Lignin reduction in jute by down-regulating the lignin biosynthetic gene(s) through RNAi technique

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CERTIFICATE

This is to certify that the research work embodied in this thesis entitled **"Lignin reduction in jute by down-regulating the lignin biosynthetic gene(s) through RNAi technique"** has been carried out both in the Molecular Biology Lab, Department of Biochemistry and Molecular Biology, University of Dhaka, Bangladesh and in the Plant Molecular Biology Group, International Center for Genetic Engineering and Biotechnology (ICGEB), New Delhi, India.

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Declaration

I hereby declare that the research work embodied in this thesis entitled **"Lignin reduction in jute by down-regulating the lignin biosynthetic gene(s) through RNAi technique"** has been carried out by me under the supervision of Dr. Haseena Khan, Molecular Biology Laboratory, Department of Biochemistry and Molecular Biology, University of Dhaka, Bangladesh and co-supervision of Dr. Neeti Sanan-Mishra, Plant Molecular Biology Group, International Center for Genetic Engineering and Biotechnology (ICGEB), New Delhi, India.

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Dedicated to

My Family

For their unconditional love and never ending support

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Knowledge is in the end based on acknowledgment – Ludwig Wittgenstein

Research is an effort of not an individual's glory but contribution of many involved in its various stages. This is a note of my gratitude to all those who made tremendous impacts in this eventful journey being the reinforcements in my belief that hard work does taste sweet on achieving success.

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Farhana Shafrin

Abstract

Lignin, a complex, three-dimensional aromatic polymer, serves as a matrix around the polysaccharide components of a plant cell wall by providing additional rigidity, comprehensive strength and hydrophobicity. As a bio-polymeric phenolic macromolecule, lignin is unusual because of its heterogeneity and lack of a defined primary structure. Plants are genetically engineered to produce less lignin so that they would be more suitable for conversion into bio-fuels or high quality paper. Jute, a naturally rich lingo-cellulosic fiber yielding crop, delineates a new promising sphere with respect to lignin manipulation. From an overabundance of experimentations, it is evident that the role of RNAi is immense in gene regulation. Likewise siRNA, artificial microRNA (amiRNA) appends a new prospect in the era of RNAi. Thus this study aimed at designing siRNA and amiRNA based hairpin constructs to introduce into jute by *Agrobacterium tumefaciens* mediated *in planta* transformation in order to develop jute variety with reduced lignin content. Apropos to this perspective, monolignoid biosynthetic genes, caffeic acid O-methyltransferase (COMT), ferulic acid 5-hydroxylase (F5H), coumarate 3-hydroxylase (C3H) and cinnamante 4-hydroxylase (C4H) were considered to be engineered. Initially, effective C3H-amiRNA and F5H-amiRNA based constructs were designed and cloned into pGEM-T entry vector. Then constructs were mobilized into destination vector pBI121. After confirmation of the functionality of the amiRNA constructs by agro-infiltration assay in tobacco, the constructs were introduced into *C. olitorius* by a tissue culture independent *Agrobacterium tumefaciens* mediated transformation process. At the same time, effectual COMT-siRNA and C4H-siRNA silencing constructs were designed and cloned into vector, pENTR11. Then the recombinant pENTR11 plasmids were mobilized into pK7GW1WG2 destination vector using LR reaction followed by *Agrobacterium tumefaciens* mediated *in planta* transformation. The transgenic generations for all four gene(s) were then analyzed through Southern, RT-PCR and northern assays. As a proof of concept, the decrease in lignin content was estimated (Klason Method) for the transgenic lines and compared with the wild type. The transgenic lines for both strategy viz. amiRNA (C3H-amiRNA & F5H-amiRNA) and siRNA (COMT-siRNA&C4H-siRNA) showed reduced level of gene expression and ~ 16-27 % reduction in acid insoluble lignin content compared to the normal control lines. The results indicate successful amiRNA/siRNA transgenesis in jute, which is likely to have far-reaching commercial implications. This study illustrates the role of RNAi (amiRNA/siRNA) with respect to jute lignomics in order to capitulate new insights into the fine-tuned alteration of lignin biosynthetic pathway of jute. Commercial usability of jute is expected to be boosted from a reduction in the lignin content of jute leading to its economic acceleration.

Key words: Lignin; Gene silencing; RNAi; Jute.

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Abbreviations

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%	Percentage
°C	Degree Celcius
μg	Microgram
μl	Microliter
μM	Micromolar
β-ME	Beta-mercaptoethanol
amiRNA	Artificial miroRNA
AGO	Argonaute
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BSA	Bovine Serum Albumin
C'-terminal	Carboxy terminal
cDNA	Complementary DNA
dATP	Deoxyadenosine triphosphate
DCL	Dicer like protein
dCTP	Deoxycytosine triphosphate
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphates
dsDNA	Double stranded DNA
dsRNA	Double stranded RNA
DTT	Dithiothreitol
EDTA	Ethylenediamine tetracetic acid

g	Gram
GITC	Guanidinium thiocyanate
GFP	Green Fluroscnence Protein
h	Hour
Kb	Kilo base pair
kDa	Kilo dalton
L	Liter
LB	Luria Bertani medium
M	Molar
mA	Milli Amperes
MCS	Mulitiple Cloning Site
mg	Milligram
min	Minutes
miRNA	MicroRNA
ml	Milliliter
mM	Mili molar
MQ	Milli Q
mRNA	Messenger RNA
N'-terminal	Amino terminal
ng	Nano gram
nt	Nucleotide
ORF	Open Reading Frame
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction

pre-miRNA   Precursor microRNA

pri-miRNA   Primary microRNA

PTGS   Post Transcriptional Gene Silencing

RDR   RNA dependent RNA polymerase

RISC   RNA Induced Silencing Complex

RNA   Ribonucleic acid

RNAi   RNA interference

RNase   Ribonuclease

rpm   Rotation Per Minute

sec   Seconds

siRNA   Small interfering RNA

ssDNA   Single stranded DNA

ssRNA   Single stranded RNA

TBE   Tris-Borate-EDTA

TE   TRIS-EDTA

TGS   Transcriptional Gene Silencing

Tm   Melting Temperature

U   Unit

VIGS   Virus Induced Gene Silencing

vsiRNA   virus-derived interfering RNAs

WT   Wild type

YEM   Yeast Extract Mannitol

CHAPTER 1

LIGNOMICS OF JUTE

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Preface:

Lignin, an abundant bio-polymeric phenolic macromolecule present in cell walls of plants is co-embedded with cellulose. It is usually derived from phenylalanine through dehydrogenative polymerization of three hydroxy-cinnamyl alcohols (monolignols), viz. p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol resulting in p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units, respectively (Vanholme *et al.* 2010). This phenolic-based polymer plays multiple roles in plants by contributing to the hydrophobicity of plant vasculature (Xu, Zhu *et al.* 2011) by providing mechanical support for plant tissues (Boerjan, Ralph *et al.* 2003) and by contributing in defense mechanisms against both biotic and abiotic stresses (Hammond-Kosack and Jones 1996). The ensemble of all phenolic metabolites are co-synthesized with lignin biosynthesis from the general phenyl-propanoid, monolignol, and (neo)-lignan biosynthetic pathways and their derivatives as well as the lignin oligomers (Wang *et al.* 2013). In lignifying tissues, the lignome comprises a significant portion of the metabolome (Vanholme, Demedts *et al.* 2010).

Jute (a naturally rich lingo-cellulosic fiber yielding crop) delineates a new promising sphere with respect to lignin manipulation. Like other bast fibers, jute has a fair amount of lignin, which makes it the strongest of them all (Del Rio, Rencoret *et al.* 2009; Stevens and Müssig 2010). However, the H:G:S composition of jute is 2:36:62 (Río Andrade, Gutiérrez Suárez *et al.* 2007) which is completely different from others. The S/G ratio is 2:1, which makes it inappropriate for various industrial purposes such as paper pulp, textile raw material, forage digestibility and biofuels (Schmidt, Alfermann *et al.* 2008; Żuk, Kulma *et al.* 2011). In spite of the importance of lignin in plant growth and development, it is evident that reduction in lignin content would result in superior fiber quality for industrial purposes and consequently better profitability (Mandal and Srimani 1987). Lignin perturbation in jute and its assessment in transgenic generations have been long overdue. An escalating number of examples points that lignin engineering can improve the use of jute as a sustainable resource for the production of bio-based materials for commercial purposes (viz. textile, paper- pulping, biofuel). Despite the extensive amount of work focused on jute lignin, effective mechanisms for its reduction need more research; to identify, characterize as well as manipulate lignin biosynthetic gene(s) in jute. In this era of

Molecular Biology, the best preference to study the biosynthetic genes would be the "silencing method".

The regulation of gene expression is at the center of all the molecular and developmental aspect of an organism. Several mechanisms have evolved in living organisms to regulate gene expression spatially and temporally, as well as to harmonize with the environmental cues, which occurs mainly at the transcriptional and post transcriptional levels (Tiwari, Sharma *et al.* 2014). Homology based gene silencing has emerged as a convenient approach for repressing expression of genes in order to study their functions. For this purpose, several antisense or small interfering RNA based gene silencing techniques have been frequently employed in plant research (Vance and Vaucheret 2001; Waterhouse and Helliwell 2003).

RNA interference (RNAi) is based on sequence-specific RNA degradation that follows the formation of double-stranded RNA (dsRNA) homologous in sequence to the targeted gene (Baulcombe 2004). Functional genomics, dependent on insertional mutagenesis or knockout method are not feasible for all plants (Page and Grossniklaus 2002). RNAi vector mediated gene silencing has increased the efficiency of genetic manipulation in plants because of its ease of application and the possibilities for genome-wide reverse genetics (Travella, Klimm *et al.* 2006). Gene constructs encoding intron spliced RNA with a self complementary hairpin (hp) structure have been shown to induce post-transcriptional gene silencing with almost 100% efficiency when directed against viruses or endogenous genes and transgenes (Smith, Singh *et al.* 2000; Travella, Klimm *et al.* 2006; Travella and Keller 2009).

In recent years, endogenous microRNAs have been described as important regulators of gene expression in eukaryotes. Artificial microRNAs (amiRNAs) represent a recently developed miRNA-based strategy to silence endogenous genes. amiRNA, an innovative strategy having enormous potential, can bind to target RNAs (mRNA) and decrease their activity, by transcript cleavage or translational repression. It is a form of miRNA, which is derived by replacing native miRNA duplexes from a natural miRNA precursor. This tool can improve plant research and crop engineering by making amiRNA a more predictable and manageable genetic and functional genomics technology. amiRNAs can be created by exchanging the miRNA/miRNA (*) sequence within a miRNA precursor with a sequence designed to match the target

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gene. The possibility of lignin modulation through RNAi strategy has proved to be an effective mechanism for the alteration of gene expression (Fire, Xu *et al.* 1998).

On the other hand, even though the groundwork of lignin chemistry has been done decades ago recent work has prompted a number of re-evaluations of the lignin biosynthetic pathway. The molecular biology of lignification has been elucidated in the past decade through full-fledged research by many scientists (Vanholme, Morreel *et al.* 2008; Bonawitz and Chapple 2010). But lignin engineering in jute is still in its infancy. So far, many lignin pathway genes have been studied to get a better understanding. Few genes which do not overlap with plant defense machinery, are considered as a good choice for gene silencing (Boerjan, Ralph *et al.* 2003).

In light of these considerations, in this study, four lignin biosynthetic genes of jute, caffeic acid O-methyltransferase (COMT), ferulic acid 5-hydroxylase (F5H), coumarate 3-hydroxylase (C3H) and cinnamante 4-hydroxylase (C4H) were chosen for engineering. Apropos to this, the RNAi technique was preferred for reducing the lignin content of jute. This study is the first ever initiative of gene silencing in jute using RNAi to find its impact on lignin reduction. It also illustrates the role of RNAi with respect to jute lignomics to capitulate new insights into the fine-tuned alteration of lignin biosynthetic pathway of jute which will certainly boost its commercial usability and economic acceleration.

Therefore, we focused on lignin reduction in jute using both hp-RNA and amiRNA based RNAi strategy. So, a broad aim of the proposed study was to introduce antisense RNA based vectors (hp-RNA and amiRNA) in jute plants for silencing targeted genes (individually) of the lignin biosynthetic pathway. The ultimate goal of the study was to develop a low lignin containing jute variety. These findings will not only append a new dimension in jute gene silencing but also provide an innovative way to improve the fiber quality of jute.

Form a procedural point of view, the following objectives were specified–

- Targeting the four above mentioned genes involved in lignin biosynthesis.
- Designing and development of both hp-RNA and amiRNA based constructs aiming at individual genes of interests.

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- Introduction of these RNAi constructs in jute plants in order to raise transgenic jute plants with reduced lignin content.
- Molecular analysis of the RNAi transgenic generations to corroborate the concept of the study.
- Quantifying lignin content in the transgenic lines as a proof of functionality.

Review of literature:

Jute: The Golden Fiber

In an illustrated document collected from as early as 16th century Rumphius (1743), one of the earliest plant scientist of India, gave a detailed account of a jute plant along with a figure of *Corchorus capsularis*. He mentioned that it was under cultivation in Bengal, the Arakan and South China. He also remarked that a fine white thread was made from the bark of this plant, which was stronger than cotton. In the history of this land jute has been one of the major cash crops. Hence jute, rightly so, has been termed as the 'golden fiber' of Bangladesh.

Jute is an elongated, flexible, and one of the most affordable vegetable fiber producing crops naturally rich in lignin. At the end of the 18th century, Roxburg identified jute (*Corchorus* spp. 2n=14, Genome size =1250 Mb) as an alternative to European hemp (*Canabis sativa* L) (Ghosh 1983). The two species of the genus *Corchorus* (family: Sparrmanniaceae), which are cultivated as jute crop include *Corchorus capsularis* L. (white jute) and *C. olitorius* L. (dark/tossa jute), although 50-60 species are widely known and 170 names are described under the genus *Corchorus* in index Kewensis (Jackson 1908; Edmonds 1990). The two major species are distinct in their growth and, branching habits and characteristics relating to leaf, flower, fruit, bast fiber and photosensitivity (Basu, Ghosh *et al.* 2004).

Generally, the bast fibers of two plant species, *Corchorus capsularis* and *Corchorus olitorius* are known as jute (Islam and Sarkanen 1993). Bast fiber develops in the phloem or bast region of the plant stem. These fibers are mainly sclerenchyma cells with copious secondary wall thickening (Sengupta and Palit 2004).

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Domain: Eukaryota
Kingdom: Plantae
Division: Magnoliophyta
Class: Magnoliopsida
Order: Malvales
Family: Sparrmanniaceae
Genus: *Corchorus*
Species:
Many species, including:
Corchorus aestuans
Corchorus capsularis
Corchorus carnarvonensis
Corchorus cunninghamii
Corchorus junodi
Corchorus olitorius
Corchorus sidoides
Corchorus tridens

Fig.1.1: Image of jute plant, flower and pod with taxonomic classification

Jute fibers are composed primarily of the plant materials cellulose (major component of plant fiber), and lignin (major components wood fiber) (Giwa and Akwu 2007). It is thus a lingo-cellulosic fiber that is partially a textile fiber and partially wood. With increasing height from bottom to top of a jute plant, the lignin content is decreased and α-cellulose content is increased (Sengupta and Palit 2004; Jahan, Kanna *et al.* 2008; Del Rio, Rencoret *et al.* 2009). The bark has higher α-cellulose, glucose and lower lignin, xylose than core (Widyorini, Xu *et al.* 2005; Jahan, Kanna *et al.* 2008; Del Rio, Rencoret *et al.* 2009).

Table 1.1: Chemical composition of jute fiber (Giwa and Akwu 2007)

Constituent	Percentage (%)
Cellulose	**59-61**
Pentose	**15-17**
Lignin	**12.5-13.5**
Polyuronide	**4.8-5.2**
Acetyl value	**2.8-3.5**
Fat and Wax	**0.9-1.4**
Nitrogenous matter	**1.56-1.87**
Mineral Substances	**0.5-0.79**

Table 1.2: Physical properties of jute fiber (Del Rio, Rencoret *et al.* 2009; Rahman 2010)

Properties	Unit Value
Thickness of Fiber	15-20 μm.
Length of Fiber	150 to 300 CM
Tensile strength of Fiber	3.5 to 5 G/Den
Elasticity of Fiber	1.8%

Although there is a strong demand for jute in both local and international markets, there are problems in increasing the productivity and profitability of jute. Developing jute varieties having low lignin content would result in better fiber quality and consequently better profitability (Bogoeva-Gaceva, Avella *et al.* 2007; Del Rio, Rencoret *et al.* 2009). This is because high lignin content hinders the processing of plants for various industrial purposes such as for the use of paper pulp, textile raw material, forage digestibility and biofuels (Kumar, Barrett *et al.* 2009; Alfermann 2010).

With the industrial revolution, extensive research yielded synthetic fibers, like nylon, rayon, acrylic, polyester and are popularized due to their easy usability and handy nature as well as low cost. However, ever since the negative impact of synthetic fibers has been realized, the search of eco-friendly fibers has gained tremendous speed. Even the world of fashion has become environmentally conscience. Style is paramount, and retailers are battling to keep costs low and shoppers tempted, but consumers are increasingly aware of the green credentials of all industries – including fashion. In their search for biodegradable fiber scientist have chosen jute as one of their probable candidates.

In terms of usage, production and global consumption, jute is second only to cotton (Roy, Bandyopadhyay *et al.* 2006). It is environmentally friendly as well as being one of the most affordable fibers; jute plants are easy to grow, have a high yield per acre and unlike cotton, have little need for pesticides and fertilizers. It is considered as the "Fiber of Future".

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Unexploited potentials of jute

Well versed as a renewable source, jute and allied fibers have long been found to be technologically sound and ideal for making various grades of papers. Being cheap and easily available in large quantities, it is an ideal lingo-cellulosic substrate for producing industrial raw materials of much higher values (Barbieri, Somigliana *et al.* 2007; Karmakar, Hazra *et al.* 2008). Natural fibers like jute have intrinsic properties – mechanical strength, low weight and low cost – that have made them particularly attractive to the automobile industry (Saha, Manna *et al.* 2010). For consumers, natural fiber composites in automobiles provide better thermal and acoustic insulation than fiberglass, and reduce irritation of the skin and respiratory system (Bergfjord and Holst 2010). The low density of plant fibers also reduces vehicle weight, which cuts fuel consumption. For car manufacturers, the molding process consumes less energy than that of fiberglass and produces less wear and tear on machinery cutting production costs. The use of natural fibers by Europe's car industry is projected to reach 100000 tons by 2010. German companies appear to lead the way. Daimler-Chrysler has developed flax-reinforced polyester composite, and in 2005 produced an award-winning spare wheel cover that incorporated abaca yarn from the Philippines. Japan's carmakers, too, are "going green" (Corrales, Vilaseca *et al.* 2007). In Indonesia, Toyota manufactures door trims made from kenaf and polypropylene, and Mazda is using a bio-plastic made with kenaf for car interiors (Plackett, Jankova *et al.* 2005).

 Not only the automobile industry, jute has entered various diversified sectors, where natural fibers are gradually becoming better substitutions (Corrales, Vilaseca *et al.* 2007; Asadullah, Rahman *et al.* 2008). Among these industries are: Celluloid products (Films), Nonwoven composites (Psydo-wood), Nonwoven textiles (for car interiors and other uses). Jute is also used as herbal medicine to control or prevent dysentery, worm and constipation etc. (Oboh, Raddatz *et al.* 2009). Jute leaves are used as health-food in Japan. Jute leaves contain anti-tumor agents as phytol and monogalactosyl-diacylglycerol. It may reduce risk of cancer (Herath, Suzuki *et al.* 2005).

Lignin: an organic polymer

Lignin is a multifaceted heteropolymer produced by the oxidative combinatorial coupling of mainly three monolignols (Zhao and Dixon 2011). In plants, lignin is found in the secondarily thickened plant cell walls and to a lesser extent, in the middle lamella region between cells (Ralph, Lundquist *et al.* 2004; Vanholme, Morreel *et al.* 2008; Zhong and Ye 2009; Vanholme, Demedts *et al.* 2010). Although virtually all plant cells are surrounded by a primary cell wall, a much thicker, lignified secondary cell wall is laid down inside the primary wall only in specialized cells collectively known as sclerenchyma (Zhong and Ye 2009; Vanholme, Demedts *et al.* 2010). Secondary wall formation proceeds only after cells have ceased elongating, under the control of a complex network of tissue-specific transcription factors, most of which are NAC or MYB domain-containing proteins (Fang, You *et al.* 2008; Zhong, Lee *et al.* 2010). Lignin deposited in the secondary wall impregnates the cellulose and Hemicellulose matrix found there, imparting upon it increased strength, rigidity, and hydrophobicity (Chabannes, Ruel *et al.* 2001; Jones, Ennos *et al.* 2001).

The majority of secondary wall formation in most plants occurs in the water-conducting cells of the xylem, which tend to be enriched in G lignin, and in the structural fibers, which in angiosperms typically contain higher levels of S subunits (Bonawitz and Chapple 2010; Goring 2012). Lignified cell walls are also found in sclereid cells, endodermal tissue in roots, and in specialized cells of anthers and some seed pods, where they are important for the dehydration-driven release (dehiscence) of pollen and seeds, respectively (Zhong and Ye 2009). The wood of both gymnosperm and angiosperm trees is composed largely of the secondary cell walls of vascular tissue and associated fibers, and as such, a substantial fraction of its weight is made up by lignin. It is important to note, however, that lignin biosynthesis is also of crucial importance in herbaceous (non-woody) species and that even in these species the majority of fluctuation through the phenylpropanoid pathway is dedicated to the synthesis of monolignols (Monties and Fukushima 2001; Vogel and Jung 2001; Bonawitz and Chapple 2010).
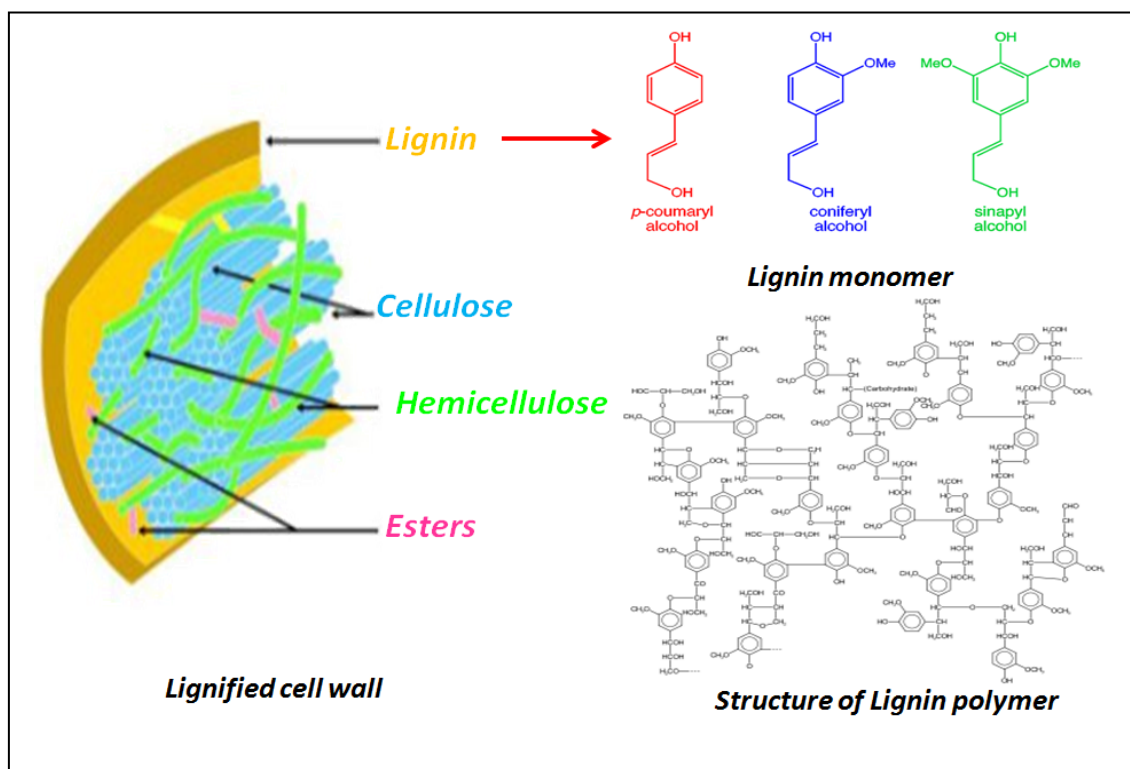
Fig.1.2: Lignified cell wall indicating major components of cell; Basic structure of lignin monomer and lignin polymer.

An overview of lignin synthesis, structure anddistribution

The pathway that leads to lignin biosynthesis can be divided into three main parts (Vanholme, Morreel *et al.* 2008). The first is the shikimate pathway that forms the aromatic amino acids phenylalanine, tyrosine, and tryptophan. The second part is the phenylpropanoid pathway that starts from phenylalanine and leads to the conversion of phenylalanine to cinnamic acid. The third part is the lignin-specific pathway that involves polymerization of monolignols via radical coupling. Monolignols are synthesized in the cytoplasm and transported to the cell wall, where they are oxidized prior to their incorporation into the polymer (Vanholme, Morreel *et al.* 2008).

