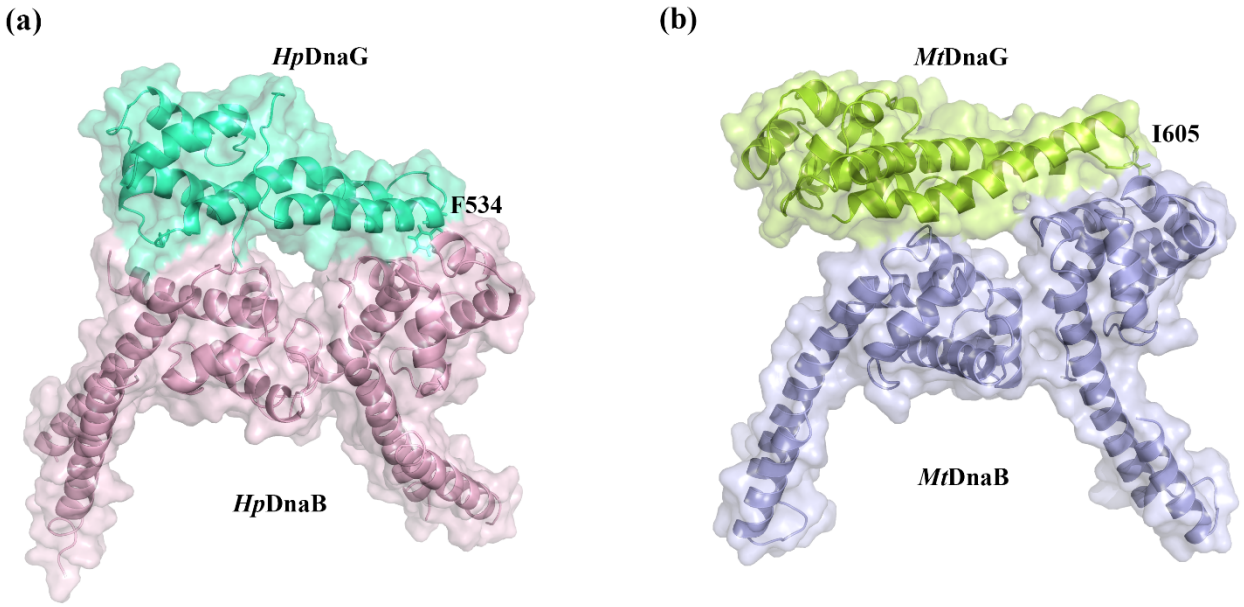

Figure. 7: DnaB-DnaG complex in (a) *Helicobacter pylori* [adapted from (Rehman et al., 2013)] and (b) *Mycobacterium tuberculosis* [adapted from (Sharma et al., 2018)], where the dimeric DnaB-NTD is modeled to interact with monomeric DnaG-CTD.

Conclusion

DnaB helicase is a hexameric replicative helicase where all the six subunits are arranged in a ring forming a central cavity. The flexible linker connected the globular N-terminal domain to the helicase motor domain. The N-terminal domain with linker region has very less sequence homology among eubacteria. The first helix of the linker is involved in hairpin structure formation with the last helix of DnaB-NTD (Kashav et al., 2009). The hairpin structure helps in head to tail dimerization of NTDs and subsequently the trimerization of dimers to form hexamers (Kashav et al., 2009). The hexameric form is stabilized by the interaction of linker region with the CTD of adjacent monomer. The helicase motor domain shows ATPase and helicase activity, and also shows more sequence conservation compare to N-terminal domain. DnaB-NTD is involved in helicase-primase interaction where DnaB-NTD binds to the C-terminal domain of DnaG. DnaB-NTD with linker

binds to DnaG-CTD with higher affinity compare to DnaB-NTD (Kashav et al., 2009). Though the different bacterial DnaG-CTDs show the insignificant sequence homology, the structural alignment of DnaG-CTDs show conservation of the folds (Rehman et al., 2013; Sharma et al., 2018). The conservations of folds mainly occur in the globular region of DnaG-CTD compare to helix hairpin region. The hairpin region of DnaG-CTD interacts to the dimer-dimer interface of DnaB-NTD. A loop, connecting both the helices of the HHR, is mainly responsible for the stability of DnaB-DnaG complex (Sharma et al., 2018). The strength of the interaction between DnaG primase and DnaB helicase also varies among eubacteria. The structural and biophysical data provided by our lab have shed new light on the structural assembly and mode of binding of DnaG-CTD with DnaB-NTD in eubacteria and unveiled mechanistic details and selectivity determinants for other eubacteria (Rehman et al., 2013, Sharma et al., 2018). Though the DnaB-NTD and DnaG-CTD do not have any sequence homology among different organisms, the structures of individual domains and their mode of complex is very similar. The non-conserved interacting domains of DnaG-DnaB complex expand the study on the aspects of the species-specific evolution of the replication system.

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