The monolignols are products of the phenylpropanoid pathway, starting from phenylalanine (Vanholme, Demedts *et al.* ; Freudenberg 1959; Whetten and Sederoff 1995; Boerjan, Ralph *et al.* 2003; Vanholme, Demedts *et al.* 2010). Lignin, a product of the plant phenyl propanoid pathway, is also required for the synthesis of other hydrophobic polymers, such as cutin, suberin, and sporopollenin, as well as an array of soluble specialized metabolites, including flavonoids, hydroxy-cinnamic acids and

esters, lignans, tannins, and stilbenes (Cooper-Driver 2001; Umezawa 2010). The synthesis of lignin diverges from primary metabolism to form cinnamic acid through the deamination of phenylalanine. Deamination comprises a series of hydroxylation and subsequent O-methylation reactions which modify the aromatic ring of cinnamic acid and its side chain is curtailed to an alcohol moiety, resulting in the production of the three most abundant lignin monomers, or monolignols: p-coumaryl (4-hydroxycinnamyl) alcohol, coniferyl (3-methoxy 4-hydroxycinnamyl) alcohol, and sinapyl (3,5-dimethoxy 4-hydroxycinnamyl) alcohol. Upon incorporation into the lignin polymer, these monomers are referred to as p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units, respectively. In addition to this, the fractions of the polymer made up by each monomer vary substantially between species, between tissues in the same plant, by subcellular location, and in response to environmental stimuli (Campbell and Sederoff 1996; Ralph, Lundquist *et al.* 2004; Rogers and Campbell 2004; Goring 2012). Owing to differences in the bonding propensities of the three monomers, their relative abundance in a particular lignin polymer can significantly impact its overall structure and thus play a major role in determining its physical properties. A few notable exceptions notwithstanding, the lignin of angiosperms consists mainly of G and S subunits, with that of some monocots possibly containing relatively more H subunits. In contrast, the lignin of non-flowering vascular plants (gymnosperms, ferns, and lycophytes) is composed mostly of G subunits, lacks S subunits (again with a few exceptions), and is intermediate between dicots and monocots in the abundance of H subunits. It is also important to point out that many molecular species other than the three canonical monolignols can participate in the formation of the lignin polymer to varying degrees in different taxa, in some cases making up a substantial fraction of the total lignin polymer (Shadle, Chen *et al.* 2007; Lu and Ralph 2008). Particularly common noncanonical subunits include ferulates (which form crosslinks between lignin and hemicellulose), coniferaldehyde, and acylated monolignols containing acetate, p-coumarate, orp-hydroxybenzoate moieties (Sederoff, MacKay *et al.* 1999; Ralph, Lundquist *et al.* 2004; Ralph, Kim *et al.* 2008).
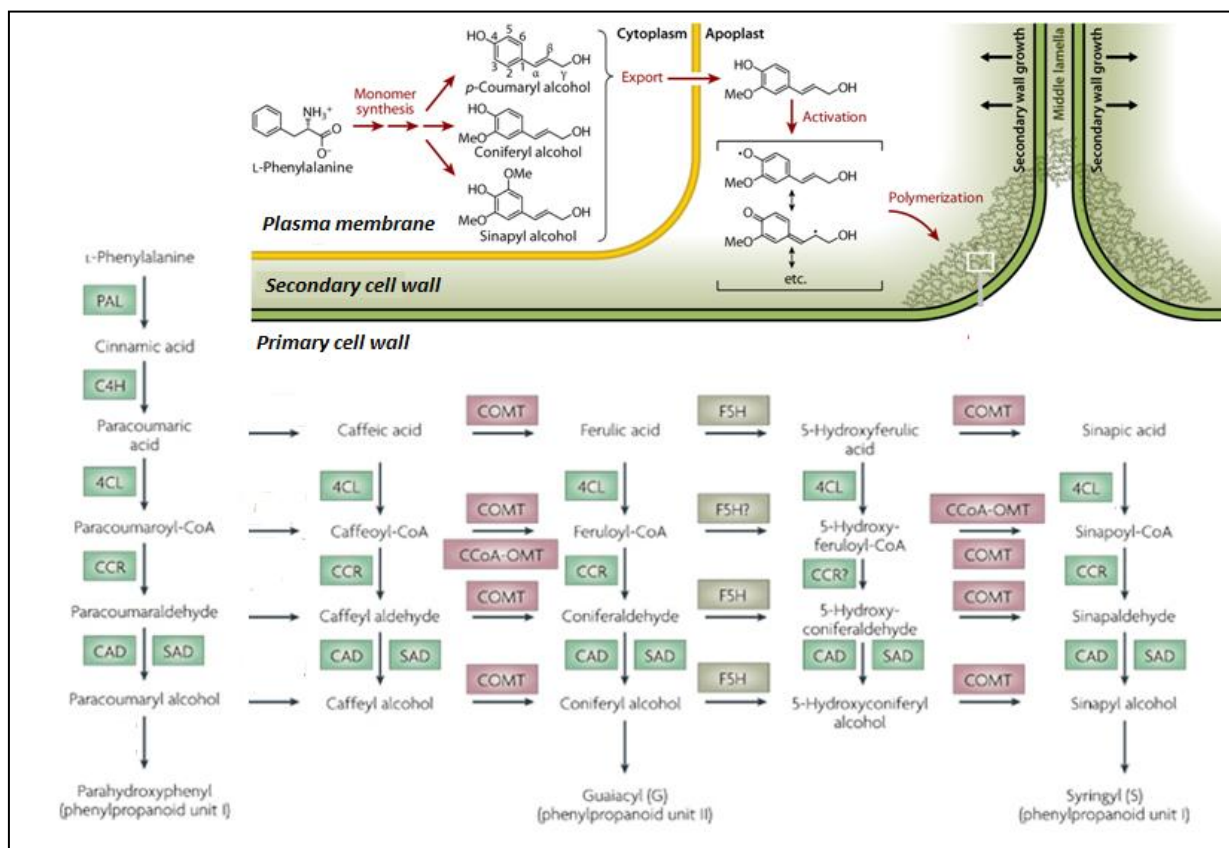
Fig.1.3: Lignin biosynthesis pathway (Sticklen 2010).

(4CL, hydroxycinnamate-CoA/5-hydroxyferuloyl-CoA- ligase; C3H, 4 hydroxycinnamate 3-hydroxylase; C4H, cinnamate 4-hydroxylase; CAD, hydroxycinnamyl alcohol dehydrogenase; CCoA-OMT, S-adenosyl-methionine caffeoyl-CoA/5-hydroxyferuloyl-CoA-O-methyltransferase; CCR, hydroxycinnamoyl-CoA:NADPH oxidoreductase; COMT, caffeate O methyltransferase; OMT: S-adenosyl-methione-caffeate/5 hydroxyferulate-O methyltransferase; PAL: phenyl ammonia lyase; SAD, sinapyl alcohol dehydrogenase)

Lignin monomers thus synthesized, are exported to the extracellular space (the apoplast) for incorporation into the lignin polymer (Whetten and Sederoff 1995; Samuels, Rensing *et al.* 2002; Boerjan, Ralph *et al.* 2003; Kaneda, Rensing *et al.* 2008). After reaching the apoplast, monolignols undergo single-electron oxidation thought to be mediated by wall bound laccases and/or peroxidases to form reactive radical species (Ralph, Lundquist *et al.* 2004). Bond formation can then occur between two such radicals (any combination of two monolignols, a monolignol and an elongating lignin polymer end, or two polymer ends) in a process known as bimolecular radical coupling. This reaction quenches both radicals, necessitating

another round of activation (i.e. oxidation or dehydrogenation) before polymer elongation can proceed (Boerjan, Ralph *et al.* 2003). Alternatively, activated monolignols may be involved in a radical transfer reaction whereby they oxidize a non-activated monolignol or oligolignol end unit (Boerjan, Ralph *et al.* 2003). The three different monolignols, and the lignin subunits derived from them, can freely form bonds with one another (Ralph, Kim *et al.* 2008). Depending on the ring substitution pattern of the molecules involved in coupling (H, G, or S) and their location (free or attached to the polymer), multiple sites may be available for bond formation, resulting in an array of potential coupling patterns (Ralph, Kim *et al.* 2008). Any time coupling involves the central (β) carbon of the monolignol tail (which is most frequently the case), one or more new chiral centers is formed, increasing the complexity of potential coupling products and the resulting lignin polymer still further. Over the last few decades, there was a huge controversy regarding the monolignol biosynthesis pathway and their complex derivatives; i.e. whether this complexity is directly controlled at the biochemical level or not (Gang, Costa *et al.* 1999; Hatfield, Ralph *et al.* 1999; Lewis 1999; Hatfield and Vermerris 2001; Rouhi 2001; Ralph, Lundquist *et al.* 2004; Davin and Lewis 2005; Davin, Jourdes *et al.* 2008; Ralph, Brunow *et al.* 2008). But the prevailing model proposes that, the coupling reactions driven lignin polymerization are dictated by the supply of monolignols in the apoplast the availability of oxidants and the activating enzymes. In addition to this, some common physical parameters also have influences which directly affect the chemical reactions viz. the stability of intermediates, steric interactions, temperature and pH (Freudenberg 1965; Hatfield and Vermerris 2001; Ralph, Lundquist *et al.* 2004; Ralph, Brunow *et al.* 2008).

Interplay between jute and lignin: Leads from other evidences

Lignin content of jute fiber (from *Corchorus capsularis)* was found to be 21.36 % (Sengupta and Palit 2004) whereas a fully grown jute plant stalk (or stick) is known to have lignin between 24 – 25% (Roy, Sen *et al.* 1991; Islam and Sarkanen 1993; Bhattacharyya 1996). Before retting, Haque and co-workers (2001) found the lignin content of the fiber to be about 15% which eventually came down to 12-13% on completion of retting (Haque, Asaduzzaman *et al.* 2001). In another study, lignin content of *Corchorus olitorius* was found much higher than *C. capsularis* (Sinha,

Sengupta *et al.* 2004; Sinha, Kar *et al.* 2011); the average fiber lignin content of *C. olitorius* was 17.058% whereas for *C. capsularis* it was 12.71%. In addition to this, a mature jute (*C. olitorius*) plant stem (stick and bast fiber) was found to have on an average 28-29% of lignin (Tanmoy, Alam *et al.* 2015).
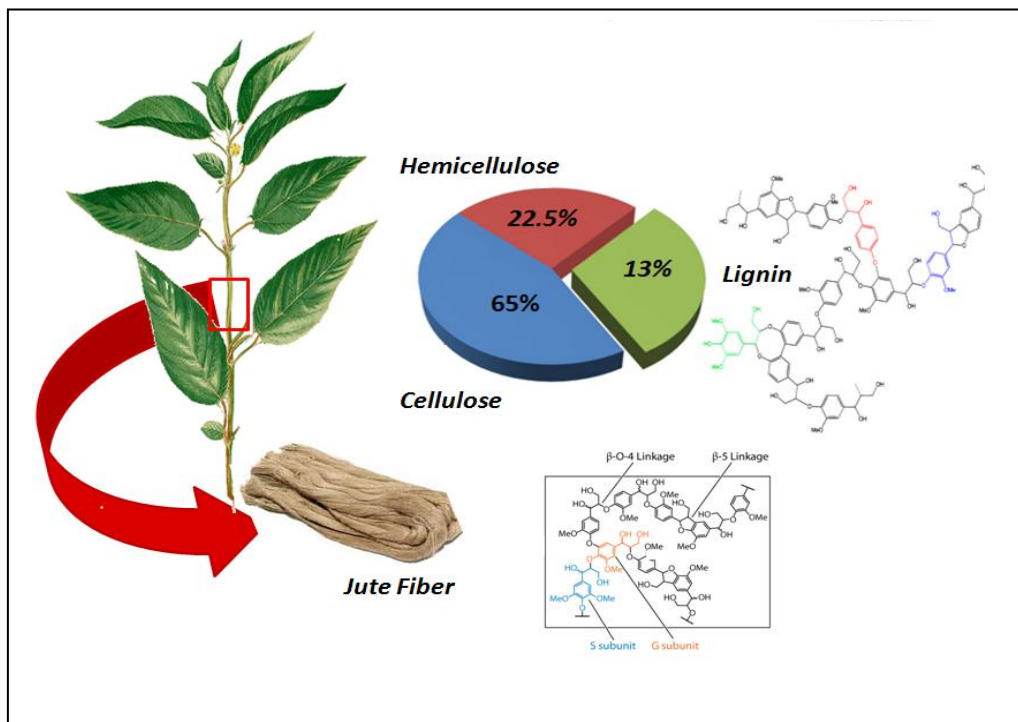

Fig.1.4: Represents major chemical constituents of jute fiber

The lignin structure has been characterized by Jose and co-workers (Del Rio, Rencoret *et al.* 2009), where the fiber lignin of *Corchorus capsularis* variety was subjected to pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS), 2D-NMR, and thioacidolysis. According to the reported data, jute fibers possess syringyl propanoid units with the S/G ratio being 2.1 and an H/G/S composition of 2:33:65. 2D-NMR of the milled wood lignin (MWL) isolated from jute fibers showed a predominance of β-O-4aryl ether linkages (72% of total side chains), followed by β-β resinol-type linkages (16% of total side chains) and lower amounts of β-5 phenylcoumaran (4%) and β-spirodienone-type (4%) linkages and cinnamyl end groups (4%). The high predominance of the S-lignin units, together with the high proportion of β-O-4aryl ether linkages, which are easily cleaved during alkaline cooking, are advantageous for pulping. On the other hand, a small percentage (ca. 4%) of the lignin side chain was found to be acetylated at the γ-carbon, predominantly over syringyl units.

As the second most abundant biopolymer on earth, lignin plays a critical role in the biogeochemical carbon cycle and affects the interaction between plants and other living organisms in a myriad of ways (Halpin, Thain *et al.* 2007). Huge variability is found in the lignin structure and content between different plant species (Halpin, Thain *et al.* 2007). Since lignin is the most recalcitrant component of a plant cell wall (Angelidaki and Ahring 2000), the higher the proportion of lignin the lower the bioavailability of the substrate (Richard 1996). Therefore the downstream processing of jute would benefit from a reduced amount of this polymer.

Lignin engineering

Lignin has a composite structure comprised entirely of natural polymers as it is mainly accountable for the strength and firmness of plant stems. Even though, lignin is an essential scaffold for plant growth and development, it is like a bane for the researchers who deal with the downstream processing of lignified crops. Many commercial processes have aimed at retrieving the polysaccharide component of plants for use in industrial applications (viz. paper pulp, textile, forage digestibility and biofuels), where lignin subtraction is an absolute necessity (Vanholme, Demedts *et al.* 2010). The non-linear aromatic and formidable polymer built with chemically diverse and poorly reactive linkages and a variety of monomer units, precludes the ability of any single enzyme to properly recognize and degrade it.

The lignin biosynthetic pathway has been the subject of many reviews (Baucher, Monties *et al.* 1998; Whetten, MacKay *et al.* 1998; Grima-Pettenati and Goffner 1999; Lewis 1999; Boudet 2000; Dixon, Chen *et al.* 2001; Anterola, Jeon *et al.* 2002; Anterola and Lewis 2002; Boerjan, Ralph *et al.* 2003), but is still being continually revised and updated to incorporate the results of new research. The processes underlying lignification and its alteration have recently been approached using both classical forward genetic screens and targeted reverse genetic approaches such as antisense suppression, RNAi and characterization of insertional mutants (Bonawitz and Chapple 2010).

The genetic control of lignin deposition has been comprehensively analyzed as an important repercussion of silencing lignin biosynthesis as a whole. In normal plants, deposition of lignin must be regulated in conjunction with plant development. The spatial and temporal control of lignification is critical in plant support, water transport

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and disease resistance. Plants that are compromised in their ability to synthesize normal quantities of lignin lose their capacity to support the plant body. Thus the risk of generating plants unable to grow well was taken into note and the gene was chosen accordingly (Rogers and Campbell 2004).

Researchers till date have elucidated the main biosynthetic routes of monolignols biosynthesis and demonstrated that lignin amounts can be engineered and that plants can cope with large shifts in hydroxyphenyl/guaiacyl/syringyl (H/G/S) lignin compositional ratios. Systems approaches, in which the plant's response to engineering of a single gene in the pathway is studied at the organismal level, are beginning to shed light on the interaction of lignin biosynthesis with other metabolic pathways and processes (Vanholme, Morreel *et al.* 2008).

Lignin biosynthetic pathway perturbation: Targeting the important lignin biosynthetic genes in other plants

The monolignol biosynthetic pathway has been characterized by *in vitro* experiments testing the alteration of transitional compounds by enzymatic reactions at every metabolic phase (Anterola, Jeon *et al.* 2002; Anterola and Lewis 2002). Transgenic plants or mutants with customized expression of monolignol biosynthesis genes have been applied to disclose the role of the genes encoding each enzyme of the lignin metabolic complexes. The shikimate pathway provides the aromatic amino acid, phenylalanine that constitutes the entry point of the monolignol biosynthesis. The doorway point enzyme to the phenylpropanoid pathway, phenylalanine ammonia lyase (PAL), was considered to be a vital first target to lessen carbon flow into the monolignol biosynthetic pathway. PAL down-regulated tobacco plants demonstrated a number of irregularities such as stunted growth, deformed flowers, and leaf epinasty with exceedingly reduced overall lignin content (Bate, Orr *et al.* 1994). Impaired vascular integrity of PAL down-regulated plants is presumable due to the reduced lignin level. The diverse functions of PAL in different metabolic processes suggest that the modification of this enzyme may cause unpredictable physiological changes not directly related to downstream reduction of lignin (Kim 2010).

Cinnamate4-hydroxylase (C4H), p-coumarate 3-hydroxylase (C3H), and ferulate 5-hydroxylase (F5H) are cytochrome P450-dependent monooxygenases, mainly involved with conversion of cinnamate into 4-hydroxy-cinnamate, introduction of a hydroxyl group into the *meta*-position of phenylpropanoid, and hydroxylation at C-5 on the aromatic ring providing the substitution pattern necessary for S unit formation, respectively. The function of these P450 genes was investigated using transgenic plants transformed with antisense genes or knockout mutant lines in arabidopsis (Franke, Hemm *et al.* 2002), tobacco (Blount, Korth *et al.* 2000) and populus (Lu, Zhou *et al.* 2006). Interestingly, down-regulation of the C4H gene by antisense constructs induced substantial reductions in lignin content, but no visible abnormalities in the growth and development of transgenic tobacco (Sewalt, Ni *et al.* 1997; Blount, Korth *et al.* 2000). The lignified regions in transgenic plants were similar to wild-type plant, although staining intensity was diminished in plants transformed with C4H antisense constructs. These results suggest that C4H is a target for the progressive and quantitative reduction in lignin content without serious compositional change. C4H is encoded at a single locus in arabidopsis and pea (Bell-Lelong, Cusumano *et al.* 1997; Franke, Hemm *et al.* 2002), whereas it has a small gene family in alfalfa (Fahrendorf and Dixon 1993), mung bean (Mizutani, Ward *et al.* 1993), and *Catharanthus roseus* (Hotze, Schröder *et al.* 1995). Expression pattern of C4H mRNA was analyzed in arabidopsis by RNA blot hybridization and C4Hpro-GUS fusion assay (Bell-Lelong, Cusumano *et al.* 1997). The highest levels were found in stem and root tissue, and the accumulation of C4H mRNA was substantially enhanced in light-grown seedlings and wounded tissue. GUS staining was strongly localized to the vein of the mature leaves and restricted to the xylem and parenchyma in stem cross-sections. Consistent with C4H mRNA accumulation, C4H-driven GUS was also induced by wounding. C4H activity was induced by a number of other treatments such as light, wounding and the application of exogeneous p-coumaric acid (Lamb and Rubery 1976; Bolwell and Dixon 1986). There are no reports of arabidopsis mutants defective in C4H activity. It is supposed that loss-of-function of C4H would be lethal due to the impact of the mutation on vascular development. C4H promoter of populus was also characterized by GUS fusion assay (Zhao, Lu *et al.* 2005). A putative C4H promoter fragment was isolated from genomic DNA of *Populus tomentosa* by PCR, and PC4Hpro-GUS construct was introduced to tobacco plants. Histochemical GUS analysis showed the expression of C4H-GUS fusion

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product to be mainly localized in the lignified tissues and its activity gradually increased from the first to the ninth internode in the stem of tobacco. Over-expression and down-regulation experiments for the C4H gene were conducted in tobacco transformed with sense or antisense constructs containing 35S promoter and alfalfa C4H cDNA (Sewalt, Ni *et al.* 1997). Reduction of C4H activity by antisense expression resulted in reduced levels of Klason lignin, accompanied by a decreased syringyl/guaiacyl monomer ratio (Coleman, Park *et al.* 2008). Treatment with safranin-O and astra-blue which stains lignin and cellulose fibers, respectively, confirmed that with the down-regulation of C4H, changes in color of distinct rows in the vascular tissue was observed with effects not evenly distributed over different cell types. C4H genes that exist as a multigene family in *Populus* were isolated and identified to understand the roles of individual C4H members (Lu, Zhou *et al.* 2006). Two C4H cDNA (PtreC4H) isolated from *Populus tremuloides* and three C4H genes (PtriC4H) identified in the *P. trichocarpa* showed different expression patterns in various tissues, and these differential expression patterns of C4H genes were associated with *cis*-acting regulatory elements such as L, P and H boxes (Lu, Zhou *et al.* 2006).

C3H, a member of P450 family (CYP98A3), is also a key enzyme participating in the formation of G and S monolignol units. The C3H gene is present in the arabidopsis genome as a single copy, and the function of this gene was identified and characterized by knock-out mutants and promoter assays (Franke, Hemm *et al.* 2002; Nair, Xia *et al.* 2002; Schoch, Morant *et al.* 2006). Expression pattern of C3H gene was similar to that of C4H gene (Schoch, Goepfert *et al.* 2001). Highest expression was detected both in stems and roots associated with lignifying tissues. C3H specific expression was also analyzed during arabidopsis development by promoter-GUS fusions. The C3H promoter showed more restricted tissue-specific expression compared to C4H (Nair, Xia *et al.* 2002). C3H was not expressed in the seeds and only expressed in the vascular tissue of petals, sepals, anthers, and stigmas, whereas C4H showed an overall expression in flower tissues and a very intense expression in seed. A mutation of C3H gene in arabidopsis displayed a dwarfed phenotype with ~20% lower lignin content than the wild type by the thioglycolic acid extraction method and ~40% of control as measured as Klason lignin (Franke, Hemm *et al.* 2002). This mutant possessed only H units, whereas wild type plants have only trace

levels. This indicates that the pathways to both coniferyl and sinapyl alcohol were blocked, and H units were made to compensate for missing G and S units in these C3H mutants. These results demonstrate that the suppression of C3H has the effect of restricting carbon flow into the monolignol and lignin pathway, and has a significant effect on lignin content and assembly. Since alfalfa C4H and C3H antisense lines driven by the PAL2 promoter showed improved digestibility with reduced lignin content without serious phenotypic changes, the early pathway genes such as C4H and C3H are considered as viable targets to achieve improvement of biomass quality by genetic modification. The application of tissue specific promoter such as PAL2 is also considered as an effective approach to avoid deleterious effects such as a dwarfed phenotype caused by the constitutive suppression of lignin biosynthesis genes.

Down-regulation of 4-coumarate: coenzyme A ligase (4CL) led to a reduction of lignin content in tobacco, arabidopsis, and aspen, but their phenotypes displayed significant species-specific differences (Kajita, Hishiyama *et al.* 1997; Lee, Meyer *et al.* 1997; Hu, Harding *et al.* 1999). 4CL down-regulation in aspen showed increased growth with no visible phenotypic change, and dwarfed growth was detected in arabidopsis and tobacco. These results indicate that different species may have differential intrinsic capabilities in tolerating large variations in lignin content and composition.

Reduction in lignin in cell walls of transgenic plants can be compensated for by an increase in other compounds that can be integrated as an essential component of polymeric lignin (Ralph, Hatfield *et al.* 1998). Since all 4CL down-regulated plants were generated by antisense constructs, differently suppressed levels of the 4CL gene might be another reason of species-specific differences in transgenic phenotypes in these species. Hence, greater suppression of the 4CL gene using RNAi silencing could be applied to characterize gene function.

Transgenic down regulation of downstream enzymes in the monolignol pathway (CAD, COMT, and CCR) exhibited relatively minor reductions in lignin content, whereas the down-regulation of upstream enzymes in this pathway resulted in a more dramatic decrease in lignin contents (Chen, Srinivasa Reddy *et al.* 2006). Simultaneous change of multiple downstream enzymes showed additive reductions in lignin content (Pinçon, Chabannes *et al.* 2001). The repression of such genes has been

all together achieved either by hybridization of single gene transformants [COMT and CCR (Pinçon et al., 2001), CAD and cinnamoyl-CoA reductase (CCR) (Chabannes, Barakate *et al.* 2001)] or by double transformation events [COMT and caffeoyl CoA O-methyltransferase (CCoAOMT) (Guo, Chen *et al.* 2001)].

The brown midrib mutations (bm1, bm2, bm3, and bm4) in maize are linked with major modifications in lignin composition and content in stover (Pedersen, Vogel *et al.* 2005). The bm1 mutation decreases cinnamyl alcohol dehydrogenase (CAD) activity (Halpin, Holt *et al.* 1998) while bm3 results in reduced caffeic acid O-methyltransferase (COMT) activity (Vignols, Rigau *et al.* 1995). Diminution in lignin content of maize has recently been accomplished by down-regulation of COMT using antisense constructs, with transgenic plants showing very similar characteristics to the maize bm3 mutant phenotype (Piquemal, Chamayou *et al.* 2002). Down-regulation of COMT to low activity levels also reduces lignin content in alfalfa, maize, and poplar by 30%, 30%, and 17%, respectively (Chabbert, Tollier *et al.* 1994; Jouanin, Goujon *et al.* 2000; Guo, Chen *et al.* 2001). The phenotypes of maize brown midrib mutants offer constructive information to approximate the probable impact of lignin reduction in grass species sharing similar stalk structures. Although maize bm3 mutants showed moderately normal phenotypes, this mutation had a negative effect on maize stalk rigidity. Stem stalk strength of bm3 mutants was reduced (17-26% decrease of crushing strength and 8-14% decrease of stalk-section weight) resulting in maize plants prone to stalk collapse and lodging (Zuber, Colbert *et al.* 1977). Sorghum brown midrib (bmr) mutants generated via chemical mutagenesis of seeds also confirm lower lignin content with reduced vigor and grain yield (Porter, Axtell *et al.* 1978). Selection of the most appropriate mutant having the lowest lignin content with highest fiber digestibility without a reduction in overall biomass yield was possible among the various bmr mutant lines (Oliver, Pedersen *et al.* 2005). These results highlight the significance of mutant pools having difference in lignin content and developmental phenotypes in order to select commercially valuable genotypes as biofuel crop resources. The following table (table 1.3) depicts a brief overview of lignin engineered plants.

Table 1.3: Overview of lignin engineered plants

Genes	Plants	Lignin Content	Lignin-Composition	References
COMT	*Nicotiana tabacum*	Reduced	Klason and acid soluble lignin reduced (S and G decreased)	(Ni, Paiva *et al.* 1994)
	Arabidopsis thaliana	No changes	No changes	(Zhong, Morrison *et al.* 1998; Goujon, Ferret *et al.* 2003; Goujon, Sibout *et al.* 2003)
	Medicago sativa	Reduced	Klason lignin reduced (S/G ratio decreased)	(Guo, Chen *et al.* 2001)
	P. tremuloides	No changes in Klason lignin	(S/G ratio decreased)	(Tsai, Popko *et al.* 1998)
	Sorghum bicolor Zea mays p. tremula x P. alba	Klason lignin reduced	S lignin decreased	(Pillonel, Mulder *et al.* 1991; Chabbert, Tollier *et al.* 1993; Suzuki, Lam *et al.* 1997)
CCoAO MT	*Medicago sativa*	Klason lignin reduced	S/G ratio decreased	(Guo, Chen *et al.* 2002)
	Nicotiana tabacum	Klason lignin reduced	S/G ratio increased; S and G lignin decreased	(Zhong, Morrison *et al.* 1998)
	P. tremula x P. alba	Klason lignin and acetyl bromide reduced	S/G ratio increased; S and G lignin decreased	(Franke, McMichael *et al.* 2000; Zhong, Morrison *et al.* 2000)
C3H	*Arabidopsis thaliana*	Klason and acid soluble lignin reduced	S/G ratio decreased; H lignin decreased	(Franke, Hemm *et al.* 2002; Franke, Humphreys *et al.* 2002)
F5H	*Arabidopsis thaliana*	Klason lignin reduced	S lignin increased; G lignin decreased	*(Sibout, Baucher et al. 2002)*

	Nicotiana tabacum	Klason and acid soluble lignin reduced	S/G ratio increased; G lignin decreased; S lignin increased	(Franke, McMichael *et al.* 2000; Zhong, Morrison *et al.* 2000)
	P. tremula x P. alba	Klason lignin reduced	G and S lignin decreased; S/G ratio increased.	(Li, Popko *et al.* 2000; Li, Zhou *et al.* 2003)
	P. tremuloides	No changes in Klason lignin	S/G ratio increased	
4CL	*Arabidopsis thaliana*	Klason and acid soluble lignin reduced	Vanillin and vanillic acid increased; syringaldehyde/vallic acid decreased	(Li, Zhou *et al.* 2003)
	Nicotiana tabacum	Klasion lignin and acetyl bromide reduced	S and G lignin decreased; H lignin increased	(Kajita, Hishiyama *et al.* 1997)
	P. tremuloides	Klasion lignin reduced	S/G ratio increased	(Li, Zhou *et al.* 2003)
PAL	*Nicotiana tabacum*	Klason and acid soluble lignin reduced	S and G lignin decreased; S/G ratio increased	(Korth, Blount *et al.* 2001)
POX	*Lycopersicon esculentum*	Klason and acid soluble lignin increased	No changes	(Dean, LaFayette *et al.* 1998)
	Nicotiana tabacum	No changes	No changes	(Chabbert, Monties *et al.* 1992; Lagrimini, Gingas *et al.* 1997; Elfstrand, Sitbon *et al.* 2002)
	Populus kitakamiensis	No changes in Klasion lignin'acetyl bromide reduced.	No changes in S/G/H lignin; thioacidolysis increased	(Yahong, Tsuji *et al.* 2001) (Christensen, Overney *et al.* 2001; Christensen, Van Montagu *et al.* 2001)
	P. tremula x P. alba	No changes in Klasion lignin'acetyl	No changes in S/G/H lignin;	Christensen *et al.*(2001)
CCR	*Arabidopsis thaliana*	Klason lignin reduced	S/G ratio decreased	(Goujon, Ferret *et al.* 2003; Goujon, Sibout *et al.* 2003)
	Nicotiana tabacum	Klason lignin reduced	S/G ratio increased; S and G lignin decreased	(Ralph, MacKay *et al.* 1997; O'Connell, Holt *et al.* 2002)

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	P. tremula x P. alba	Klason lignin reduced	S/G ratio increased;	(Boerjan, Ralph *et al.* 2003)
CAD	*Arabidopsis thaliana*	Klason lignin reduced	S/G ratio decreased; S lignin increased ; G lignin decreased	(Baucher, Monties *et al.* 1998; Sibout, Eudes *et al.* 2003)
	Medicago sativa	No changes in total Klason lignin content	S/G ratio decreased	(Baucher, Monties *et al.* 1998)
	Nicotiana tabacum	Klason lignin reduced	S/G ratio increased; S and G lignin decreased	(Chabannes, Barakate *et al.* 2001)
	Pinus taeda	Klason lignin reduced	No changes in S/G/H lignin	(MacKay, O'Malley *et al.* 1997; Ralph, MacKay *et al.* 1997)
	P. tremula x P. alba	Klason lignin reduced	No changes in S/G/H lignin ratio.	(Baucher, Chabbert *et al.* 1996)
	Sorghum bicolor	Klason and acid soluble lignin decreased	S and G lignin decreased	(Suzuki, Lam *et al.* 1997) (Chabbert, Tollier *et al.* 1993)
	Zea mays	Klason lignin reduced	S and G lignin decreased	(Pillonel, Mulder *et al.* 1991; Halpin and Boerjan 2003)
C4H	*Nicotiana tabacum*	Klason lignin reduced	S and G lignin decreased	(Sewalt, Ni *et al.* 1997)
LAC	*Liriodendron tulipifera*	No changes in total Klason lignin content	No changes	(Dean, LaFayette *et al.* 1998)
	P. tremula x P. alba	No changes	No changes	(Ranocha, Chabannes *et al.* 2002)

RNA interference: a powerful tool for genetic manipulation

RNA interference (RNAi) has been a breakthrough of the gene modulation age. It has emerged as a boon for molecular biologists since first reported by Rich Jorgensen in 1990 with his spectacular petunia experiment. The discovery of the phenomenon of RNA silencing is the result of a unexpected unearthing related to loss of pigmentation in petunia flower (Napoli, Lemieux *et al.* 1990). Other foundationally related

experiments shed enough light for the understanding of the RNAi phenomenon, including unraveling the mystery of individual gene function. RNA silencing is an occurrence that refers to the regulation of gene expression through nucleotide-sequence specific interaction mediated by small non-coding RNAs (Agrawal, Dasaradhi *et al.* 2003; Baulcombe 2004). Since then it outgrew as a rapidly developing field of biology and a wealth of knowledge has accumulated regarding the mechanisms and machineries of RNAi. A major contribution of RNAi technology is that it gives scientists the liberty to silence genes on a sequence specific manner which is often not the case in insertion and deletion based mutant library. The technology has become a prevailing tool to understand the functions of individual genes and is also useful for molecular breeders to produce improved crop varieties with specific traits, particularly in situations where existing methods failed.

RNAi: The History and Overview

Prior to the discovery of RNAi, scientists applied various methods such as insertion of T-DNA elements, and transposons, treatment with mutagens or irradiation and antisense RNA suppression to generate loss-of-function mutations (Williams, Clark *et al.* 2004). These approaches allowed scientists to study the functions of a gene or gene family of interest in an organism. Apart from being time-consuming, the above methods did not always work satisfactorily. For instance, transposons and T-DNA elements were found to occasionally insert randomly in the genome resulting in highly variable gene expression (Liu, Mitsukawa *et al.* 1995). Furthermore, in many instances the particular phenotype or a trait could not be correlated with the function of a gene of interest. It is in this backdrop that the RNAi phenomenon was discovered. Eventually leading to the discovery of RNAi, antisense RNA suppression was an early form of RNA silencing employed mainly by plant scientists.

This process involved the introduction of the antisense strand of RNA into a cell that corresponded to the target mRNA, the transcript intended to be silenced (Knee and Murphy 1997; Brantl 2002). After entry into the cell, the introduced antisense RNA and the native target mRNA would bind via complementary base pairing preventing the translation of mRNA. This is due to the inability of ribosomes to bind to dsRNA

(Brantl 2002; Arenz and Schepers 2003). This process, however, did not always result in a loss of function of a targeted gene. This led concerned scientists to continue the search for other methods of gene silencing. Fire *et al.* (1998) took the antisense silencing approach a step further, in *C. elegans*, with simultaneous introduction of both the sense and antisense strands of the targeted mRNA resulting in a 10-fold higher potency at silencing the gene than treatment with the antisense or sense strand alone. By injecting the two strands (sense and antisense) simultaneously the scientists were, in fact, creating the double stranded RNA required for starting the RNA interference pathway.

In plants, RNA silencing phenomena was first depicted during over-expression of chalconesynthase (CHS) in pigmented petunia petals by introducing a chimeric petunia CHS gene. Instead of enhanced expression it unexpectedly created a block in endogenous anthocyanin biosynthesis (Napoli, Lemieux *et al.* 1990). It was later confirmed that the introduction of the CHS transgene led to the inhibition of endogenous gene expression. This phenomenon was termed as co-suppression. Further studies have established that such inhibition is not due to the reduced transcription but due to the degradation of the transcript through a partial mRNA duplex formation. In another study, transgenic tobacco plants expressing untranslatable parts of the plant viruses proved to be highly resistant to the homologous virus, but not to related viruses (de Haan, Gielen *et al.* 1992; Lindbo and Dougherty 1992). It was later shown that, even non-transgenic plants that recovered from a viral infection showed resistance against an unrelated virus provided it carried a sequence insert from the first inoculated virus (Ratcliff, MacFarlane *et al.* 1999). These observations were subsequently described as 'co-suppression', 'post-transcriptional gene silencing' or 'virus induced gene silencing' (Smyth 1997; Hammond, Bernstein *et al.* 2000; Lu, Martin-Hernandez *et al.* 2003). Similar phenomenon was described in *Neurospora crassa*, where introduction of homologous RNA sequences caused sequence-specific RNA degradation or 'quelling' of the endogenous gene (Romano and Macino 1992). Similarly it was observed that in *C. elegans* introduction of sense or antisense RNA resulted in the degradation of the endogenous messenger (Guo and Kemphues 1995). The breakthrough came with the observation that injection of dsRNA in *C. elegans* resulted in the degradation of

endogenous mRNA. This phenomenon was introduced as 'RNA interference' (RNAi) by Fire *et al.* (1998).

It has since been implicated in natural virus resistance and basic biological processes such as development, gene regulation and chromatin condensation (Fagard and Vaucheret 2000; Bucher and Prins 2006). The emerging view is that RNA silencing is a part of a sophisticated network of interconnected pathways for cellular defense (pathogen resistance and stabilization of mobile genetic elements), RNA surveillance (chromatin remodelling, genome organization and stability) and development. This RNA regulated silencing can inhibit gene expression at the transcriptional level (Transcription Gene Silencing, TGS) or at the post-transcriptional level (Post Transcription Gene Silencing, PTGS). The TGS operates in the nucleus and limits gene expression by inducing mostly the cytosine methylation of DNA and post-transcriptional modifications of histone proteins (e.g. H3-methylation at lysine-9). On the other hand PTGS is mainly a cytoplasmic event that acts at the transcript level by way of repression of translation or cleavage of the target mRNA (Hammond, Caudy *et al.* 2001; Vaucheret, Béclin *et al.* 2001; Escobar and Dandekar 2003).

Biochemical studies indicate that RNAi proceeds via a two-step mechanism. In the first step, long dsRNAs, are produced locally or taken up by the cells, and cleaved by the RNase III-like nuclease DICER, which generates 21-23 nt duplex RNAs called short interfering RNA (siRNA). In the second step, siRNAs are incorporated into a RNA-induced silencing complex (RISC). The antisense strand of the duplex serves as a guide that directs RISC to recognize the cognate mRNA in the PTGS pathway (Hannon 2002). The siRNA can also guide the RNA-induced initiation of TGS (RITS) complex to switch off gene transcription (Verdel, Jia *et al.* 2004).
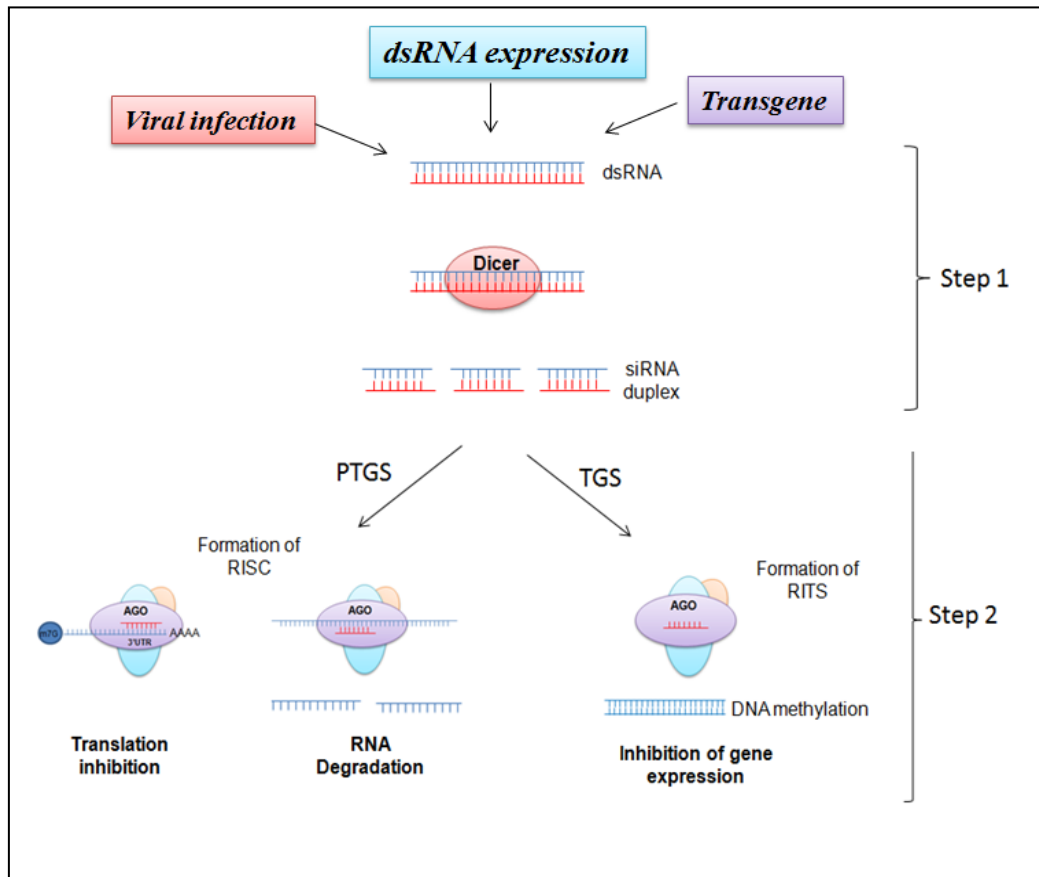
Fig.1.5: Mechanism of RNAi

Due to biochemical dissection of components of the silencing pathway in several model organisms, such as *Arabidopsis thaliana, Caenorhabditis elegans* and *Drosophila melanogaster* the general understanding about RNA silencing mechanisms have greatly advanced in recent years and led to an exponential expansion of research in silencing involving the sequence-specific cellular degradation of target RNAs (Agrawal, Dasaradhi *et al.* 2003). The silencing process is very efficient, a few dsRNA molecules can trigger inactivation of a continuously transcribed target mRNA for prolonged periods of time (Travella, Klimm *et al.* 2006). This inactivation persists through cell division and in some organisms can spread to untreated cells and tissues; when the RNA silencing spreads into germ line cells it can even be inherited by subsequent generations (Grossniklaus, Kelly *et al.* 2013; Kelly 2014). Amplification and spreading of RNA silencing has been demonstrated in plants, nematodes and *in vitro* in Drosophila extracts, but has not yet been observed in mammalian systems.

The trigger of RNA silencing

It is crucial to understand the trigger of RNA silencing mechanism. The RNA silencing can be triggered by different classes of double-stranded RNA (dsRNA) viz. endogenously by inverted repeats, transposons, intragenic regions and exogenously by transgene, viral infection etc. (Liu, Mitsukawa *et al.* 1995). During RNA virus replication, folding of the replicated ssRNA and partial or complete annealing of positive and negative strand RNAs can all lead to the formation of dsRNA (Donaire, Barajas *et al.* 2008). For DNA virus, dsRNA is produced by bidirectional transcription or terminal repeats. For retro-transposons, gene silencing happens when they are integrated into the vicinity of host genes and read-through transcription will lead to the formation of dsRNA. While dsRNA is formed in a cellular system, the bimolecular or folded monomolecular dsRNA is recognized by an effector referred to as DICER, a dsRNA specific RNase III-type endonuclease. DICER processes dsRNAs into siRNAs that vary in length from 20-24nt (Fire, Xu *et al.* 1998; Hamilton and Baulcombe 1999; Elbashir, Harborth *et al.* 2001; Wesley, Helliwell *et al.* 2001). Animals usually encode a single type of DICER to generate various classes of siRNA with exceptions of Drosophila and *C. elegans* each encoding two DICERs. Plants on the other hand have proteins referred to as DICER like proteins. Arabidopsis possess 4 DCLs whereas rice possesses 6 different DCLs.

Virus induced gene silencing (VIGS): Virus as a target of RNA silencing

RNA silencing emerged as an ancient RNA surveillance system that is conserved amongst the eukaryotes and acts as a natural defense against invasive nucleic acids, including viruses. RNA silencing plays a major role in antiviral defense mechanism in plants. Most plant viruses are RNA viruses, which replicate via double stranded replication intermediates, acting as a trigger for RNA silencing. Further viral mRNA recognized by the host plant as aberrant are also converted into dsRNA by plant RNA-dependent RNA polymerase (RdRPs). Double stranded RNA of virus is processed by DCLs into siRNAs, and then recruited into host Argonaut like protein AGOs to form RISC.

There is a good deal of genetic support for the importance of RNAi in antiviral defense (Cullen 2002; Lecellier, Dunoyer *et al.* 2005; Wilkins, Dishongh *et al.* 2005; Obbard, Gordon *et al.* 2009; Ding 2010). Arabidopsis strains defective in post transcriptional gene silencing are more susceptible to virus infections, and a substantial number of plant viruses encode proteins that counter silencing (Mourrain, Beclin *et al.* 2000). It has been shown that *Drosophila melanogaster* strains defective in RNAi core components, DICER2 (essential for miRISC directed translational repression) and AGO-2, are highly hypersensitive to infection, resulting in a 1000-fold increase in virus production (Galiana-Arnoux, Dostert *et al.* 2006; van Rij, Saleh *et al.* 2006; Wang, Aliyari *et al.* 2006; Zambon, Vakharia *et al.* 2006).

RNA silencing as an antiviral defense mechanism is now being exploited to design vectors for studying plant gene functions and raise virus resistant plant systems (Waterhouse and Helliwell 2003; Robertson 2004; Becker and Lange 2010; Senthil-Kumar and Mysore 2011). Virus Induced Gene Silencing (VIGS) is a virus based vector technology that exploits the defense mechanism of RNA. The first report of the VIGS was published as long back as 1929 by McKinney where tobacco plants infected with green strains of Tobacco Mosaic Virus (TMV) was protected against infection by yellow strain of TMV. Later this mechanism was explained as cross protection (Wilson 1993). Interestingly it turned out to be a RNA mediated cross protection (Ratcliff, Harrison *et al.* 1997). Knocking out genes to create mutant phenotype for investigating the function of a gene is the most frequently used strategy of reverse genetics. Two insertional mutagenesis approaches for gene disruption which have been predominantly used in plants are transferred DNA (T-DNA) (Krysan, Young *et al.* 1999) and transposons tagging (Parinov, Sevugan *et al.* 1999; Speulman, Metz *et al.* 1999). VIGS is a simple, rapid and transformation free technology. VIGS serves as a powerful tool for studying gene functions and metabolic pathways. VIGS vectors are mainly derived from viruses such as tobacco mosaic virus (TMV), potato virus X (PVX), tobacco rattle virus (TRV), cabbage leaf curl virus (CaLCuV), tomato yellow leaf curl china virus (TYLCV) (Unver and Budak 2009). Depending on the host, a VIGS vector can trigger siRNA based silencing in diverse plant species including dicots such as *Arabidopsis thaliana, Solanum lycopersicum, Nicotiana benthamiana, Pisum sativum* (Dalmay, Hamilton *et al.* 2000; Liu, Ishitani *et al.* 2000) and some monocot species like *Hordeum vulgare, Triticum*

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aestivum and *Zea mays* (Hein, Barciszewska-Pacak *et al.* 2005; Ding and Voinnet 2007; Ma, Liu *et al.* 2009). In the last few years, VIGS has frequently been applied for elucidation of gene function in eudicot species *Papaver somniferum, Eschscholzia californica* and *Cysticapnos vesicaria* (Hileman, Drea *et al.* 2005; Wege, Scholz *et al.* 2007; Hidalgo, Bartholmes *et al.* 2012) and to study genes involved in vindoline biosynthetic in *Catharanthus roseus* (Liscombe and O'Connor 2011). One of the major limitations of this approach is to identify an appropriate virus that can infect a wide range of hosts without causing any disease symptoms (Senthil-Kumar and Mysore 2011). However, solution for such a limitation has been developed to some extent with the advancement of this technology. A vector has been devised by modifying CaLCuV-based vectors, named as MIR VIGS, which carried endogenous miRNAs (miR156 and miR165) (Tang, Wang *et al.* 2010). By using MIR VIGS, several endogenous genes have been silenced in *Nicotiana benthamiana* (Tang, Lai *et al.* 2013). Nevertheless owing to few salient features for instance, accuracy, efficiency, and ease in handling, sRNAs-mediated gene silencing became popular in a very short time (Ossowski, Schwab *et al.* 2008).

Small RNA (sRNA): as a prevailing regulator of gene expression

Over the couple of last decades, small RNAs (sRNAs) have been recognized as important regulators of developmental and physiological programs of animals and plants (Fire, Xu *et al.* 1998; Lau, Lim *et al.* 2001; Lee and Ambros 2001; Reinhart, Weinstein *et al.* 2002; Ossowski, Schwab *et al.* 2008). Apart from slicer activity, sRNAs can affect normal gene expression at several steps, for example minimizing promoter activity and inhibiting protein synthesis of the transcribed mRNAs (Baulcombe 2004; Brodersen, Sakvarelidze-Achard *et al.* 2008). Depending on nucleotide length, biogenesis and mode of action, sRNAs are heterogeneous and versatile molecules, and are classified in two major classes termed as small interfering RNAs (siRNAs) and microRNAs (miRNAs) (Carthew and Sontheimer 2009; Ghildiyal and Zamore 2009).

siRNA

The siRNAs are short (21-24nt) double stranded RNAs with characteristic 3' two nucleotide overhang and a 5' phosphate. The foremost requirement for the siRNA generation is long, linear perfectly base paired double stranded RNA, introduced directly into the cytoplasm or taken up from the environment. Defining features of small RNAs are their short lengths i.e. 21-24 nucleotides and their association with AGO family of proteins, another key feature defining their function.

In the last couple of years, several genome sequencing studies revealed the presence of new cohort of sRNAs. In addition to defined groups of sRNAs, some other categories of sRNAs such as trans-acting sRNAs or phased siRNAs (phasiRNAs) and natural antisense transcript siRNAs (NAT-siRNAs) are also well described in plants. The mechanism of biogenesis of these siRNAs is almost conserved in plants except for phasiRNAs. Synthesis of phasiRNAs start with miRNA mediated cleavage of nascent precursor RNA transcript at both sides (Yoshikawa, Peragine *et al.* 2005; Yoshikawa, Iki *et al.* 2013).

siRNAs are regulatory molecules that besides protecting cell from intrusion of any exogenous nucleic acid (like viruses), are involved in maintaining genome integrity by silencing transcription from undesired loci (retro-transposon, repeat sequences). siRNAs originate from long, linear, perfectly paired dsRNA which are commonly generated from loci containing inverted and direct repeat sequences, retro-transposon, foreign RNA or aberrant sources (Meister 2007; Carthew and Sontheimer 2009). Hence, they play a natural role in genome defense against viruses or transgenes (Mello and Conte 2004). They guide direct transcriptional gene silencing via precise slicing (Tomari and Zamore 2005).

Biogenesis

These siRNAs duplexes produced by DICER make complex with RISC (siRISC). RISC most likely involves the participation of one or more Argonaute-like (AGO) proteins (Baumberger and Baulcombe 2005), which form the catalytic component of the RISC. The incorporated siRNA functions as a search-and-strike module to specifically position RISC onto RNAs that are complementary to the RISC-bound siRNA. The strand that directs silencing is called the guide strand whereas the other

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strand, which is ultimately destroyed, is called the passenger strand. The 5' end of the guide strand sets the rule for the target-RNA cleavage, as the cleavage occurs between 10th and 11th nucleotide upstream of the 5'end (Elbashir, Lendeckel *et al.* 2001; Leuschner, Ameres *et al.* 2006; Carthew and Sontheimer 2009). The duplex siRNAs are unwound by helicase activity of Argonaute (AGO), a protein recruited by DICER. The relative thermodynamic stability of 5'ends of the two siRNA strand in the duplex determines the identity of the guide and passenger strands (Khvorova, Reynolds *et al.* 2003; Schwarz, Hutvagner *et al.* 2003). The hallmark of the siRNA is its remarkable potency: only a few molecules of dsRNA per cell can induce a robust response. The key initiator molecule (dsRNA) can be delivered exogenously or produced *in vivo* by RNA dependent RNA polymerases (RdRP) or by transcription either through inverted repeats or from converging promoters or short hairpin RNA (shRNA).
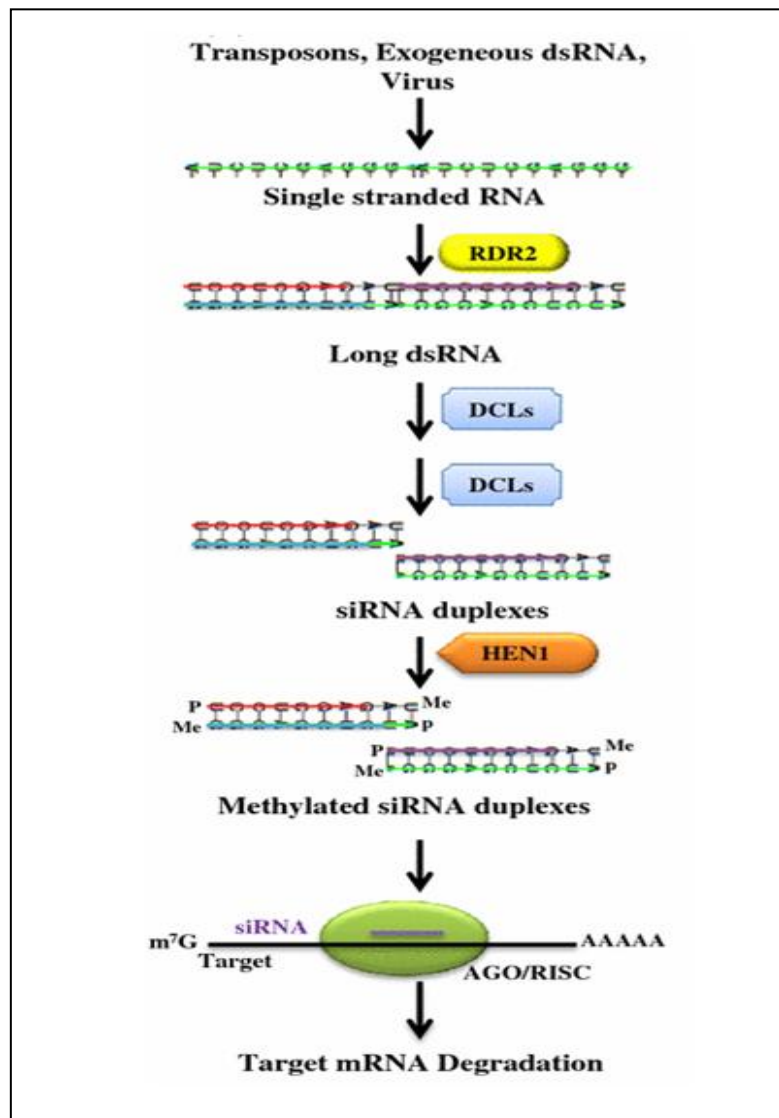

Fig.1.6: siRNA biogenesis pathway

Function of siRNAs

The functionality of siRNA is the consequence following its binding to target sequences and this is governed by a critical region within the siRNA sequence called "seed region" (Lewis, Burge *et al.* 2005). The ribonucleotides encompassing the 2-7 positions (with reference to 5' end) of siRNA constitute the "seed region" and are critical to confer siRNAs their target specificity. It is through the "seed region" that RISC lands onto, anneals and consequently brings about target cleavage/repression. Since siRNAs bind to the sequences from which they are derived, they are not under any kind of selection pressure. It may be noted that although the seed region is important in target recognition, the complementarity in other region of siRNA is critical during the cleavage event. siRNAs have been involved in almost all possible nucleic acid regulatory pathways like target cleavage (Hammond, Bernstein *et al.* 2000; Elbashir, Harborth *et al.* 2001), transcriptional gene silencing (Mette, Aufsatz *et al.* 2000; Aufsatz, Mette *et al.* 2002; Taverna, Coyne *et al.* 2002; Volpe, Kidner *et al.* 2002) and DNA elimination (Liu, Mochizuki *et al.* 2004; Mochizuki and Gorovsky 2004). Additionally they are involved in systemic spread of silencing and RNA-directed methylation of homologous genomic DNA. The small RNAs produced from transgene are of two types: short (21-22nt) and long (24-26nt) (Hamilton, Voinnet *et al.* 2002). Long siRNA is dispensable for sequence-specific mRNA degradation, but correlates with systemic silencing and methylation of homologous DNA. Also long siRNA is not a guide for the RISC. Conversely, the short siRNA are incorporated into RISC and are involved in mRNA degradation but not with systemic signaling or methylation. Moreover, long siRNAs (lsiRNAs; 30-40nt) from plants have been shown to behave functionally similar to animal miRNAs where the siRNA binding rather than the cleavage leads to decapping of target transcripts (Katiyar-Agarwal, Gao *et al.* 2007). The exonuclease (XRN4) then acts on decapped mRNAs and bring about target cleavage. siRNAs with lesser complementarity has been demonstrated to suppress the targets at the translation level (Doench, Petersen *et al.* 2003).

Hairpin RNA interference (hpRNAi)

During the advent of gene silencing, many studies showed that efficient repression of target genes can be induced by expressing self complimentary hpRNA constructs. This phenomenon is called hpRNAi (Watson, Fusaro *et al.* 2005). hpRNA constructs contain a portion of the desired target gene in both the sense and antisense direction linked by a non-complementary spacer sequence (similar to intron). Both the ends of transcribed RNA hybridize with each other and form a single stranded loop, hpRNA, which is subsequently processed by multiple actions of DCLs into siRNAs. siRNAs derived from hpRNA in turn get associated with AGO proteins, and create RISC complex which later represses the target genes based upon nucleotide complementarities (de Felippes, Wang *et al.* 2012). In this way, siRNAs generated by hpRNAs induce highly efficient PTGS in transgenic plants and have been successfully utilized for conferring resistance against a variety of viruses in different plant species such as tobacco, plum, banana, sweet orange, tomato (Zrachya, Kumar *et al.* 2007; Reyes, De Francesco *et al.* 2011; Shekhawat, Ganapathi *et al.* 2012).

For easing the application of hpRNAi, several cloning strategies have been devised which include traditional ligase based vector method as well as direct amplification of intron-containing hairpin RNA (DA-ihpRNA) method, where ihpRNA construct is directly amplified from genomic DNA (Xiao, Yin *et al.* 2006). The introduction of Gateway compatible RNAi destination vectors like pHELLSGATE, pIPK and pANDA simplified the cloning and hpRNA construct preparation to only few steps (Wesley, Liu *et al.* 2003). Gateway system is based on site specific recombination between flanking sequences, and the DNA fragments are joined in predefined orientation and frame. In addition, overlap extension (OE) PCR method help to generate ihpRNA constructs in which two inverted repeat fragments of the target genes are associated to an intron. In the same context, Golden Gate cloning based restriction-ligation method has evolved for developing such constructs (Yan, Shen *et al.* 2012). This method is one-step, cost effective, and provides a high-throughput platform for carrying large scale functional analyses. For measuring the silencing efficacy, it is advised to develop hpRNA constructs targeting green fluorescent protein (GFP) as an indicator. Recently, such a verification strategy for developing hpRNA constructs has been used during preparation of constructs (Harmoko, Fanata *et al.* 2013; McHale, Eamens *et al.* 2013). Despite the frequent use of hpRNAi in

silencing of target genes, there are many drawbacks associated with this technology, specifically, generation of aberrant siRNAs. These siRNAs, after combining in RISC, result in silencing of the unintentional genes and this is referred as 'off-target' effects. Because of having high off targets, hpRNAi appear to be highly unsuitable for further use as well as limits the scope of application in plant research.

MicroRNA

MicroRNAs (miRNAs) are endogenously transcribed non-coding small RNA molecules, ranging in length from 21- to 24-nt that regulate gene expression by binding to their cognate target gene (Carrington and Ambros 2003; Bartel 2004; Kim 2005) in a sequence dependent manner. They comprise one of the most abundant classes of sRNA (Bartel 2004). microRNAs role in growth, development, hormone signaling, genome methylation, morphogenesis, and stress responses of plants has been recognized and is still expanding rapidly (Mallory and Vaucheret 2004; Jones-Rhoades, Bartel *et al.* 2006; Zhang, Pan *et al.* 2006; Mishra and Mukherjee 2007; Zhou, Liu *et al.* 2010).

History:

Two decades ago, the existence of microRNA was completely unknown. Also the classical central dogma (DNA-RNA-Protein) put aside the study of all non-protein coding genes. The scientific community was majorly focused on protein coding genes. Only after 1993, when the first microRNA lin-4 was discovered in the screen of genes that was required for post-embryonic development in *C. elegans* (Lee, Feinbaum *et al.* 1993;Wightman, Ha *et al.* 1993), the importance of miRNA started to be revealed.

Animals with lin-4 loss-of-function mutations were unable to lay eggs and reiterate early development programs at inappropriate late larval stages (Horvitz and Sulston 1980; Chalfie, Horvitz *et al.* 1981; Lee, Feinbaum *et al.* 2004). The lin-4 locus produces a 22-nucleotide small RNA which is partially complementary to the 3'UTR of its regulatory target, the lin-14 mRNA (Lee, Feinbaum *et al.* 1993; Wightman, Ha *et al.* 1993; Lee, Feinbaum *et al.* 2004). After almost 7 years, another small temporal RNA, namely let-7 was identified in nematode. It was conserved across animals and was found to regulate the protein levels of various other transcripts due to its binding

potential at the 3'UTR of these targets   (Pasquinelli and Ruvkun 2002; Reinhart, Weinstein *et al.* 2002). The second microRNA discovered was let-7 in 2000, after seven years of the finding of the first miRNA (Reinhart, Slack *et al.* 2000). Since 2000, direct cloning followed by sequencing and computational predictions led to the identification of various plant miR families mainly from arabidopsis (Llave, Xie *et al.* 2002; Park, Li *et al.* 2002; Reinhart, Weinstein *et al.* 2002; Park, Wu *et al.* 2005).

Plant microRNA biogenesis

miRNA biogenesis is a complex process and is compartmentalized among nucleus and cytoplasm. miRNAs are transcribed from their own genes by RNA polymerase II (Pol II) (Lee, Kim *et al.* 2004). Unlike most metazoan miRNA genes, which are mainly found within exon or intronic regions, most characterized plant miRNAs are encoded by sequences previously annotated as intergenic regions (Reinhart, Weinstein *et al.* 2002; Griffiths-Jones, Saini *et al.* 2008; Zhang, Pan *et al.* 2008). The distribution patterns of the basic *cis*-elements for transcriptional control (i.e. the transcription start site, the TATA box, and the CAAT box) on the miRNA promoters were demonstrated to be identical to the protein-coding genes (Xie, Allen *et al.* 2005;Kim, Zheng *et al.* 2011). The primary-microRNAs (pri-miRNAs) are stabilized by the addition of a 5' 7-methylguanosine cap (Xie, Allen *et al.* 2005) and a 3' polyadenylate tail (Jones-Rhoades and Bartel 2004; Zhang, Pan *et al.* 2005). These pri-miRs are then presumably stabilized by an RNA binding protein DAWDLE (DDL) (Yu, Bi *et al.* 2008) and processed into stem loop precursor-miRNAs (pre-miRs). The processing occurs in the dicing bodies (D bodies) or small nuclear RNA binding protein D3 bodies (SmD3-bodies) (Kurihara, Takashi *et al.* 2006; Fang and Spector 2007; Fujioka, Utsumi *et al.* 2007) and is mediated by a microprocessor complex containing the RNase III enzyme DICER-like 1 (DCL1) (Schauer, Jacobsen *et al.* 2002). The presence of DCL1 and pri-miRNAs led to their designation as dicing bodies (Fang and Spector 2007; Fujioka, Utsumi *et al.* 2007). Dicing bodies are present in several miRNA processing mutants (Fang and Spector 2007), but may vary in number and composition.
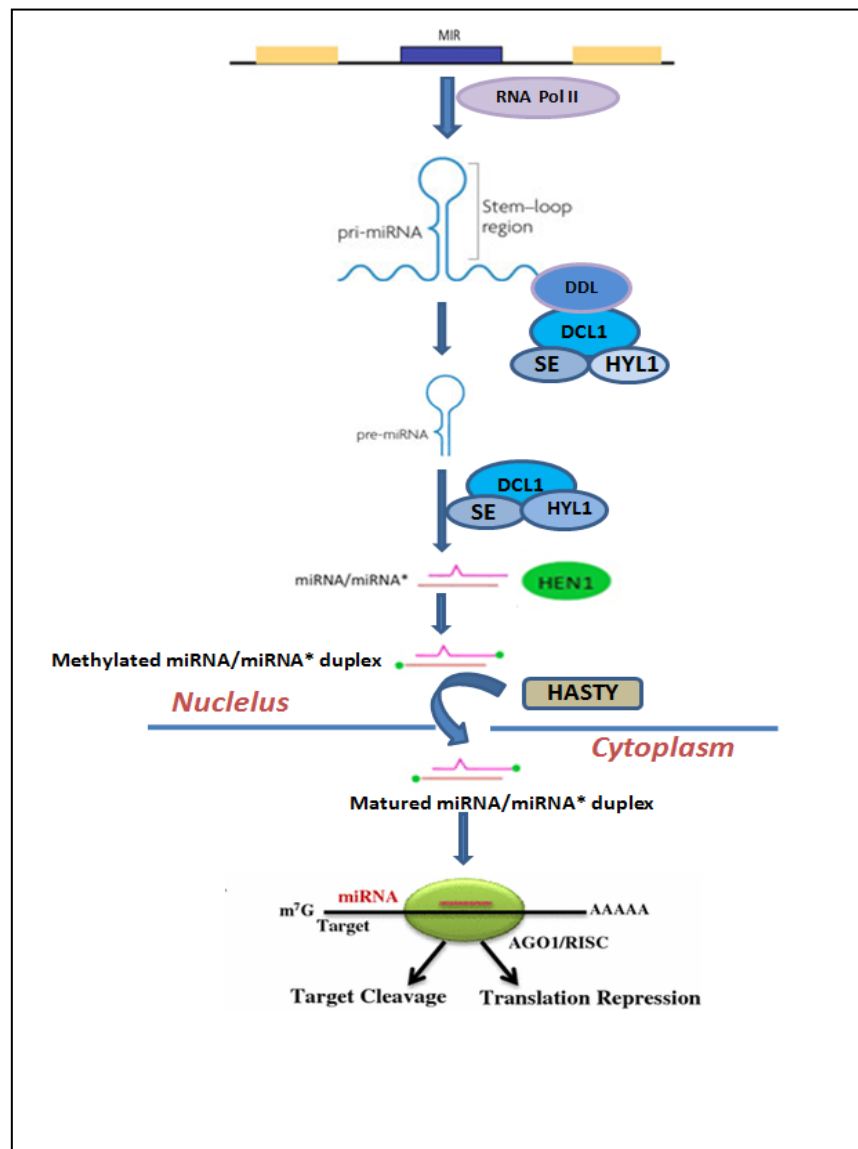
Fig.1.7: Plant microRNA biogenesis pathway

The other partners identified in subnuclear foci by biomolecular fluorescence complementation (Fang and Spector 2007; Fujioka, Utsumi *et al.* 2007; Song, Han *et al.* 2007; Manavella, Hagmann *et al.* 2012; Ren, Xie *et al.* 2012) includes the dsRNA-binding protein HYPONASTIC LEAVES (HYL1/DRB1) (Han, Goud *et al.* 2004; Vazquez, Gasciolli *et al.* 2004), the C2H2-zinc finger protein SERRATE (SE)(Grigg, Canales *et al.* 2005; Yang, Liu *et al.* 2006), the G-patch domain protein TOUGH (TGH) (Ren, Xie *et al.* 2012),C-TERMINAL DOMAIN PHOSPHATASE-LIKE1 (CPL1) and the nuclear cap-binding protein complex (CBC) (Kim, Yang *et al.* 2008).

The TGH, SE and HYL1/DRB1 can bind to RNA and are required for pri-miRNA processing and miRNA accumulation. It has been shown that TGH binds ssRNA (Ren, Xie *et al.* 2012), SE binds pri-miRNA (Machida *et al.* 2011) possibly at ssRNA/dsRNA junctions, and HYL1 binds dsRNA (Hiraguri, Itoh *et al.* 2005; Rasia, Mateos *et al.* 2010; Yang, Chen *et al.* 2010). HYL1 and SE promote the accuracy of pri-miRNA processing by DCL1, while TGH enhances DCL1 activity in pri-miRNA processing and plays no role in its processing accuracy (Ren, Xie *et al.* 2012). HYL1 is a phospho-protein and its phosphorylation status is affected by a mutation in SE (Manavella, Hagmann *et al.* 2012). SE interacts with CPL1 (Manavella, Hagmann *et al.* 2012) which is also required to maintain the hypo-phosphorylated state of HYL1 (Manavella, Hagmann *et al.* 2012). This suggests that CPL1 is recruited to the DCL1 complex by SE, where it regulates HYL1 function through de-phosphorylation. Although CPL1 possesses DRB domains, it is not known if these direct CPL1 to pri-miRNAs. Although a network of physical interactions connects DCL1, HYL1, SE, and TGH but it is not known if these interactions represent a stable plant microprocessor complex. The model which emerges indicates that multiple RNA binding proteins may associate with distinct regions of the pri-miRNA during processing to recruit DCL1 or maintain the structural determinants directing DCL1 activity.

The miRNA/miRNA* duplexes are then methylated on the 3' ribose of the last nucleotide by the methyltransferase activity of HUA ENHANCER 1 (HEN1). This prevents the RNA duplexes from uridylation and subsequent degradation (Li and Ding 2005; Yu, Yang *et al.* 2005; Yu, Bi *et al.* 2008). The miRNA/miRNA* duplexes or the mature miRNAs are transported from the nucleus to the cytoplasm by the plant exportin-5 orthologue HASTY (HST) and other unknown factors (Park, Wu *et al.* 2005). The guide strand of the miRNA/miRNA* duplexes is then incorporated into the RISC protein complex containing AGO1 protein as the core component to regulate target gene expression by mRNA cleavage, translational repression or epigenetic modification (Jones-Rhoades, Bartel *et al.* 2006).

Mechanisms of biogenesis and function of miRNAs in animals and plants are very similar but with small differences. Plant pre-miRNAs are longer (70-400 nt) compared to animals (60-90 nt) and have more complicated secondary structures (Reinhart, Weinstein *et al.* 2002; Bartel 2004). The terminal loop of plant pre-miRNA

hairpin (20-75 nt compared to 7-12 nt in animals) are often branched (Iwai and Naraba 2005; Voinnet 2009). These, unlike the metazoan ones, are conserved only in the region of the miRNA (Jones-Rhoades and Bartel 2004). In animals, excision of pre-miRNA from pri-miRNA takes place in the nucleus and of miRNA from pre-miRNA in the cytoplasm involving DROSHA/PASHA and DICER/DGCR8 proteins, respectively, while in plants the entire processing occurs in the nucleus and is catalyzed only by DCL (Papp, Mette *et al.* 2003).

Functions of miRNA:

The discovery of abundant miRNAs in diverse species raised the obvious question relating to their regulatory targets in the cell. So far near-perfect complementarity has been the exclusive criteria for target identification. Plant mRNAs contain only one miRNA binding site, with rare exceptions (Bartel 2004; Pillai, Artus *et al.* 2004; Alemán, Doench *et al.* 2007). The miRNA mediated regulation could be deconvoluted into two theoretically separable states. In the first state, miRNA would predominantly operate through transcript cleavage and in the second regulatory state; miRNA would mainly repress target protein production in a reversible manner.

AGO1 was shown to be the main slicer of the target mRNA (Qi and Hannon 2005) and miRNA-targeted mRNAs are up-regulated in AGO1 mutants correspondingly (Vaucheret, Vazquez *et al.* 2004). Plant miRNAs are highly complementary to targets throughout their length, and the high degree of complementarity is a requirement for effective target slicing by AGO proteins. The PIWI domain (a protein domain found in PIWI proteins that bind and cleave RNA) of AGO proteins forms an RNaseH-like fold with a slicer endonuclease activity capable of cleaving (Liu, Carmell *et al.* 2004). Slicer activity has been demonstrated for arabidopsis AGO1, AGO2, AGO4, AGO7, and AGO10 (Mi, Cai *et al.* 2008; Montgomery, Howell *et al.* 2008; Takeda, Iwasaki *et al.* 2008; Ji, Liu *et al.* 2011; Maunoury and Vaucheret 2011).

The discrepancy between target mRNA level and the corresponding protein level suggests that miRNAs also regulate target expression by translational repression (Aukerman and Sakai 2003; Chen 2004; Bari, Pant *et al.* 2006; Gandikota, Birkenbihl *et al.* 2007). A recent report showed that SQUINT (SQN), the orthologue of

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immunophilin cyclophilin 40 (Cyp40) in arabidopsis, is required for miRNA-mediated repression by promoting AGO1 activity (Smith, Al Hashimi *et al.* 2011). Some proteins like AGO1, AGO10, the microtubule-severing enzyme KATANIN, the decapping component VARICOSE (VCS)/Ge-1, 3-hydroxy-3-methylglutaryl CoA reductase (HMG1), sterol C-8 isomerase HYDRA1 (Brodersen, Sakvarelidze-Achard *et al.* 2012), and SUO (Yang, Wu *et al.* 2012) are shown to be required for miRNA-mediated translational repression. In addition, miRNA and AGO1 are associated with polysomes (Lanet, Delannoy *et al.* 2009). These observations suggested that translational repression is distinct from slicing and is more widespread in plants than previously thought. However, the mechanism underlying miRNA-mediated translational repression still remains largely unknown in plants. While the population of a given target mRNA has been shown to be simultaneously regulated by both slicing and translational repression, the relationship between the two modes of miRNA action is still unknown. It was hypothesized that regulation of target mRNA by cleavage was important in regulating developmental processes, which require permanent determination of cell fates. In contrast to on-off switching of cleavage, the mode of translational repression enables fine tuning of targets, and might be important in reversible modulation of the negative regulators of stress responses. By repressing translation of negative regulators, it is guaranteed that expression of the regulators will reappear when the stress disappears and ensures reducing the fitness loss due to prolonged stress response activation (Voinnet 2009). This idea was supported by miRNAs controlling phosphate starvation (Sunkar and Zhu 2004) and basal defense against bacterial infection (Navarro, Dunoyer *et al.* 2006).

In addition to PTGS, miRNAs are also capable of TGS by DNA methylation (Wu, Zhou *et al.* 2010). In rice, DCL3-dependent 24 nucleotides long miRNA are specifically sorted into AGO4 clade proteins and trigger cytosine DNA methylation at their own loci in *cis* and at their target genes in *trans* (Wu, Zhou *et al.* 2010). Similar to heterochromatic siRNAs, AGO4-loaded 24 nt miRNAs are likely to trigger cytosine methylation by RNA-directed DNA methylation (Ye, Wang *et al.* 2012).

Artificial microRNA (amiRNA): a new horizon in gene silencing

Artificial microRNA (amiRNA), delineates a new facet in the fascinating world of microRNAs. With an overabundance of experimentations, it is now evident that small non-coding RNAs like microRNAs (miRNAs) play a vital role in gene regulation in all types of eukaryotic organisms. Gene regulation offered by miRNA is considered as one of the highly conserved mechanisms in plant kingdom. Studies indicate that mutation in 21 nt mature sequences in hairpin stem loop did not hamper the biogenesis of miRNAs (Schwab, Ossowski *et al.* 2006; Sablok, Pérez-Quintero *et al.* 2011). This finding opened a new possibility which laid the emergence of amiRNA technology. amiRNA, an innovative strategy having enormous potential, can bind to target RNAs (mRNA) and decrease their activity, by transcript cleavage or translational repression (Sablok, Pérez-Quintero *et al.* 2011). It is a form of miRNA which is derived by replacing native miRNA duplexes from a natural miRNA precursor.

This concept was validated in a study and commercialized with the aim to develop virus resistant transgenic arabidopsis plants through manipulating miR159a precursor backbone (Niu, Lin *et al.* 2006) The amiRNA strategy has been used for efficient gene silencing in arabidopsis (Michniewicz, Zago *et al.* 2007; Ossowski, Schwab *et al.* 2008; Molnar, Bassett *et al.* 2009), rice (Warthmann, Chen *et al.* 2008), maize (Meng, Muszynski *et al.* 2011), barrel clover (Medicago) (Haney and Long 2010), eggplant (Toppino, Kooiker *et al.* 2011), poplar (Shi, Yang *et al.* 2010), grapevine (Jelly, Schellenbaum *et al.* 2012), *Lemna minor* (Cantó-Pastor, Mollá-Morales *et al.* 2015) etc.

Owing to certain attributes like uniqueness, effectiveness and preciseness, gene silencing through amiRNAs is considered as a second generation RNAi technology (Warthmann, Chen *et al.* 2008).
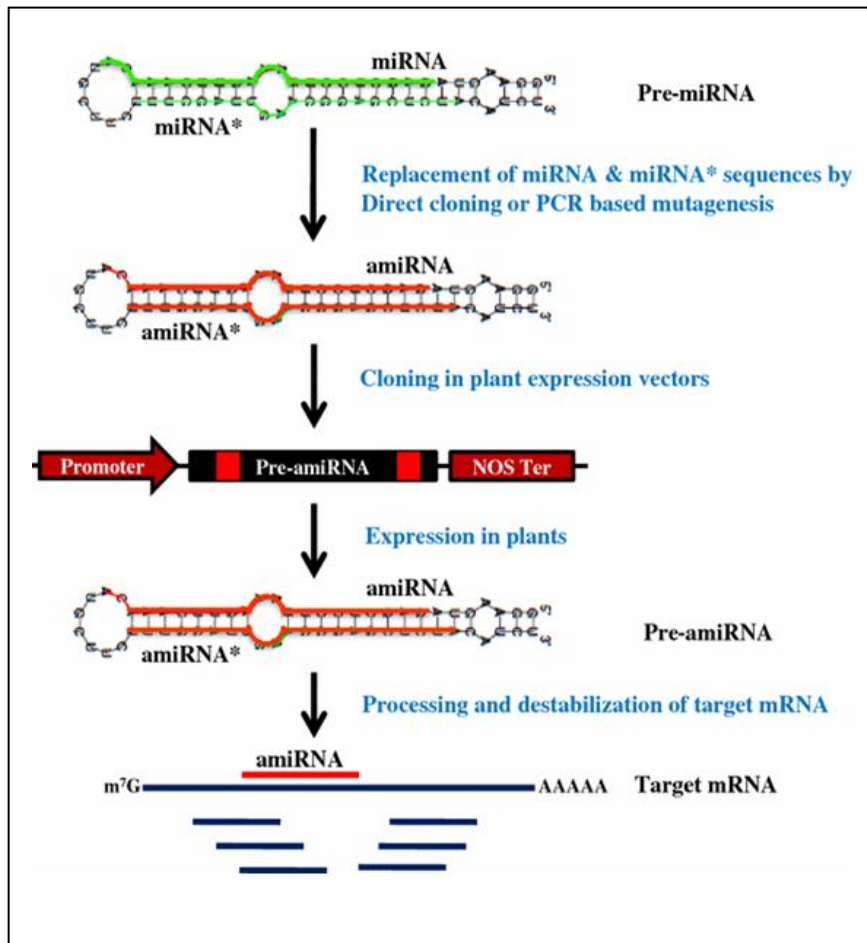
Fig.1.8: Systematic representation of amiRNA cloning and action. Designed amiRNA is inserted into endogenous miRNA precursor by replacing miRNA/miRNA* sequence with respective amiRNA-amiRNA* nucleotides. This pre-amiRNA is processed by series of DCL1 slicing events to generate mature amiRNA/amiRNA* duplex. One strand of amiRNA gets incorporated into RISC and guides RISC to inhibit target mRNA expression either by mRNA translation inhibition or degradation of mRNA.

Advances in amiRNA cloning

The amiRNA technology retains several advantages over hpRNAi, in particular, minimizing off-target effects and effectiveness. Moreover, it is easy to optimize amiRNA sequences for targeting one or several mRNAs without disturbing the expression of other genes at a time with single amiRNA, which enhances the robustness of this technology (Schwab, Ossowski *et al.* 2006). Few studies were also

conducted to assess the preciseness of amiRNA through genome-wide expression analyses, which revealed that amiRNAs are accurate in gene silencing and degrade genes without affecting expression of other genes (Khraiwesh, Ossowski *et al.* 2008). Considering the feasibility of potential implications of amiRNAi, remarkable improvements have been incorporated in this technology for instance, in cloning, construct preparation and delivery in plants (Carbonell, Takeda *et al.* 2014). In most of the earlier studies, pre-miRNA was manipulated in such a way that endogenous mature miRNA/miRNA* sequences of duplex were substituted with corresponding amiRNA/amiRNA* sequences by PCR based mutagenesis (Liang, He *et al.* 2012). Additionally, to further simplify the procedures many bioinformatic tools have been developed for making amiRNA approach more effective and convenient to be utilized in future studies (Liang, He *et al.* 2012; Hauser, Chen *et al.* 2013). These tools can be freely accessible and highly useful for designing a candidate amiRNA of desired gene with least off-target effects in a genome as well as to know other possible targets. Some of these tools comprise WMD (http://wmd3.weigelworld.org/cgi-bin/webapp. cgi), PsRNAtarget (http://plantgrn.noble.org/psRNATarget/) and Viena RNA package (http://www.tbi.univie.ac.at/RNA/), which are helpful to determine minimum free energy of hybridization. Moreover, screening of potent amiRNA designed through *in silico* analysis is another challenging task. However, a recent study described the epitope tagged protein-based amiRNA (ETPamir) method for screening of most efficient candidate sequence among the predicted amiRNAs, and which is thought to be much helpful to minimize this complexity (Li, Zhang *et al.* 2014). Consistent with ETPamir, the use of green fluorescence protein (GFP) sentinel system in hpRNAi constructs has proved its utility enormously while measuring the efficiency of constructs and rapid identification of transformants in fungi (Youseff and Rappleye 2012).

Uracil excision based cloning has been another effective method used for cloning of amiRNAs that leads to direct generation of amiRNA containing precursors from PCR products (Ossowski, Schwab *et al.* 2008). Despite the easiness, few major limitations persist in the above described approaches that include unwanted modifications to the miRNA backbones and complicated primer designing. For removing these limitations, a simple amiRNA vector has been developed which requires one step PCR amplification and ligation reaction without changing the miRNA backbone (Wang, Yang *et al.* 2012). Similarly, another group developed universal amiRNA vectors

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(pUAs) for arabidopsis and rice which are suitable for almost all kinds of miRNA backbones (Zhou, Yu *et al.* 2013). These modifications further simplified the cloning procedure and it can be used for high-throughput or genome-scale construction of amiRNA expression vectors.

A highly efficient and rapid approach for the construction of amiRNA expression vectors has been developed that uses seamless enzyme-free cloning (SEFC) and mating-assisted genetically integrated cloning (MAGIC) system (Yan, Gu *et al.* 2012). One-step MAGIC approach was applied for the transfer of amiRNA cassette to the binary recipient plasmid through homologous recombination (Yan, Shen *et al.* 2012). A very recent study has described the fast, cost effective method to clone amiRNAs as well as multiplexed syn-tasiRNAs where miRNA390a backbone was used, and the oligonucleotides directly annealed and ligated into gateway compatible expression vectors having various selectable markers (Carbonell, Takeda *et al.* 2014).

Implications of amiRNA technology

The amiRNA-mediated gene silencing has facilitated in deciphering functional importance of many genes. This technology has been exploited for improvement of agronomic performance and nutritional value in various crops. A plethora of examples are already available which describes the potential and magnitude of amiRNA technology, and provides a glimpse of leads taken through this technology in plant research.

Plant growth and development

The implication of amiRNA technology in identifying the genes and transcription factors regulating growth and development processes of plants has flourished in the recent past. Occurrence of circadian clock is a distinctive and well conserved attribute amongst eukaryotes, and various circadian clock modifiers have been characterized through gene silencing (Ouyang, Andersson *et al.* 1998; Dodd, Salathia *et al.* 2005). Many circadian clock identifiers (ELF3, GI and ZTL) were down-regulated through transient expression of specific amiRNAs in arabidopsis mesophyll protoplasts, which helped to uncover involvement in circadian regulation of flowering (Kim and Somers 2010).

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Similarly, amiRNA approach has been successfully employed in understanding the molecular mechanism of flowering and associated genes for instance, FT (Flowering Locus T) of arabidopsis (Schwartz, Balasubramanian *et al.* 2009; Yeoh, Balcerowicz *et al.* 2011) and terminal flower/ Centroradialis (ZCN) of maize (Meng, Muszynski *et al.* 2011). In addition to determining the precise role of flowering regulators, amiRNAs have helped in developing specific knockdown lines of many growth unrelated genes (Bartel 2009). Identification of involvement of the heat shock proteins (HSPs) in growth and development is such an example(Latijnhouwers, Xu *et al.* 2010). Further, role of arabinogalactan proteins (AGPs) in pollen and pistil development were elucidated through targeting these genes by amiRNAs (Coimbra, Costa *et al.* 2009). The knockdown lines of amiRNA-FLOT2 and amiRNA-FLOT4 (flotillin-like gene) in *Medicago truncatula* significantly impeded the nodule formation which consequently decreased the symbiotic interactions with nitrogen fixing bacteria (Haney and Long 2010).

Male sterility is often considered as a useful agronomic trait for reducing self crossing. A complete male sterility of pollen was reported by targeting anther-specific TATA box binding protein (TBP)-associated factor genes (SmTAF10 and SmTAF13) in *Solanum melongena* (Toppino, Kooiker *et al.* 2011) using amiRNA. In addition to providing function of individual genes, amiRNA technology has also been applied to repress an entire gene family simultaneously. Two subsets of phenylalanine ammonia lyase (PAL) genes namely subset A (PAL2, PAL4 and PAL5) and subset B (PAL1 and PAL3) of *Populus trichocarpa* have been identified using this strategy (Shi, Yang *et al.* 2010).

Conserved occurrences of few miRNAs across several plant species indicate that a specific conserved pre-miRNA for example; miR159, miR319 and miR169 backbone can be used for the production of amiRNAs in distantly related plants. This single feature extended a wide adaptability of this approach in various non-model organisms, whose genome sequences are not yet known. Many successful silencing efforts have previously been made in mosses (*Physcomitrella patens*) to repress the expression of genes like PpFtsZ2-1 required for chloroplast division, and PpGNT1 encoding an N-acetyl-glucosaminyltransferase (Khraiwesh, Ossowski *et al.* 2008). Gene silencing has also been frequently performed in unicellular alga, *Chlamydomonas reinhardtii* to deregulate cytochrome c-oxidase subunit (COX90), phytoene synthase (PSY), pyruvate formate lyase (PFL1) and DICER-like 1 (DCL1) genes using the

endogenous pre-miRNA1157 (Molnar *et al.* 2009). A detailed list of previous studies related to amiRNA and plant development is summarized in the following table.

Table 4: Artificial microRNA along with their targets and subsequent outcomes from its use in different plants (Tiwari, Sharma *et al.* 2014)

Plants	Artificial microRNA	Target genes	Findings	References
Arabidopsis thaliana	amiR-P69159	P69	Resistant to TYMV and TuMV	(Niu, Lin *et al.* 2006)
	amiR-HC-Pro159	HC-Pro		
	amiR-lfy	LFY	Floral defects	(Schwab, Ossowski *et al.* 2006)
	amiR-white	GUN4	Arrested growth as white seedlings	
	amiR-ft	FT	Late flowering under long days	
	amiR-trichome	TRY, CPC, ETC2	Highly clustered trichomes on leaf blades and petiole	
	amiR-mads	SOC1, MAF1-3, ANR1, AP1	Floral morphology with leaf-like organs along with increased number of cauline leaf	
	amiR-yabby	INO, YAB3	Polarity defects in cauline leaves	
	ProAGP6:AGP6 + AGP11 amiRNA	Arabinogalactan proteins (AGP6 and AGP11)	Pollen grains failed to develop normally, leading to their collapse	(Coimbra, Costa *et al.* 2009)
	DM1-amiRNA	NBS–LRR (DM1)	Normal phenotype was restored	(Bomblies, Lempe *et al.* 2007)
	amiR-ELF3	ELF3	Transfected cells showed arrhythmicity	(Kim and Somers 2010)

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	amiR-ZTL	ZTL	Transfected protoplasts	
	amiR-GI	GI	Transfected protoplasts had longer (~2.5 h) periods	
	amiR-FT	FT	Very late flowering	(Yeoh, Balcerowicz *et al.* 2011)
	FAD-2-amiRNA159	FAD-2	Increased oleic acid from the normal levels of ~15 % to as high as 63.3 and reduced total PUFA content from 46.8 to 4.8 %	(Belide, Petrie *et al.* 2012)
	FAE1-amiRNA159	FAE1	Reduction of eicosenoic acid to 1.9 from 15.4 %	
	FATB1-amiRNA159	FATB1	Reduction of palmitic acid (16:0) to 4.4 % from 8.0 %	
	amiRNA14	MYB14 transcription factor	Knock-down AtMYB14 lines showed increased tolerance to freezing stress	(Chen, Jiang *et al.* 2013)
Oryza sativa	Spl11-amiRNA	Spl11	Spotted leaves	(Warthmann, Chen *et al.* 2008)
	Pds-amiRNA	Pds	Albino phenotype	
	Eui1-amiRNA	Eui1	Elongated upper-most internode	
	Hsc-amiRNA	Hsc70	Transformants showed white and severely stunted phenotype	(Latijnhouwers, Xu *et al.* 2010)
	Ami-BEIIb	SBEIIb	Rice grain showed increase in the proportion of long amylopectin and intermediate chains	(Butardo, Fitzgerald *et al.* 2011)
	Ubi::BADH2-amiRNA	OsBADH2	Grain 2AP content elevated	(Chen, Wei *et al.* 2012)

Glycine max	amiRNA GmLRR-K	Glyma18g02 680.1 gene	No Significant Impact on SCN (Soybean cyst nematode) Resistance	(Melito, Heuberger *et al.* 2010)
Nicotiana benthamiana	amiR-2b	CMV 2b	Transgenic tobacco plants showed effective resistance to CMV infection	(Qu, Ye *et al.* 2007)
	amiR-LA amiR-LB1 amiR-LB2 amiR-LC amiR-LD amiR-LE	Single L (replicase) motif A B1, B2, C, D of E	Single amiRNA transgenic lines expressing amiR-LB2 or amiR-LD showed resistance to WSMoV by delaying symptom development. The amiR-LA- and amiR-LB1-expressing lines were susceptible to WSMoV while amiR-LC and amiR-LE lines did not provide resistance to WSMoV	(KUNG, LIN *et al.* 2012)
	amiR-LB2DC	Triple L (replicase) motifs AB1E	Does not provide resistance to WSMoV	
	amiR-LAB1E	Triple L (replicase) motifs B2DC	Provided complete resistance against WSMoV	
Physcomitrella patens	PpFtsZ2-1-amiRNA	PpFtsZ2-1	Impaired chloroplast division and formation of macrochloroplasts	(Khraiwesh, Ossowski *et al.* 2008)
Solanum melongena	amiRNA-SmTAF10	SmTAF10	Transgenic eggplants are completely male sterile	(Toppino, Kooiker *et al.*

	amiRNA-SmTAF13	SmTAF13		2011)
Nicotiana tabacum	amiR-HC-Pro	HC-Pro	Highly specific resistance against PVY or PVX infection	(Simón-Mateo and García 2006)
	amiR-p25	TGBp1/p25 (p25)		
	amiFLS1	NtFLS	Enhanced anthocyanin synthesis, rutin content reduced and due to this insect tolerance also reduces	(Misra, Pandey *et al.* 2010)
Chlamydomonas reinhardtii	pChlamiRNA1-COX1 pChlamiRNA2-COX-2	Cytochrome c oxidase	Reduced growth in darkness	(Molnar, Bassett *et al.* 2009)
	pChlamiRNA1-PSY	Phytoene synthase (PSY)	Albino or pale green phenotype	
	pChlamiRNA2-DCL1	DICER-like nuclease 1 (DCL-1)	Accumulated slightly less retrotransposon-specific siRNA	
	HSF1-amiRNA	HSF1	HSF1-amiRNA exhibited a thermosensitive phenotype	(Schmollinger, Strenkert *et al.* 2010)
	Pfl1-KD1 Pfl1-KD2	Pyruvate formate lyase (PFL1)	Decreased production of formate and enhanced production of ethanol and lactate	(Burgess, Tredwell *et al.* 2012)
Medicago truncatula	FLOT2-amiRNA FLOT4-amiRNA	FLOT2 FLOT4	Decreased nodule number, infection events, and nodules that do form are non-functional	(Haney and Long 2010)
Triticum	FGmiR395	Conserved regions of WSMV (5′ UTR region, pipo region of P3 cistron, P1 gene, P3 cistron, HC-PRO gene)	Resistance to Wheat streak mosaic virus	(Fahim, Millar *et al.* 2012)
Solanum lycopersicum	amiR-2a/b	2a and 2b genes	Plants exhibited a higher antiviral ability due to suppression of viral invasion and movement	(Zhang, Li *et al.* 2011)

	amiR- 3′UTR	Conserved 3′UTR of CMV	Resistance against wide range of the members of Cucumo virus	
Solanum tuberosum	amiR- CBP80	CBP80 gene	Transgenic plants displayed a higher tolerance to drought, ABA-hypersensitive stomatal closing. Also showed increased leaf stomata and trichome density, and compact cuticle structures with a lower number of microchannels	(Pieczynski, Marczewski *et al.* 2013)
Populus trichocarpa	amiRNA-palA	PAL2, PAL4 and PAL5 genes	Reduced transcript level up to 20–60 %	
	amiRNA-palB	PAL1 and PAL3 genes	Reduced transcript level up to 9–23 %	
Vitis vinifera	amiR^{CP}-1	CP-1	Induced cleavage of viral RNA in GFLV-infected cells.	(Jelly, Schellenbaum *et al.* 2012)
	amiR^{CP}-2	CP-2		

Crop improvement

Development of new crop varieties with increased productivity is highly needed to meet the need of increasing global human population. In this context, genetic engineering has successfully proven its worth; more importantly, application of amiRNA-mediated gene silencing has also been exemplified in several reports.

Considering crop plants, silencing of endogenous genes like phytoene desaturase (pds), spotted leaf 11 (Spl11), and elongated uppermost internode 1/CYP714D (Eui1) was achieved through amiRNA technology in rice which is thought to be one of the beneficial aspects to improve grain quality (Warthmann, Chen *et al.* 2008; Chen, Jiang *et al.* 2013). In an attempt to increase grain quality and economic value of rice, starch branching (SBEIIb) gene was targeted through amiRNA. SBEIIb is associated with certain features such as elevated amylase, increased peak gelatinization temperature and decreased proportion of short amylopectin branches (Butardo,

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Fitzgerald *et al.* 2011). Interestingly, rice genotype having aroma in grains was developed from non-fragrant variety by reducing expression of BADH2, which inhibits the function of 2-acetyl-1-pyrroline (2AP), the principal compound responsible for grain fragrance using amiRNA (Chen, Wei *et al.* 2012). Development of wheat streak mosaic virus (WSMV) resistant transgenic lines of wheat is another remarkable success story of amiRNA approach (Fahim, Millar *et al.* 2012). A report described that suppression of genes involved in fatty acid biosynthesis, such as fatty acid desaturase 2 (FAD2), fatty acid elongase (FAE1) and fatty acyl-ACP thioesterase B (FATB), led to the elevated seed oil composition in arabidopsis (Belide, Petrie *et al.* 2012). Nevertheless, this result needs to be translated in oil yielding crops for getting more advantage of amiRNAi in the coming days.

Elucidation of gene function of metabolic pathways

In addition, one of the most noteworthy applications of amiRNA is to elucidate function of any candidate gene in a very short time as compared to conventional methods. Although, VIGS is an extensively used technique for deducing function of genes in metabolic pathways but efforts are now also being made to use amiRNA technology for such purposes due to the retention of some advantageous features.

amiRNA approach has successfully been applied for modulating flavonoid biosynthesis through degrading NtFLS (flavonol synthase), a key structural gene of flavonoid biosynthetic pathway, and confirmed the role of flavonols in providing insect resistance (Misra, Pandey *et al.* 2010). Transgenic tobacco plants expressing both AtMYB12 and amiFLS miRNA transgenes displayed a deep red flower color and reduced rutin content in contrast to white flower of AtMYB12 expressing transgenic plants. This study provided evidence related to insecticidal property of flavonols, especially rutin. Subsequently, this information led to the development of AtMYB12 expressing transgenic tobacco callus culture for large scale production of rutin with biopesticidal potential (Pandey, Misra *et al.* 2012). Although not many reports are available in relation to pathway engineering using amiRNA, but it seems

that insertion of precursor miRNA backbone in VIGS vectors may open a new dimension in the near future.

amiRNA and biotic stress tolerance

Environmental stresses such as heat, cold, drought, salinity, redox stress and pathogen attack adversely affect plant growth and crop yield (Khraiwesh, Zhu *et al.* 2012). The identification of miRNAs associated with various abiotic and biotic stress related genes, and their likely involvement in overcoming these stresses prompted researchers to exploit amiRNAs in developing plant tolerance against biotic and abiotic stresses (Kruszka, Pieczynski *et al.* 2012; Lima, Loss-Morais *et al.* 2012).

Amongst common biotic stresses, different virus borne diseases are considered as major threats to crop production, and are responsible for huge loss of economically important crops every year around the world. Studies indicate that endogenous miRNAs-mediated gene silencing is one of the mechanisms which potentially contribute to prevent virus invasion in plants (Li, Wei *et al.* 2012). Numerous miRNAs for instance, miR156 and miR393 have been illustrated to inhibit the invasion of foreign genetic elements specially plant viruses through destabilizing their target expression (Xin, Wang *et al.* 2010; Zhang, Li *et al.* 2011). amiRNA technology has been successfully implemented to develop transgenic arabidopsis lines resistant to plant virus (Niu, Lin *et al.* 2006). The study demonstrated that amiRNAs can specifically silence turnip yellow mosaic virus and HC-Pro of turnip mosaic virus and consequently conferred virus resistance. Similarly, cucumber mosaic virus resistant transgenic arabidopsis plants has been designed by modifying the plant miRNA sequence to target viral genomes (Niu, Lin *et al.* 2006; Duan, Wang *et al.* 2008). Through amiRNA, virus resistant varieties of tobacco and tomato have been developed by using the same approach (Ai, Zhang *et al.* 2011; Zhang, Li *et al.* 2011).It is also noted that this kind of resistance is credibly hereditable.

Surprisingly, transgenic tobacco plants *(N. benthamiana)* expressing an amiRNA, that target silencing suppressor 2b (SS 2b) of the cucumber mosaic virus (CMV), showed various degree of responses towards CMV infections ranging from resistant, recovery, delayed infection, and to susceptible phenotype (Qu, Ye *et al.* 2007). Thereafter, several concurrent studies have been conducted to raise resistance for watermelon

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silver mottle virus in tobacco (Kung *et al.* 2012) and tomato leaf curl New Delhi virus in tomato (Van Vu, Choudhury *et al.* 2013).

Besides targeting viruses, deregulating the endogenous defense related genes of the host can be another way for developing disease resistance in plants. In a quantitative trait locus (QTL) map, Rhg1 (resistance to *Heterodera glycines*) was identified for the soybean cyst nematode (*Heterodera glycines*) (SCN) resistance in Glycine max, and that was verified through knocking down the expression of Rhg1 (Melito, Heuberger *et al.* 2010). On the other hand, conversion of a dominant allele XAL3 (resistant gene for bacterial blight caused by *Xanthomonas*) into recessive xa13 allele through tissue-specific expression of amiRNAs resulted in a dominant trait for bacterial blight resistance in transgenic rice (Li, Wei *et al.* 2012). Molecular mechanisms underpinning the hybrid necrosis of progenies obtained from intra-specific cross of *Arabidopsis thaliana* were explored with the help of amiRNAs and demonstrated the role of NB-LRR (nucleotide-binding site–leucine-rich repeat) in autoimmune responses like hybrid necrosis (Bomblies, Lempe *et al.* 2007). amiRNA technology has also been used to validate insecticidal property of molecules. Transgenic plants co-expressing AtMYB12 and amiFLS showed reduced rutin content in comparison with AtMYB12 expressing transgenic lines, and provided evidence related to insecticidal property of flavonols, especially rutin (Misra, Pandey *et al.* 2010). In general, pathogen resistant phenotype is a reflection of crosstalk of many genes, which are recruited at a time to impede the invasion of pathogen and to minimize the deleterious effects. Manipulation of single or set of genes seems inadequate to obtain the tolerance phenotype of plant until they can govern a dominant allele, and thus could not be an appropriate alternative. However, relevance of this technology appears to be inevitable during the dissection of underlying molecular processes of biotic stresses in plants.

amiRNA and abiotic stress tolerance

Apart from biotic factors, few abiotic stresses like heat, cold, and drought comprise the major challenges for plant growth. These stresses affect growth, development and productivity of plants (Sunkar, Li *et al.* 2012; He, He *et al.* 2014). Plants are sessile organisms and several adaptive mechanisms have evolved at different levels to combat or overcome these stresses during the evolutionary course. A plethora of

documented reports explain the efforts made in the past to know the underlying adaptive physiological and molecular processes in plants (Jover-Gil, Candela *et al.* 2005). Meanwhile, the additive input of miRNAs in stress combating regulatory circuits of cells is essential and well proven. Majority of the present miRNA research have led to the identification of stress-related sRNAs.

Role of heat shock factor 1 (HSF1), a key regulator of thermo tolerance in Chlamydomonas, was demonstrated using amiRNA (Schmollinger, Strenkert *et al.* 2010). It is reminiscent from several studies that C-repeat binding factor (CBF) proteins play crucial roles in cold tolerance of plants, and regulation of CBFs are believed to be under tight control of MYB14 transcription factor. This interaction has been revealed by knocking down AtMYB14 expression which caused higher CBF expression and eventually increased the freeze tolerance (Chen, Jiang *et al.* 2013). Surprisingly, silencing of another nuclear cap-binding protein, CBP80 in a potato (*Solanum tuberosum*) cultivar through amiRNA resulted in improved drought tolerance in transgenic lines (Pieczynski, Marczewski *et al.* 2013). These reports are few examples of potential use of amiRNA for improving health of a plant grown under challenging environments, and this technology will be replicated for betterment of crop plants in future that can robustly face these stresses.

Other applications of amiRNAs in plants

In addition to the use of amiRNAs in elucidating function of genes or developing stress resistant plants, this technology has provided few novel applications in plants. High degree of functional redundancy amongst the members of gene families often exists in the organisms. In a traditional way, functional analysis of gene is mostly performed based on overlapping orthologous function in other life forms. Recently, a study was conducted to identify all proteins-coding genes in an arabidopsis genome via amiRNAs silencing approach (Hauser, Chen *et al.* 2013). The efficacies and robustness of amiRNA was harnessed for testing 122 amiRNAs targeting different transcription factor, protein kinase and protein phosphatase families. Diverse morphological phenotypes were observed analogous to known phenotypes of single and double/triple mutants in that study. It was suggested that amiRNA approach could be useful in addressing the function of gene families on large and small scales, as well as to provide a resource of novel gene function irrespective of the overlapping

functions. On the other hand, for studying roles of miRNAs, different groups are generally relying on increasing the miRNA activity through transgenic approach due to a lack of MIR knockout mutants, except for a very few miRNAs (Palatnik, Allen *et al.* 2003; Eamens, Agius *et al.* 2010). Nonetheless, some indirect alternative approaches such as target mimic (Yan, Gu *et al.* 2012; Tang and Tang 2013; Zhang, Liu *et al.* 2013), and targeting promoters of MIR genes through RNAi constructs (Vaistij, Elias *et al.* 2010) have received attention. In the same context, Eamens *et al.* (2011) have successfully implemented amiRNA technology to repress MIR gene in arabidopsis (Eamens, Agius *et al.* 2010; Eamens and Wang 2011). Similar approach was also employed for functional characterization of miR408 in arabidopsis. The constitutive expression of amiRNA targeting miR408 resulted in significant reduction of miR408 level which ultimately led to higher expression of target genes of miR408 (Zhang and Li 2013). The phenotypic differences of transgenic lines were also found equivocal to that of T-DNA insertion lines in miR408 locus. The report suggests that loss of function allele of any MIR gene can be assessed by amiRNA technology. Such a use of amiRNA may become a promising tool for determining contribution of specific miRNA in growth, development and other biological aspects in plant.

Thus, superfluity of research findings supports the immense role of RNAi in gene regulation. Likewise siRNA, artificial microRNA (amiRNA) appends an impending prospect in RNAi era. Both strategy retain high degree of specificity, effectiveness, and can hold many key features particularly in credibility, genome stability and hereditability of phenotypes. These are certain interests that need to be understood in future to confiscate the tailbacks in the success rate as well as to enhance the reliability of the technology in plant research. Information generated in these areas will further ensure robustness of RNAi technology by adapting more innovative encroachment.

CHAPTER 2

METHODS AND MATERIALS

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Materials and Chemicals

Table 2.1 Materials and their sources

Material	Source
Plant materials	
Jute *(Corchorous olitorius)* Variety 0-9897	Bangladesh Jute Research Institute (BJRI)
Tobacco variety *Nicotiana tabacum* L. cv. Xanthi	ICGEB, New Delhi
Bacterial strains	
Escherichia coli (DH5α)	Invitrogen Life Technologies, Carlsbad, USA
Agrobacterium tumefaciens (LBA4404)	Molecular Biology Laboratory, University of Dhaka, ICGEB, New Delhi.
Agrobacterium tumefaciens (EHA105)	ICGEB, New Delhi
Vectors	
pGEMT ^{Easy}	Promega Life Science, Madison, WI, USA
pRS300	Detlef Weigel, Max Planck Institute for Developmental Biology, Tübingen, Germany
pBI121	Clontech, USA
pCR2.1 TA vector	Invitrogen, USA
pENTER11	VIB, Life Science research institute, UGent, Belgium.
pK7GW1WG2D(II)	VIB, Life Science research institute, UGent, Belgium.
Markers	
1 kb Ladder	Fermentas International Inc, Ontario, Canada
100bp Ladder	Fermentas International Inc, Ontario, Canada
Low Range Ladder	Fermentas International Inc, Ontario, Canada
Membranes and filter papers	
Nylon membrane, nitrocellulose	Amersham Biosciences, USA

Radioisotopes	
$[\alpha^{32P}]$dCTP, $[\gamma^{32P}]$dATP	Perkin-Elmer Life Sciences Inc., Boston, USA
Restriction endonuclease and DNA modifying enzymes	
Restriction enzymes (FastDigest), T4 DNA ligase and Taq polymerase, T4 polynucleotide kinase, *Pfu* DNA polymerase, DreamTaq™ DNA polymerase, dNTPs	Fermentas International Inc, Ontario, Canada
Kits	
QIAprep Spin Miniprep Kit	Qiagen, Germany
Qiaprep Filter Plasmid Midi Kit	Qiagen, Germany
QIAquick Gel Extraction Kit	Qiagen, Germany
QIAquick PCR Purification Kit	Qiagen, Germany
SuperScript^{TM} III RT	Invitrogen(Life Technology), Sigma
QIAquick gel extraction column	Qiagen, Germany
Others	
General chemicals reagents	Sigma Chemical Company, St. Louis, USA; Serva, Heidelberg, Germany; USB (Amersham International plc.), Buckinghamshire, UK; Amersham Biosciences, United Kingdom; BIO-RAD Labs, Hercules, CA, USA; Promega Life Science, Madison, WI, USA, GIBCO Invitrogen Corporation, New York, USA
Plant hormones; Acetosyringone; β-D Glucuronide (X-Gluc);	Sigma, USA; DUCHEFA Biochemie B.V., The Netherlands
TRIZOL® ; DEPC	Invitrogen (Life Technology), Sigma
Primers	Sigma Chemical Company, St. Louis, USA and Integrated DNA Technologies, Coralville, USA

Designing of primers

Primers were designed using primer blast (http://www.ncbi.nlm.nih.gov/tools/   primer-blast/) or Primer-3-input (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www. cgi) using default parameters. Oligo Analyzer software (http://www.idtdna.com) was used to check any probability of self or heterodimer formation in the designed set of primers. These primers were rechecked for their uniqueness via nucleotide blast at NCBI database. Designed primers were synthesized by Sigma-Aldrich, India and Integrated DNA Technologies (IDT), Coralville, USA.

Gene identification approach

To amplify the full length target gene sequence, conserved domains of genes (of different plants) responsible for lignin biosynthesis were studied in order to design degenerate primers. TA vector (Invitrogen) mediated cloning was carried out to determine the sequence of the degenerate primer-based amplicons by sequencing. On the basis of sequence data, specific primers for particular genes were designed. Sequences so amplified were subsequently cloned into TA vector followed by sequencing to determine the full length sequence of the desired COMT, C4H, F5H and C3H genes.

Table 2.2 List of gene specific and degenerate primers together with their sequences

Sl.No.	*Primers Name*	*Sequence (5'-3')*	**Tm(^{0}C)**
1	COMT_GSP_For	taCCCGGGATGGGTTCAACTGC	60.3
2	COMT_GSP_Rev	tgGAGCTCTTAAACAGTTTTGAGGAACTCC	58.0
3	C4H_GSP_For	atCCCGGG ATGGATCTTCTCTTCCTGGAG	61.2
4	C4H_GSP_Rev	atGAGCTCTTAAAAACTGCGTGGCTTAC	59.0
5	C3H_GSP_For	agTCTAGAATGGGTCTTCTTTTAATAATTACC	57.3
6	C3H_GSP_Rev	taCCCGGGTTATATGTCAACAGCTACACG	55.4
7	F5H_GSP_For	taCCCGGGATGTCTTCTTTAGAACAACTCA	54.7

8	F5H_GSP_Rev	atGAGCTCTTAGAGAGGACAAATCAGGC	58.5
11	NPT(ll) For	CCGTAAAGCACGAGGAAGTC	55.0
12	NPT(ll) Rev	ATGGGGATTGAACAAGATGG	58.0
13	COMT_Deg_For	ATGRGTTCRACYGGTKAAAC	60.0
14	COMT_Deg_Rev	TTATRTGYCAACARCTACACG	61.0
15	C4H_Deg_For	ATGGRTCTYCTCTTCCTRGAG	59.0
16	C4H_Deg_Rev	TTAARAACYGCGTGRCTTAC	58.0
17	C3H_Deg_For	ATGGRTCTTCYTTTMATRATTACC	57.8
18	C3H_Deg_Rev	TTATATRTYAACAGCTMCACG	56.0
19	F5H_Deg_For	ATGTCYTCTTTAGRACAMCTCA	60.9
20	F5H_Deg_Rev	TTAGARAGGACARATCAGGC	59.0
21	Actin For	TGGTATTGGATGTTG GAGAT	60.0
22	Actin Rev	GGTATTGACTTAATGCTGCT	58.0

Artificial microRNA constructs

With the assistance of artificial microRNA designing tool WMD3 (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi) 21mer amiRNA sequences were retrieved from the target gene sequences, which were to be silenced.

After identifying transcripts of target genes through sequence homology with *Arabidopsis thaliana*, WMD3 was used to generate 21nt mature amiRNA candidate sequences that resemble natural miRNAs while minimizing possible off-target effects to other transcripts. First the target gene sequences (i.e. jute specific C3H and F5H gene) were incorporated in the WMD3 target search tool individually. A list of probable amiRNA sequences was provided by WMD3 against a particular target gene of interest. To facilitate the selection of amiRNA, hybridization energy and the target site of the amiRNA sequences were analyzed and one particular amiRNA was then selected for each gene. Next, WMD3 primer designer tool was used to generate oligos against each amiRNA. Four different oligos for each gene were suggested by the program. Overlapping PCR was set up with plasmid, pRS300 (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi) as a template containing miR319 precursor backbone sequence of *Arabidopsis thaliana* (ath-miR319a). The jute specific amiRNA and its complementary (amiRNA*) sequences were introduced into

pSR300 by site-directed overlapping PCR amplification replacing the amiRNA and its complementary (amiRNA*) sequence of *Arabidopsis thaliana* (Schwab, Ossowski et al. 2006). Overlapping PCR products were then mobilized into pGEM-T vector and incorporation of jute specific miRNA and miRNA* in the mir319 backbone was confirmed by sequencing individually for both C3H and F5H gene. After sequence-based confirmation, *Smal* and *Sacl* restriction enzymes were used to transfer the C3H-amiRNA and F5H-amiRNA precursor cassette into plant expression vector pBl121 (Chen, Wang et al. 2003).

Table 2.3 List of amiR primers and their sequences used for over-lapping PCR

Sl. No.	Oligos provided by WMD3	Sequences of the primer (5'-3'))	Tm(^{0}C)
1	I amiR-C3H s	gaTAAGTAATCCCTTACTTGCAAtctctcttttgtattcc	60.9
2	II amiR-C3H a	gaTTGCAAGTAAGGGATTACTTAtcaaagagaatcaatga	60.1
3	III amiR*-C3H s	gaTTACAAGTAAGGGTTTACTTTtcacaggtcgtgatatg	59.9
4	IV amiR*-C3H a	gaAAAGTAAACCCTTACTTGTAAtctacatatatattcct	58.9
5	I amiR-F5H s	gaTAACATTAATGGATTTCCCAGtctctcttttgtattcc	60.0
6	II amiR-F5H a	gaCTGGGAAATCCATTAATGTTAtcaaagagaatcaatga	59.9
7	III amiR*-F5H s	gaCTAGGAAATCCATAAATGTTTtcacaggtcgtgatatg	61.0
8	IV amiR*-F5H a	aAAACATTTATGGATTTCCTAGtctacatatatattcct	60.1
9	Oligo A	CTG CAA GGC GAT TAA GTT GGG TAA C	60.2
10	Oligo B	GCG GAT AAC AAT TTC ACA CAG GAA ACA	59.8

hp-RNAi constructs

Once the sequence was known, the RNAi primers were designed from the conserved coding region to amplify the RNAi products. TA vector mediated cloning was used to determine the RNAi amplicon for the target gene by sequencing. Then RNAi amplicon was incorporated into pENTER11 and the recombinant pENTER11 plasmid was mobilized into pK7GWIWG(II) destination vector using LR reaction to introduce the hairpin construct (Wu shuchi 2013).

Table 2.4 List of RNAi primers together with their sequences

Sl.No.	Primers_Name	Sequence (5'-3')	Tm(^{0}C)
1	COMT_RNAi_For	ACCCAATTCACTCCAACTCAAG	59.9
2	COMT_RNAi_Rev	GAGATTAAGGGCGGAAAGAGCG	58.0
3	C4H_RNAi_For	ATGATATCCCCGCTGAGAGC	60.0
4	C4H_RNAi_Rev	ATACCAAGGATGGGCAAAGC	57.8

Plants materials and growth conditions

The gene bank of Bangladesh Jute Research Institute (BJRI) has one of the world's largest jute and allied fiber (JAF) germplasm collection of about 6000 accessions. The seeds of *Corchorus olitorius* (var. 0-9897), one of the two cultivated jute species were collected from the Physiology Department, Bangladesh Jute Research Institute (BJRI), Dhaka. Jute seeds were germinated in small pots containing vermiculite or in MS media under sterile conditions with proper antibiotic selection. Fifteen days post germination the plantlets were transferred to big pots with nutrient rich soil. Throughout, the jute plants were grown in a greenhouse (16-hr-light/ 8-hr-dark cycles) at 28°C to 30°C.

Similarly for tobacco, the seeds were germinated in small pots containing vermiculite or in MS media under sterile conditions with proper antibiotic selection. Fifteen days post germination the plantlets were transferred to big pots with nutrient rich soil. *Agrobacterium* infiltration was carried out in the young leaves of 1 month old plant for the gene silencing experiments. Transient replication studies were carried out in the mature leaves of 25-30 day old plants. Throughout the experiment tobacco plants were maintained in a green house at 24°C and 14 hr light cycle.

Chemically competent cell preparation

E. coli DH5α:

From a glycerol stock, bacteria were spread on LB agar plate and incubated overnight at 37°C. A single colony was picked and inoculated in a test tube containing 5-10ml of liquid LB medium. The tube was incubated overnight at 37°C in a shaking incubator (200rpm). Secondary culture was made by inoculating the primary culture in fresh liquid LB at 1/50 ratio (e.g. 1ml of primary culture in 49ml of LB). The secondary culture was incubated in the shaking incubator at 37°C for 3-4 hr so as to have a value of 0.8 to 1.0 of OD600 (optical density at 600nm). The culture was pre-chilled on ice for 30 min (from this step onwards the bacterial cells were always kept in the cold) and centrifuged at 5,000rpm for 10 min at 4°C in order to collect the cells. The cell pellet was washed by adding 10ml of cold $CaCl_2$ (100mM) and was centrifuged at 5,000rpm for 10 min at 4°C. The aqueous phase was poured off and then the cells were resuspended in cold $CaCl_2$ (200mM): cold glycerol (50%) (1:1). 100µl of the suspension was added to a pre-chilled 1.5ml eppendorf tube and the tube was quickly frozen in liquid nitrogen. The tubes were stored at -70°C till further use.

Agrobacterium tumefaciens LBA4404:

From a glycerol stock of *Agrobacterium tumefaciens* LBA4404, the bacteria were spread on LB agar plate and the plate was incubated overnight at 28°C. A single colony was picked and inoculated in a test tube containing 5-10ml of liquid YEM + 50mg/l of streptomycin + 20mg/l of rifampicin. The tube was incubated overnight at 37°C in a shaking incubator (200rpm). Next day, a secondary culture was made by inoculating the primary culture in fresh liquid YEM + 50mg/l of streptomycin + 20mg/l of rifampicin at 1/10 ratio (e.g. 5ml of primary culture in 45ml of YEM). The secondary culture was incubated in the shaking incubator at 28°C for 4-5 hr so as to have an OD of 0.8 to 1.0 of at 600nm. The culture was pre-chilled on ice for 30 min (from this step onwards the bacterial cells were always kept in the cold) and centrifuged at 6,000rpm for 10 min at 4°C to collect the cells. The cell pellet was washed by adding 10ml of cold $CaCl_2$ (50mM) and was centrifuged at 5,000rpm for 10 min at 4°C. The aqueous phase was poured off and the cells were resuspended in cold $CaCl_2$ (40mM): cold glycerol (50%) (1:1). 100µl of the suspension was added to

a pre-chilled 1.5ml eppendorf tube and the tube was quickly frozen in liquid nitrogen. The tubes were stored at -70°C till further use.

Bacterium transformation by heat shock

E. coli DH5α:

Two different tubes containing either the competent cells or the plasmids were incubated on ice for 10 min. Plasmids (10ng-100ng) were added to the tube containing competent cells and the tube was mixed well by hand. The mixture was incubated on ice for 30 min and the tube was subjected to heat shock by placing the tube in a water bath at 42°C for 45 seconds and put on ice immediately afterward. The tube was then kept on ice for 5-10 min and 750μl of SOC was added to the tube. The tube was incubated in a shaking incubator for 1 hour at 37°C. The cells were then pelleted by centrifugation at 6,000rpm for 5 min. 700μl of aqueous phase was poured off, the cells were resuspended in the remaining medium and were spread on LB agar plate containing suitable antibiotic for selection of plasmid containing colonies.

Agrobacterium tumefaciens LBA4404:

Tubes containing the competent cells and plasmids were incubated on ice for 10 min. Plasmids (100ng-1μg) were added to the competent cell tube and the tube was mixed well by hand. The mixture was incubated on ice for 30 min and was quickly frozen in liquid nitrogen. The cells were given heat shock by putting the tube in water bath at 37°C for 5 min and the tube was put on ice immediately afterward. The tube was kept on ice for 5-10 min and 750μl of SOC or LB medium was added into the tube. The tube was incubated in a shaking incubator for 3-4 hour at 28°C. The cells were collected by centrifugation at 6,000rpm for 5 min. The aqueous phase was then poured off and the cells were resuspended in the remaining medium and spread on LB agar plate containing suitable antibiotic for selection of plasmid containing colonies.

Restriction digestion and DNA ligation

Digestion reactions were carried out using the manufacture's protocols. About 1-5μg of DNA samples were digested and resolved on agarose gel (1.2 % wt/vol.). The

vector and insert fragments were purified from agarose gel using gel extraction kit of RBC (RBC, Taiwan) or Qiagen, using the manufacture's protocol.

Ligation of DNA(s) was/were carried out using 50-100ng of plasmid vector and the corresponding amount of insert which was calculated on the ratio of insert:vector (3:1) by the following formula:

[The amount of insert in ng] $= 3 \times \frac{\text{[the size of the insert in kb]}}{\text{[the size of the vector in kb]}} \times$ [the amount of the vector in ng]

The ligation reactions were set up using the manufacture's protocol in a 1.5ml tube and the tube was then incubated overnight at 16ºC.

Transient assay

Transient assays for expression of both C3H-amiRNAs and F5H-amiRNAs were done by *Agrobacterium*-mediated transient expression in *Nicotiana tabacum* L. cv Xanthi leaves achieved through pressure infiltration, as described by Karjee and co-workers (Karjee, Islam et al. 2008), with minor modification. *Agrobacterium tumefaciens* LBA4404 cells were transformed separately with pre-amirRNA containing recombinant vectors. An *Agrobacterium* culture was grown until an optical density of 1.0 at 600 nm (OD600) was reached. The culture was treated with 200 µM acetosyringone (AS) for one hour prior to infiltration. Total RNA was isolated from the infiltrated zones at 7-21 days post-infiltration (dpi). To estimate the degree of functional potency of precursors to form amiRNA, infiltrated zones at different dpi were subjected to small RNA northern analysis using the reverse complementary sequence of corresponding amiRNAs as probes, end labeled with $[\gamma^{32P}]$ (6000 Ci/mmol; Perkin Elmer Life Sciences, USA). Another strategy for transient assay was followed in order to assess amiRNA functionality upon expression of a particular gene. In this case, tobacco leaf (*Nicotiana tabacum* L. cv Xanthi) was infiltrated in a quadrant fashion with four different *Agrobacterium* suspensions containing (i) amiR-construct, (ii) full length gene construct, (iii) mixture of gene and amiR construct and (iv) MES (2-(N-morpholino) ethanesulfonic acid) respectively. After 21dpi, the infiltrated zones were excised and subjected to reverse transcriptase (RT) using

SuperScript® II (Invitrogen,) followed by RT-PCR with gene specific primers to amplify the full length transcript. This strategy proves down-regulation of target gene expression if the amiRNA construct is functional.

Agrobacterium mediated in planta transformation

A tissue culture independent *Agrobacterium tumefaciens* mediated gene transformation protocol established for jute (Sajib, Islam et al. 2008) was used to introduce both siRNA and amiRNA based hairpin constructs into *C. olitorius* var 0-9897. According to this protocol, shoot tips of young jute plants (15–20 cm in height) are injured with a fine needle. After an hour, the injured region is infected with a few drops of *A. tumefaciens* suspension, having an O.D. of 0.80 in YMB medium containing the respective constructs. This is followed an hour later by a second infection in the same region. The infected plants are next incubated in dark for 3 days at 28^0C. Then the plants are allowed to grow under normal conditions in light.

Plant DNA isolation (CTAB method)

To check for the presence of the transgene in putative transformants, genomic DNA was isolated from leaves of hygromycin-resistant and non-transformed rice plants. The genomic DNA was extracted using the CTAB (N-acetyl-N,N,N-trimethylammonium bromide) method (Murray and Thompson 1980).

1. 2X CTAB buffer (Cetyl trimethylammonium bromide)

2 g of CTAB was dissolved in 70 ml water; 8.18 g of NaCl was added and dissolved. Then 10 ml of Tris (1 M, pH 8.0) and 4 ml of EDTA (0.5 M, pH 8.0) were added. The volume was made up to 99 ml with water. After autoclaving, 1 g of PVP was added and dissolved accordingly.

2. 5% CTAB

1.25 g of CTAB was dissolved in 20 ml water. 0.5 g of NaCl was added and dissolved. The volume was made up to 25 ml with double distilled water and autoclaved.

3. CTAB precipitation buffer

1 g of CTAB was dissolved in 90 ml double distilled water. 5 ml of 1 M Tris (pH 8.0) was added. And the volume was made up to 100 ml and autoclaved.

4. High salt TE buffer

5.84 g of NaCl was dissolved in 80 ml water. 1 ml of 1M Tris (pH 8.0) and 200 μl of 0.5 M EDTA (pH 8.0) were added. The final volume was made up to 100 ml with water and autoclaved.

5. Choloroform: isoamylalcohol (24:1)

For 50 ml 48 ml of choloroform and 2 ml of isoamylalcohol were taken.

Quality check and quantification of DNA

The quality of DNA was estimated by taking spectrophotometric absorbance at a wavelength of 260 nm and 280 nm. Concentration of DNA was calculated using OD_{260nm} formula ($OD_{260} = 1$, corresponds to 50 μg/ml of dsDNA). Quality of DNA was considered to be good if A_{260}/A_{280} ratio was above 1.8 for DNA. Integrity of DNA was further checked by running on 0.8% agarose gel.

Polymerase chain reaction

Genomic DNA/Plasmid PCR

PCR amplification was carried out in a 0.2 ml PCR tube (Axygen Inc., USA) containing 100 ng of template DNA, 100 ng each of forward and reverse primers (IDT, USA/Sigma-Aldrich, India), 200 μM dNTPs (Amersham Pharmacia Biotech, England), 1.5 mM $MgCl_2$ and 1U *Taq* DNA polymerase (Genotex, India) in 25 μl of reaction volume in a thermal cycler (M.J. Research, USA). The programming of Applied Biosystem thermal cycler for the PCR amplification was as follows: First cycle consisted of initial denaturation of 95^0C for 5 min. Then, 35 cycles were carried

out with each cycle having denaturation at 95^0C for 30 sec, annealing at 55^0C to 62^0C for 1 min and extension at 72^0C for 30 sec to 3 min. A final extension at 72^0C for 7 min was run at the last cycle. The annealing temperature was calculated based on the melting temperature (Tm) of the primers used in the corresponding PCR reactions.

Colony PCR

Each bacterial colony was picked by a sterile toothpick and mixed with 50 μl of sterile water in an eppendorf tube. The same toothpick was touched on an LB media plate and incubated at 37ºC to obtain a replica. The tubes containing colonies were kept in a boiling water bath for 10 min to lyse the bacterial cells and a quick short spin was given to pellet down the residual colonies and call debris. 20 μl of supernatant from this mixture was used as template for PCR amplification. 50 ng each of vector specific forward and reverse primers (flanking the cloning sites), 200 μM dNTPs (Amersham Pharmacia Biotech, England), 1.5 mM $MgCl_2$ and 1U *Taq* DNA polymerase (Genotex, India) was added and the PCR reaction was carried out in a Peltier thermal cycler.

PCR for screening putative transgenic plants

To check for the presence of the transgene in the putative transformants (COMT-hpRNA, C4H-hpRNA lines, C3H-amiRNA and F5H-amiRNA lines), genomic DNA was isolated from leaves of kanamycin-resistant and non-transformed jute plants. Genomic DNA was extracted using the CTAB (N-acetyl-N, N, N-trimethylammonium bromide) method (Murray and Thompson 1980). To check for the presence of the reporter gene, NPT (II), PCR was performed by using the primers, NPT (II) forward (5'-CCGTAAAGCACGAGGAAGTC-3') and NPT (II) reverse (5'-ATGGGGATTGAACAAGATGG-3'). The PCR reaction profile included initial sample denaturation at 95^0C for 5 min followed by 30 cycles of strand separation at 94^0C for 1 min, annealing at 56^0C for 30 s and extension at 72^0C for 30 s. The program ended with a final extension step for 7 min at 72^0C. Amplification products of approximately 350 bp were analyzed on a 0.8 % agarose gel.

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Southern hybridization

20µg of genomic DNA from PCR-positive amiRNA lines i.e. C3H and F5H together with the wild type (WT) plant for particular transgenic lines were digested with *Bam*HI/*Hin*dIII and hp-RNA lines i.e. COMT and C4H along with their respective WT were digested with *Xho*l. A 0.8% agarose gel containing electrophoresed digested DNA was stained for 30 min in ethidium bromide (1 µg/ml) solution. The gel was soaked in 250 ml of denaturation solution and left on a rocking platform for 90 min. The gel was washed briefly with sterile double distilled water (4 to 5-times) and soaked in 250 ml neutralization solution for 90 min. The agarose gel was subjected to capillary blotting (Southern, 1975) in saline sodium citrate buffer (20X SSC) (as described by Maniatis *et al*., 1982) for the capillary transfer of DNA to the Hybond N+ membrane (GE Healthcare Life Sciences, UK). The transfer was carried out for 12-16 h. The membrane was removed and rinsed briefly for 20 sec in 2X SSC. DNA was fixed to the nylon membrane by UV-cross linking.

1. **Denaturation solution (500 ml)**

Sodium chloride (29.22 g) and NaOH (10.0 g) were dissolved in 400 ml of double distilled water and made up to 500 ml so that the final concentration is 1 M NaCl and 0.5 M NaOH. The solution was autoclaved and stored at room temperature.

2. **Neutralization solution (500 ml, pH 7.0)**

NaCl (43.83 g) and Tris (30.28 g) were dissolved in 300 ml of double distilled water. pH was adjusted to 7.0 with concentrated HCl. The volume was made up to 500 ml, autoclaved and stored in room temperature. The final concentration was 1.5 M NaCl and 0.5 M Tris.

3. **20X SSC (1000 ml, pH 7.0)**

NaCl (175.3 g) and sodium citrate (88.2 g) were dissolved in 800 ml of double distilled water. pH was adjusted to 7.0 and the volume was made up to 1000 ml. The final concentration was 3 M NaCl and 0.3 M sodium citrate. The solution was autoclaved and stored at room temperature.

4. **Labeling of DNA fragments by radioactive method**

The probes were labeled with [α ^{32P}] dCTP (PerkinElmer Life Sciences, USA). Random primer labeling was carried out according to the manufacturer's

instructions (Gibco BRL), in a 50 µl reaction mixture containing 25 ng DNA template, 15 µl buffer, 2 µl each of dATP, dGTP, dTTP, and 5 µl [α^{32P}]dCTP and 1 µl of Klenow enzyme. This mixture was incubated at 25°C for 1 h. The reaction was stopped by adding 5 µl of stop reaction buffer. Resulting reaction mixture was boiled for 5 min and kept on ice for probe purification. A ~250 bp hp-RNAi construct was used as a probe for hp-RNA lines and for amiRNA lines ~250 bp amiRNA construct containing pre-miroRNA backbone was used as a probe (Sambrook, Fritsch et al. 1989).

5. Purification of labeled probe

The purification of radio-labeled probe has been done using G25 column (GE Healthcare Life Sciences, UK). The bottom of a 1 ml syringe was plugged with autoclaved glass wool using a plunger. The syringe was placed in a 14 ml culture tube containing a decapped eppendorf tube at its bottom. The syringe was filled with Sephadex G-50 beads till the column volume was around 0.9 ml and then centrifuged at 3,000 rpm for 3 min. The packed column was washed with 50 µl of TE buffer pH 8.0 and centrifuged at 3,000 rpm for 3 min. This procedure was repeated until the volume of TE eluted was equal to the volume of TE loaded (50 µl). Radiolabeled DNA was loaded onto the spin column and centrifuged at 3,000 rpm for 3 min. The radiolabeled DNA probe was eluted out from spin column whereas the free radioactive label was retained in the spin column.

6. Hybridization

The nylon membrane with bound DNA (blot) was placed inside the hybridization bottle using a sterile forcep and pre-warmed (65^{0}C) pre-hybridization solution was added. Pre-hybridization was carried out for 1 h at 65^{0}C in a hybridization oven (Bachoefer, Germany). Radio labeled DNA was denatured for 5 min by placing in a boiling water bath and plunged immediately on ice for 10 min. Pre-hybridization solution (10 ml) was decanted and refilled with fresh solution (10 ml) and the denatured probe was then added. Hybridization was performed for 12 h at 65^{0}C.

Pre-hybridization solution (1000 ml)

20 X SSC	: 250	ml
50% Dextran sulphate	: 100	ml
1.0 M sodium phosphate pH 7.2	: 50	ml
50 X Denhardts solution	: 100	ml
0.5 M EDTA	: 5	ml
20% SDS	: 20	ml
Distilled water	: 475	ml

The solution was prepared freshly before use from autoclaved reagents.

Denhardt's solution (50 X stock solution)

Ficol	: 5g
PVP	: 5g
BSA	: 5g
H_2O to 500 ml	

7. Post- hybridization washes

Post- hybridization washes were performed using high stringency washing conditions. The temperature was kept at 65^0C and the salt concentration was decreased at each subsequent washes. The hybridization solution was removed and the blot was washed successively with 2X SSC/ 0.1% SDS, 0.5X SSC/ 0.1% SDS and 0.1X SSC/ 0.1% SDS.

Each wash was done at 65^0C for 30 min in the hybridization oven. After the completion of the washes, the blot was briefly rinsed with 2X SSC at room temperature and air dried on Whatmann 1 filter paper. The membrane was wrapped in saran wrap and was exposed to the storage phosphor screen for a desired period of time and then the presence or absence of band(s) in the membrane was visualized by scanning the screen using a TYPHOON phosphor imager (GE Healthcare Life Sciences, UK) and the intensity of the band(s) was analyzed by using Imagequanta TL program.

RNA Isolation from plant

(A) TRIzol® reagent method

RNA was isolated from plant tissue by TRIzol® reagent method (Gibco BRL). The tissue was homogenized in liquid nitrogen; TRIzol® reagent was added to the homogenate (1 ml per 100 mg of tissue) and was incubated at room temperature for 5 min. To the mixture, 200 µl of chloroform per 1 ml of TRIzol® reagent was then added and mixed by vigorous shaking for 15 seconds and incubated again at room temperature for 2-3 min. The homogenate was centrifuged at 12,000 X g for 15 min at 4°C. To precipitate RNA 500 µl isopropanol was added to the supernatant and centrifuged at 10,000 X g for 10 min. The pellet was washed with 75% ethanol made in DEPC treated water and air dried and dissolved in DEPC treated water.

(B) GITC method

1. **DEPC treated water (1l):**

   1L milli Q water and 1 ml DEPC were used. DEPC was added drop wise till a bubble was seen at the bottom. Then it was again autoclaved.

2. **GITC buffer (RNA extraction buffer) 500 ml**

GITC	:	250g in 250ml of DEPC treated water
Sodium citrate	:	3.225g
10% Sarcosyl	:	26.4 ml

   Add β mercaptoethanol 7µl/ml (at the time of crushing)

3. **Phenol (water saturated)**

   - Phenol + DEPC treated water in equal volume were kept to continuous vortexing.
   - When phase separation came, upper layer was discarded. It has been repeated twice.
   - The saturation was cross checked by pH, which should be approx 5.0.

4. **Chloroform: Isoamylalcohol (24:1)**

5. **3M sodium acetate (pH 5.2),** pH was adjusted with glacial acetic acid

6. **Isopropanol**

7. 75% ethanol with DEPC treated water

Total RNA was extracted from WT and kanamycin screened 0-9897 jute seedlings of amiRNA transgenic lines (C3H and F5H) and hp-RNA (COMT & C4H) lines using the guanidium thiocynate extraction method (Chomczynski and Sacchi 1987). Approximately 5g of plant tissue was ground in liquid nitrogen to a fine powder with mortar and pestle. The powdered tissue was transferred to an Oakridge tube and the liquid nitrogen was allowed to evaporate. Approximately 5ml of guanidium thiocyanate buffer (β -mercaptoethanol freshly added 8 µl/ml buffer) was added to each Oakridge tube and kept at RT with intermittent vortexing for 15 min. Centrifugation was applied at 11,000 rpm for 10 min using SS-34 tube rotor at RT. The supernatant was collected to a fresh tube separately and to each tube equal volume of water saturated phenol and chloroform isoamylalcohol (24:1) was added, then vortexed immediately and kept for 10 min at RT. After centrifugation at 11,000 rpm for 10 min using a SS-34 tube rotor the aqueous phase was collected. The phenol-chloroform extraction was repeated for 2-3 times till a clear inter phase appeared. Extraction was carried out with equal volume of chloroform-isoamyl alcohol mixture. To the aqueous phase $1/10^{th}$ volume of 3M sodium acetate was added, mixed and then 1 volume of isopropanol was added to the tube. The reaction mix was kept at –20°C for 2 hr and centrifuged at 10,000rpm for 10 min to precipitate the nucleic acids in pellet form. The pellet was washed with 75% DEPC-treated ethanol, air-dried and dissolved in DEPC-treated water (0.5-1ml/g of tissue). After completely dissolving the pellet $1/4^{th}$ volume of 10N LiCl (final concentration 2N) was added, mixed properly and kept at 4°C for 12-14 hr (RNA was selectively precipitated where as DNA and polysaccharides remained in the solution) and centrifuged at 10,000 rpm for 10-15 min at 4°C. The pellet was collected and dissolved in 1-3ml of DEPC-water. Next $1/10^{th}$ volume of sodium acetate was added and the RNA was re-precipitated with 0.6 volume of isopropanol. The pellet was washed with 75% DEPC-treated ethanol. Fresh 80% ethanol was added and stored in –70°C till further use.

(C) Isolation of low molecular weight RNA

The RNA pellet obtained in GITC method, was dissolved in 0.5 – 1ml of DEPC-H$_2$O depending upon the pellet size. The steps below were used with little modification of the protocol described by Hamilton and Baulcombe (1999) to separate the low molecular weight RNA containing siRNA. The RNA samples were heated at 65ºC for 10 min to disrupt the association between larger and smaller size RNA molecules; it also quickened the dissolving of the pellet. It was immediately placed on ice and PEG (MW=8000) was added to a final concentration of 5% and NaCl to a final concentration of 0.5M. After mixing properly it was kept on ice for 30 min and subsequently centrifuged at 10,000Xg for 10 min to pellet down the high molecular weight nucleic acids. The supernatant was collected; (which contains mainly siRNA, tRNA and rRNA) in an eppendrof tube and to it was added three volumes of ethanol and kept at–20°C for at least 2 hr. It was then centrifuged at 10,000Xg for 10 min and the pellet was collected; (which contains mainly tRNA, rRNA and also siRNA). The pellet was washed with DEPC-treated 75% ethanol, air dried and dissolved in DEPC-H$_2$O before using.

Quality check and quantification of RNA

The integrity and quality of RNA was monitored by both spectrophotometric and electrophoresis methods. Concentration of RNA was calculated using OD$_{260nm}$ formula (OD$_{260}$ = 1, corresponds to 40 μg/ml of RNA). A ratio of A260/A280 should be 2.0, for high quality pure RNA. To check the integrity of isolated RNA, it was run in a 1 % denaturing formaldehyde agarose gel in 1X MOPS buffer. Before loading, 5 μg of RNA samples were mixed with formaldehyde loading dye (for 1.5 ml, glycerol- 100 μl, formamide- 720 μl, 10X MOPS- 160 μl, 37% formaldehyde- 260 μl, and nuclease-free water -260 μl, a pinch of ethidium bromide (EtBr) and heated at 65^{0}C for10 min to make the RNAs single stranded. After cooling on ice, RNA was run at 5 V/cm for 45 min in an electrophoresis unit (Biorad, USA) and the gel was photographed using gel documentation system (Alpha imager, USA). Intact total RNA run on a denaturing gel would have two sharp bands corresponding to 28S and 18S rRNA. Besides that, in RNA from photosynthetic tissues, two additional bands corresponding to 26S and 16S plastidial rRNAs were often visible. The 28S rRNA band should be approximately twice as intense as the 18S rRNA band. This 2:1 ratio (28S:18S) is a good indication for the intact RNA.

Northern blotting

For high molecular weight RNA

For northern blot of the transcripts 30μg of total RNA from each plant sample was fractionated on 1.2% formaldehyde denaturing agarose gel. After completion of the run, gel was rinsed in DEPC-treated water to remove the formaldehyde from the gel and saturated with 10 X SSC for 45 min. RNA was transferred to a nylon membrane (GE Healthcare Life Sciences, UK) by capillary transfer in 10 X SSC (Sambrook, Fritsch et al. 1989). The RNA blots were cross-linked to the membrane in UV-Cross-linker (Stratagene). The probe was prepared according to a described protocol (Sambrook, Fritsch et al. 1989) using [α ^{32P}] dCTP labeled cDNA of COMT, C4H, C3H and F5H gene separately that spanned the entire coding sequence of the corresponding genes. Purification of radio-labeled probe has been done using G25 column (GE Healthcare Life Sciences, UK) according to manufacturer's protocol. Pre-hybridization and hybridization conditions for the RNA blot were same as described for Southern hybridization.

(a) Gel composition (100 ml)

1% agarose	1.0g
0.66M formaldehyde	5.4ml (37% w/v)
1XMOPS buffer	10ml (10X)

(b) Loading buffer (1.5 ml)

Formamide	720 μl
10XMOPS	160 μl
37% formaldehyde	260 μl
dH$_2$O	180 μl
80% glycerol	100μl
Bromophenol Blue	80μl (saturated solution)

Hybridization:

The membrane was then placed in a hybridization bottle in which 10 ml pre-hybridization buffer (buffer composition is given below) with 10mg/ml of sheared carrier DNA (viz. salmon sperm DNA) was added and allowed to pre-hybridize for 5-6 hr at 65°C. Random priming or nick translation kit was used to radiolabel the probe. The probe was denatured by boiling for 10 min and then was immediately placed on ice before adding to the pre-hybridization solution. Hybridization was done for 12-14 h at 65°C with constant shaking.

Washing the blot:

The hybridization buffer was discarded and the membrane was washed in 2X SSC, 0.1% SDS, twice (10 min each time) at RT and then twice with 0.5X SSC, 0.1% SDS at 65°C for 15 min each time. The membrane was regularly checked for counts using a Gieger Muller counter and final washes of 0.2X SSC were given for 20 min at RT. The membrane was wiped to remove extra buffer and placed on an equal sized Whatman sheet. The membrane was wrapped in saran wrap and was exposed to the storage phosphor screen for a desired period of time. The presence or absence of the band in the membrane was then visualized by scanning the screen using a TYPHOON phosphor imager (GE Healthcare Life Sciences, UK) and the intensity of the band was analyzed by using the ImagequantaTL program.

For small RNA

Low molecular weight RNA containing siRNA were resolved overnight in 15% acrylamide urea gel at 50 voltage and the nucleic acids were transferred to nylon membrane by electro-blotting (Hamilton and Baulcombe, 1999). While setting the transfer the membrane and Whatman sheets were soaked in 0.5XTBE and the transfer was carried out at 100 volt for 40 min. Then the nucleic acids were cross-linked by a UV-cross-linker and pre-hybridized in hybridization buffer for 4-5 hr with mild agitation at 37°C. After hybridization for 14-16 hr the blot was washed twice at 37°C using 0.5XSSC, 0.1%SDS for 30 min. The blot was wrapped with a saran wrap and exposed to phosphor-imager screen for subsequent analysis.

Composition of denaturing urea-PAGE gel

    15% polyacrylamide (50 ml)

        40% acrylamide:bisacrylamide (37.5:1)   : 18.75 ml

        Urea   : 21g

        1xTBE (filtered)   : 5ml

        DEPC Water up to 50ml

    For 10 ml

        5μl TEMED

        80μl 10% APS

        Running buffer: 1x TBE (diluted from 10x with autoclaved water)

Northern blot for hpRNA

For northern blot of hpRNA, total RNA was isolated from non-transgenic and transgenic hpRNA (COMT and C4H) lines using TRIzol® reagent and following the manufacturer's protocol (Invitrogen). 20 μg of each sample was resolved on a 12% denaturing urea-PAGE gel. The RNA was then blotted onto a Hybond-N+ membrane (GE Healthcare Life Sciences, UK) by Semi-Dry Transfer Cell (Bio-Rad Laboratories, Inc, CA). Probes were prepared according to a described protocol (Sambrook et al. 1989) using [α ^{32P}] dCTP labeled cDNAs that covered the respective hp-RNAi amplicon specific sequences of COMT and C4H genes. The probes were purified in a G25 column (GE Healthcare Life Sciences, UK) according to the supplier's protocol. Hybridization was carried out at 42^0C using a standard protocol. The membranes were subjected to autoradiography using a TYPHOON phosphor imager (GE Healthcare Life Sciences, UK).

Northern blot for amiRNA

For northern blot of artificial microRNA, total RNA was isolated from non-transgenic and transgenic amiRNA lines using TRIzol® reagent and following the manufacturer's protocol (Invitrogen). 20 μg of each sample was resolved on a 12%

denaturing urea-PAGE gel. The RNA was then blotted onto a Hybond-N+ membrane (GE Healthcare Life Sciences, UK) by Semi-Dry Transfer Cell (Bio-Rad Laboratories, Inc, CA) and hybridized with the following amiRNA antisense sequences: 5' GGGAAGCGTTTTATGAACTA 3' for C3H-amiR and 5' CTGGGAAATCCATTAATGTTA 3' for F5H-amiR. The probes were end labeled using 6000 Ci/mmol [γ^{32P}] ATP (PerkinElmer Life Sciences, USA) using T4 polynucleotide kinase (T4 PNK – Fermentas, Lithuania) and purified in a G25 column (GE Healthcare Life Sciences, UK) according to the supplier's protocol. Hybridization was carried out at 37^0C using a standard protocol. The membranes were subjected to autoradiography using a TYPHOON phosphor imager (GE Healthcare Life Sciences, UK).

Reverse transcriptase polymerase chain reaction (RT-PCR) analysis

For RT PCR, cDNA was prepared in 20 µl reactions, using 50U of SuperScriptTM II reverse transcriptase (Invitrogen) and random hexamers. The first strand cDNA of total RNA were subjected to DNaseI treatment for 30 min. Approximately 2 µg of total RNA from the non-transgenic and transgenic jute seedlings were used for first-strand cDNA synthesis, followed by gene specific PCR. One microliter of the cDNA mixture was used as a template for PCR amplification with gene specific primers (COMT, C4H, C3H and F5H) to amplify the respective genes. PCR for the amplification of particular genes was carried out at an initial sample denaturation at 95°C for 5 min followed by 30 cycles of strand separation at 94°C for 1min, annealing at 56°C to 60°C for 30 s and extension at 72°C for 30 s. The program was extended for 7 min at 72°C. PCR of the same cDNA samples was carried out using gene-specific primers for actin gene in order to semi-quantify the gene transcripts present. The RT-PCR band intensities were quantified using the ImageJ software (http://rsbweb.nih.gov/ij/index.html).

Pigment measurement

Chlorophyll content from the leaf tissues were measured spectrophotometrically after extraction in 80% acetone according to Lichtenthaler and Buschmann, 2001, and Arnon, 1949 (Arnon 1949; Lichtenthaler and Buschmann 2001). Experiments were repeated thrice with three replicates each time (n = 3). 100 mg of leaf tissue was homogenized thoroughly in 1 ml of 80% acetone and centrifuged at 3000 rpm for 2-3 min. The supernatant was retained and absorbance (A) was recorded using a spectrophotometer at 663 nm and 647 nm respectively for chlorophyll-A (Chl-A), chlorophyll-B (Chl-B). Their amounts were determined using the following equations-

Chl-A (µg/ml) = 12.25A663-2.79A647

Chl-B (µg/ml) = 21.50A647-5.10A663

Chemical method of lignin estimation

Klason lignin estimation method (Moore and Johnson 1967, Ehrman and Himmel 1994, Templeton and Ehrman 1995) was used in our study to estimate the acid insoluble lignin (*AIL*). Lignin content of jute whole stem and fiber was measured to determine the amount of acid insoluble lignin in jute for both transgenic and non-transgenic plants. To get the average lignin value of a plant, only the middle section (~16 cm) of the stem was used in this study. A modified Klason lignin estimation method was used in our study to estimate the acid insoluble lignin or, AIL (Tanmoy, Alam et al. 2015). According to the estimation procedure, plant stems were first collected and cut off according to length. Then, they were dried at 105° C to get the dry weight (Ehrman 1994) and ~ 0.5 gm of sample was taken for estimation, denoted as W_1.

These dried samples were subjected to 72% H_2SO_4 hydrolysis at room temperature, followed by 4% H_2SO_4 hydrolysis at boiling temperature. The hydrolyzed solutions were then vacuum-filtered using filtering crucibles (glass crucibles with silica filter). After filtration, the crucibles with the residual content were heated at 105° C in a

heating oven. Then, the weight of the crucibles with the dried residual content was taken, and denoted as W_2.

The next step was to heat the filtering crucibles with residual contents at $575 \pm 25°$ C in a muffle furnace (carried out at the Bangladesh Council of Scientific and Industrial Research, BCSIR). The crucibles were next cooled in a desiccator, weighed and the weight designated as W_3.

With these three different weights, the percentage of acid-insoluble lignin was calculated using the following formula (Templeton and Ehrman 1995):

$$Acid\ Insoluble\ Lignin\ (\%) = \{(W_3 - W_2)/W_1\} \times 100$$

CHAPTER 3

RESULT AND DISCUSSION

Pages 79 ~ 120

The current trend of alteration of lignin biosynthetic pathway with a view to reducing the amount of lignin is largely effective to enhance the profitable usability of lignified plant-based resources. Jute, a lingo-cellulose-rich fiber yielding crop, delineates a new promising sphere with respect to lignin manipulation. In the light of several literature reports, it can be assumed that lignin engineering can reinforce the use of jute as a sustainable resource of bio-based materials for commercial purposes (viz. textile, paper- pulping, biofuel). This study emphasizes on reducing lignin content in jute, using state-of-the-art RNAi techniques (amiRNA and siRNA) for fine-tuned alteration of lignin biosynthetic pathway of jute. The results obtained in this study using two different RNAi approaches are described below.

Gene identification approach

The first challenge of this study was the unavailability of candidate gene sequences for jute, because of no deposition of related information in public database. Because RNAi method is especially sequence specific, retrieving the very sequence of jute gene was imperative. For this purpose, the traditional gene walking method was used with degenerate primers designed from species with sequence similarity to jute like *Populus trichocarpa, Arabidopsis thaliana*, *Nicotiana tabacum*, *Gossypium arboretum* etc (Samira, Moosa *et al.* 2010) followed by cloning and sequencing. TA (Invitrogen) vector mediated cloning was carried out to determine the sequence of the degenerate primer-based amplicons followed by sequencing. Figure 3.1 depicts particular gene specific primer (GSP) based amplicons for the corresponding genes. Full length sequence of the selected genes in the lignin biosynthetic pathway namely, caffeic acid O-methyltransferase (COMT), ferulic acid 5-hydroxylase (F5H), coumarate 3-hydroxylase (C3H) and cinnamante 4-hydroxylase (C4H) were thus retrieved.
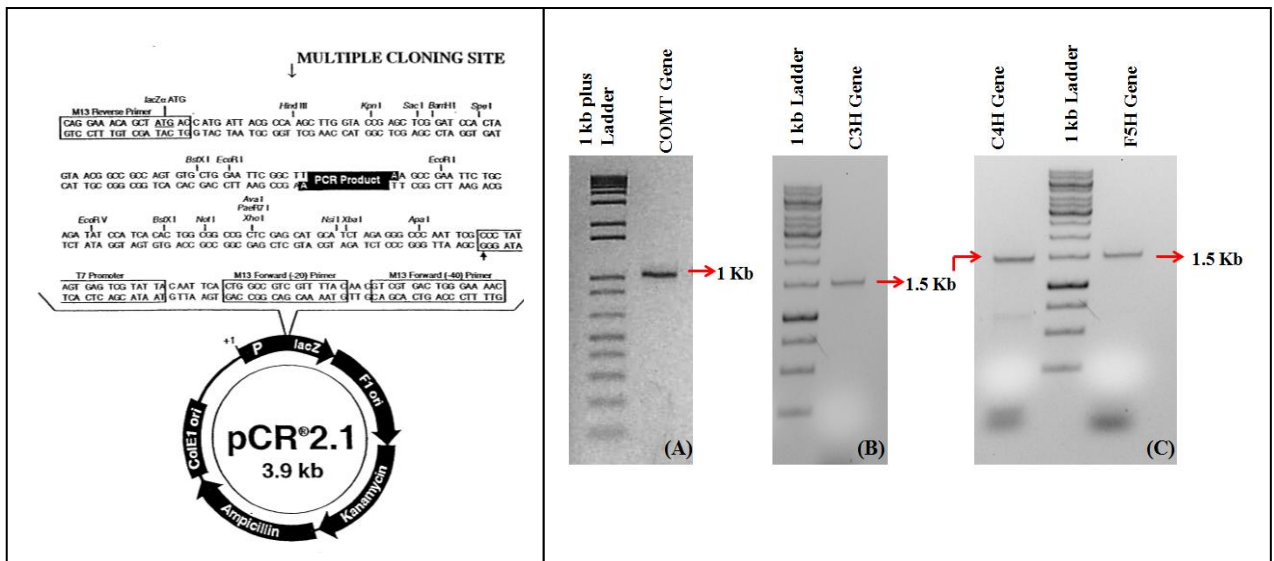
Fig.3.1: Map of pCR2.1 vector used for TA cloning (Left). Agarose gel electrophoreses of gene specific primer (GSP) based PCR amplicons of COMT gene (A), C3H gene (B), C4H and F5H gene(C) respectively (Right).

Down-regulation of lignin biosynthetic gene(s) of jute using hp-RNA

Antisense RNA technology has previously been adopted to reduce the expression of targeted genes in plants. Many researches on small RNAs (sRNAs) have exceeded exponentially in the past years, and it has been concluded that sRNAs control genome expression as well as uphold the integrity by means of an extensively known 'homology dependent gene silencing' method (Araújo 2015). In plants, sRNAs regulate genes engaged in organ polarity, morphogenesis, floral transition, leaf growth, hormone signaling, nutrient deprivation, biotic and abiotic stresses, and are thereby considered as the master regulators of eukaryotic gene expression. (Ruiz-Ferrer and Voinnet 2009; Ding, Chen et al. 2011). With the advent of gene silencing techniques, many studies have shown that efficient repression of target genes can be induced by expressing self complimentary hp-RNA constructs, known as hpRNAi (Watson, Fusaro *et al.* 2005). The hpRNA constructs contain a portion of the desired target gene in sense and antisense direction linked by a non-complementary spacer sequences (similar to intron). Both the ends of transcribed RNA hybridize with each other and form a single stranded loop, hpRNA, which is subsequently processed by RNAi machinery (de Felippes, Wang *et al.* 2012). Successful utilization of siRNAs

engendered by hpRNAs has been reported in many transgenic plants, where they have been shown to work very efficiently in PTGS conferring resistance against a variety of viruses (Zrachya, Kumar et al. 2007; Reyes, De Francesco et al. 2011; Shekhawat, Ganapathi et al. 2012).

At this point, we report, the down-regulation of two lignin biosynthetic genes, cinnamate 4-hydroxylase (C4H) and caffeic acid O-methyltransferase (COMT) by the technique of hp-RNAi (Wesley, Helliwell *et al.* 2001).

Designing of hp-RNA construct:

The first step of target gene silencing involved designing siRNA based hairpin constructs capable of reducing expression of COMT and C4H genes. Conserved domains of the two genes were used to design primers in order to amplify that very region. COMT-RNAi and C4H-RNAi products were amplified by the primers followed by TA vector mediated cloning to determine the RNAi amplicons (Fig. 3.2A). Then these amplicons were incorporated into pENTER11 plasmid and finally the recombinant pENTER11 plasmids (COMT-pENTER11 & C4H-pENTER11) were mobilized into pK7GWIWG (II) destination vector using LR reaction (Wu and Zhao 2013). Figure 3.2B represents the vector map of both entry and destination vector used in this study.
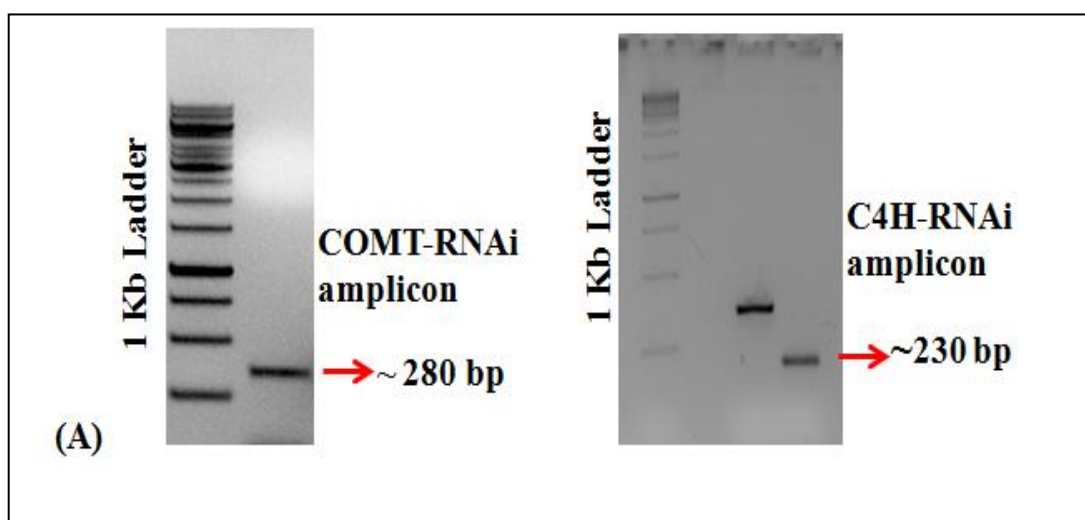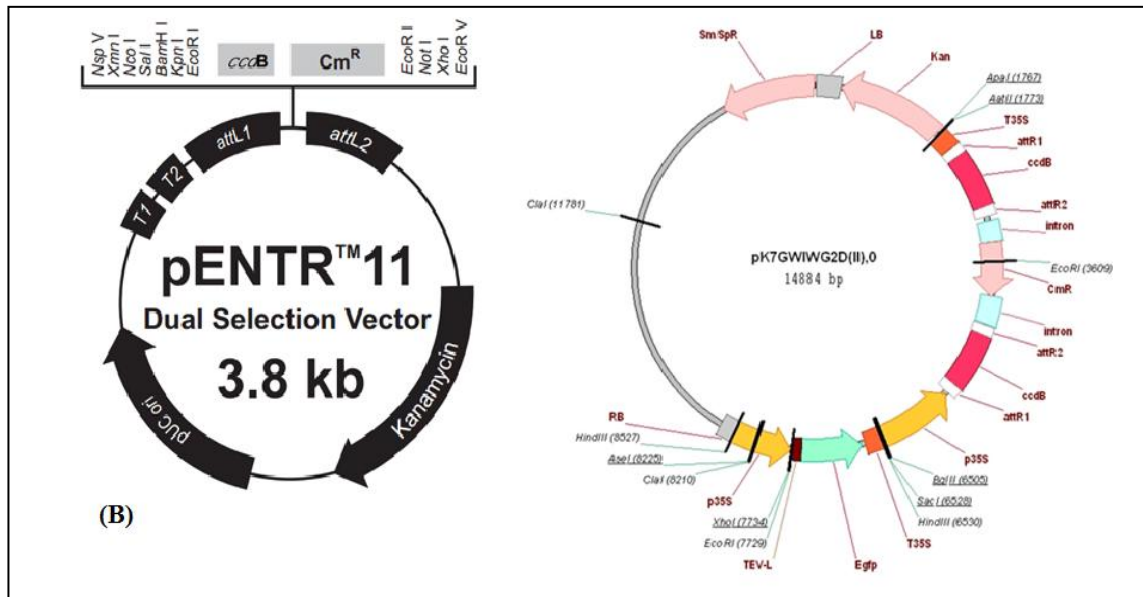
Fig.3.2: (A) Agarose gel electrophoreses illustrates the RNAi-primer based amplification of COMT and C4H gene (B) presents the vector map of pENTER11 and pK7GWIWG (II) used as entry vector and destination vector respectively.

Construct validation:

Initial COMT-pENTER11 and C4H-pENTER11 constructs were confirmed by colony PCR and restriction digestion with enzymes (*BamHI*/*XhoI*), using the respective cutting sites present in the constructs and then mobilized into the destination vector pK7GWIWG (II). A set of colony PCR and restriction digestion from the recombinant destination vectors revealed the presence of the full cassette (35s Promoter and Nos Terminator spanning the RNAi amplicon). Correct insertion and inverted orientation of the two copies of COMT-hpRNA and C4H-hpRNA sequences in the destination vectors was validated by full-length sequencing of the recombinant destination vectors. Figure 3.3 depicts the colony PCR results and correct insert release upon restriction digestion for COMT gene and figure 3.4 represents the same for C4H gene.
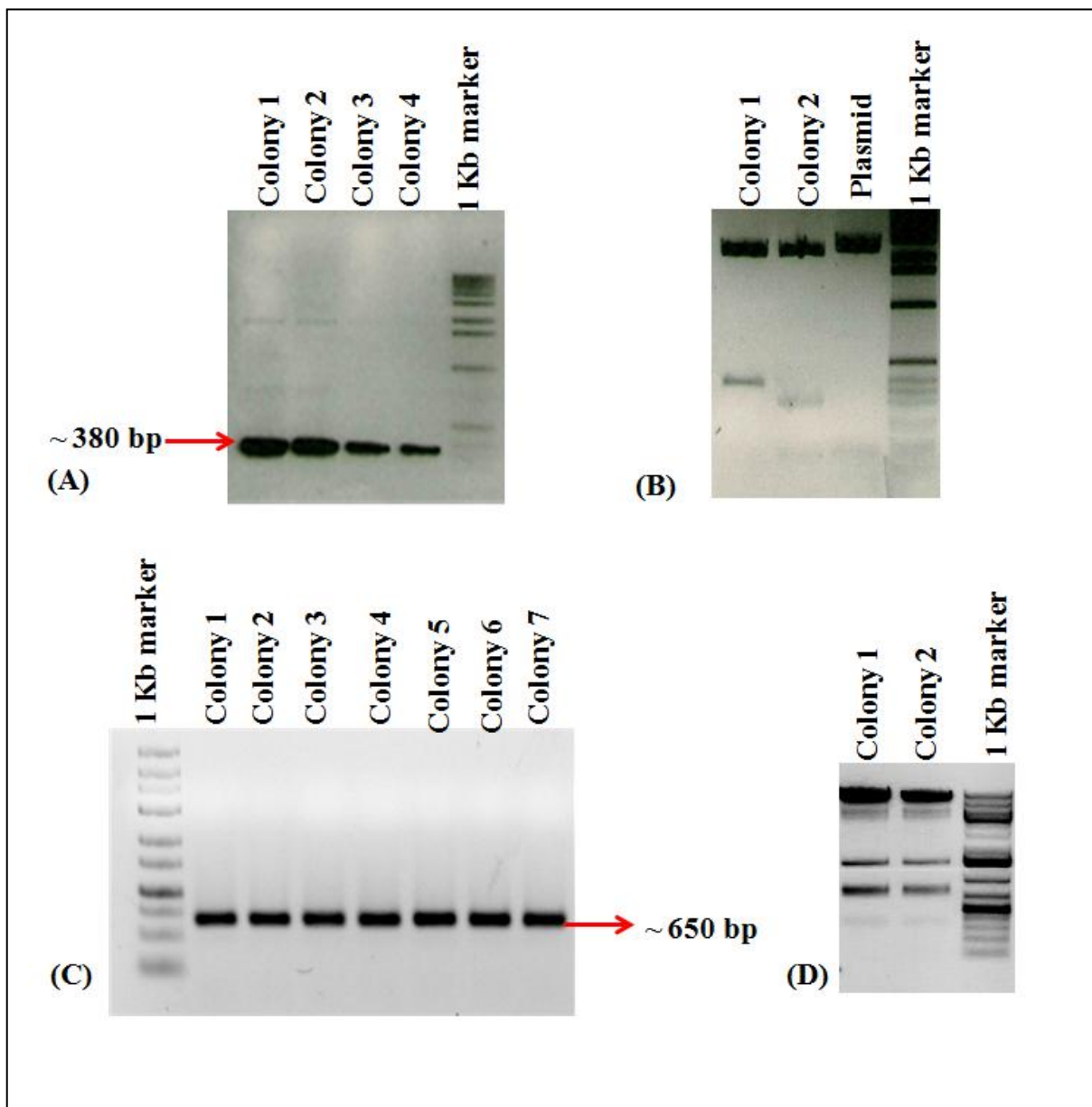
Fig.3.3: Represents the cloning confirmation for COMT-RNAi (A) Colony PCR from pENTER11 (B) COMT-RNAi insert release from pENTER11 (C) Colony PCR from destination vector pK7GWIWG (II) (D) Insert release from destination vector upon restriction digestion with *BamHI/XhoI*.
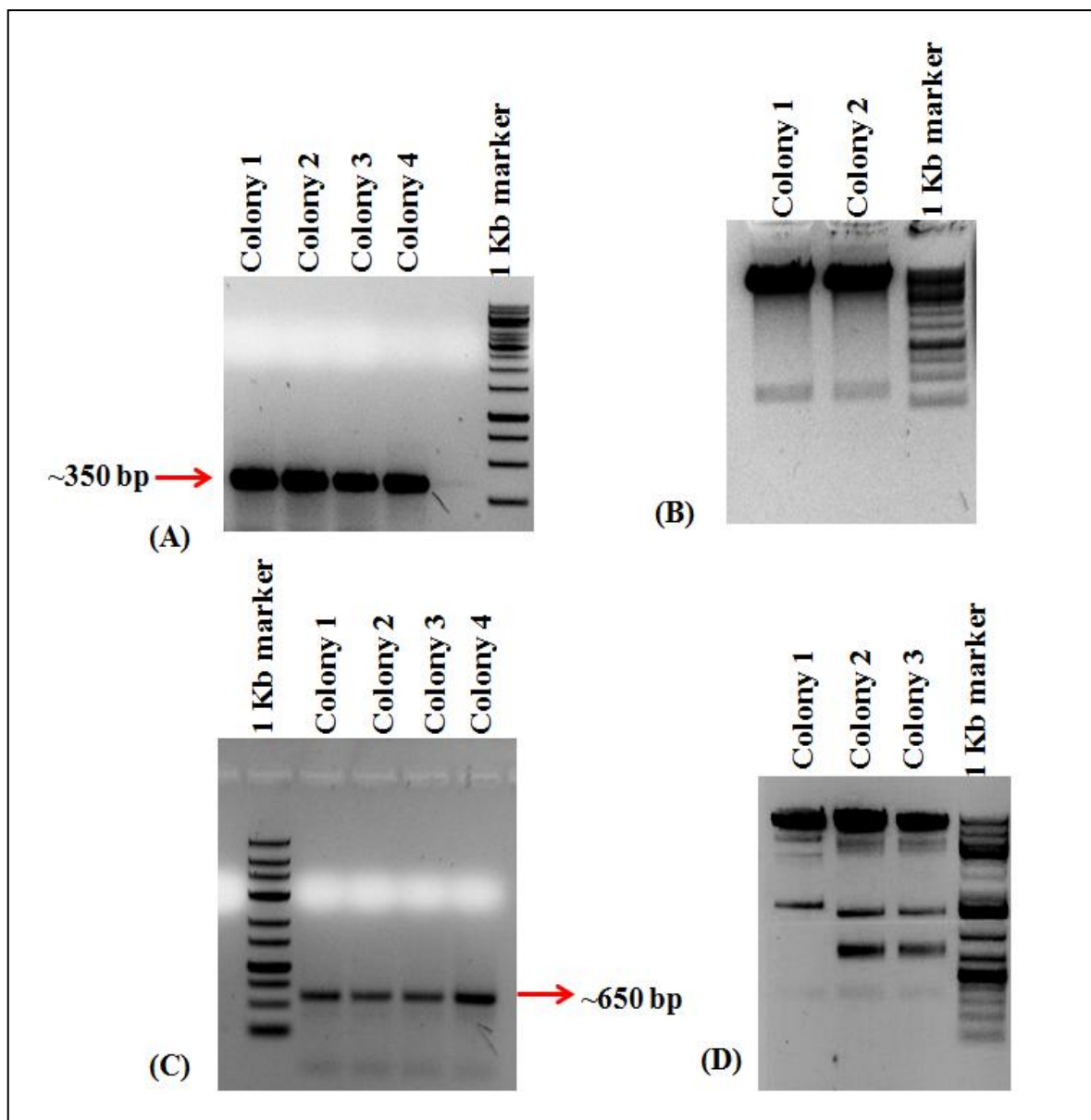
Fig.3.4: Shows the cloning confirmation for C4H-RNAi (A) Colony PCR from pENTER11 (B) COMT-RNAi insert release from pENTER11 (C) Colony PCR from destination vector pK7GWIWG (II) (D) Insert release from destination vector upon restriction digestion with *BamHI/XhoI*

Plant transformation:

Agrobacterium mediated transformation remains a powerful and preferred method for genetic modification of plants. This is due to its high efficiency of transformation, integration of small numbers of copies of T-DNA into chromosomes, minimal rearrangement of transgene and transfer of relatively large segments of DNA upon transformation (Birch 1997; Hansen and Wright 1999). In mid 1990s, *Agrobacterium* mediated gene delivery, via tissue culture involving callus initiation and regeneration, emerged as a powerful tool for introducing foreign gene(s) into rice (Hiei, Ohta et al. 1994; Hiei, Komari et al. 1997). Since then several protocols and their modified versions are available for efficient transformation in other plants (Cho, Chung et al. 1998; Hansen and Wright 1999; Rakoczy-Trojanowska 2002). However, the success of this method is limited by the efficiency and duration of callus induction, transformation and plant regeneration. For this reason this method is not effective for jute. Regeneration after callus transformation has not been possible for this plant since it is recalcitrant to tissue culture (Smith and Drew 1990; Saha and Sen 1992; Birch 1997; Sarker, Al-Amin et al. 2007).

In planta transformation technique has emerged as an alternative and fast route to overcome the complexities associated with tissue culture. This method offered convenience and broader applicability to a wide genotype range. Several *in planta* transformation protocols are available for a variety of plants including maize (Chumakov, Rozhok et al. 2006; Mamontova, Velikov et al. 2010), arabidopsis (Zhang, Henriques et al. 2006; Li, Park et al. 2009), wheat (Supartana, Shimizu *et al.* 2006) and rice (Das and Sanan-Mishra 2015).

A simple, fast, efficient and reproducible method for *Agrobacterium* mediated *in planta* transformation protocol has been developed for jute (Sajib, Islam *et al.* 2008). This tissue culture independent *Agrobacterium tumefaciens* mediated gene transformation was used to introduce both COMT-hpRNA and C4H-hpRNA based hairpin constructs into *C.olitorius* var 0-9897 (Fig. 3.4). Screening of primary transformants was carried out by growing on antibiotic selective media containing 400 mM kanamycin. Seedlings tested positive were subjected to molecular analysis and plants used in these analyses were randomly selected to rule out screening biasness.
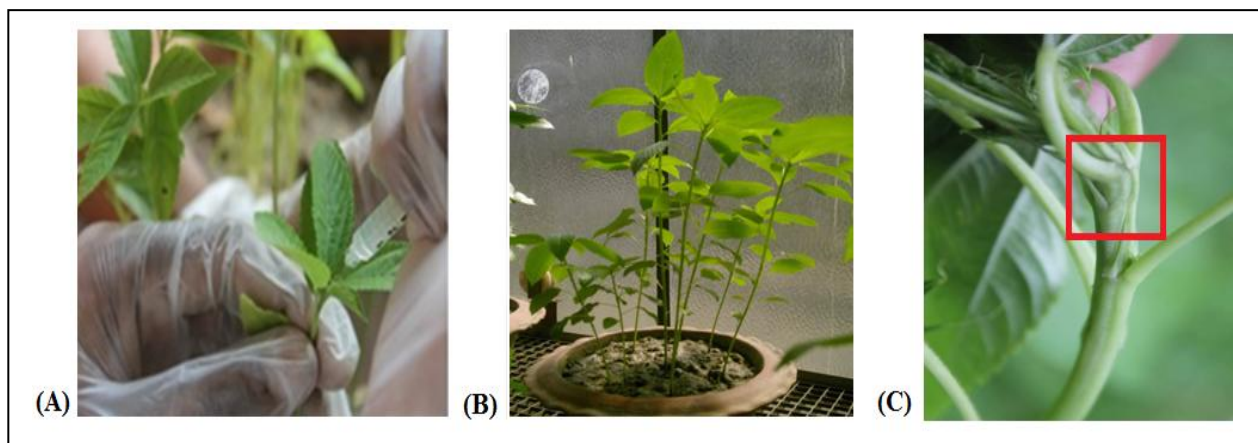
Fig. 3.4: (A) Represents *in planta Agrobacterium tumefaciens* mediated transfection (B) shows incubation of transfected plants in dark @ 28^0C (C) depicts stem thickening due to *Agrobacterium* accumulation in the infected area.

Stable inheritance of transgene(s):

To investigate the presence of the hp-RNAi gene, the antibiotic screened jute plants were randomly selected and subjected to molecular confirmation. Initial transgenesis of COMT-hpRNA and C4H-hpRNA constructs in the T_1 progenies were confirmed by amplification of the reporter gene present in the corresponding construct. Transgenic lines showed an expected size of 350 bp, ubiquitous signal for NPT (II) gene whereas it was negative for WT (Fig. 3.5 A & B). The integration of the hp-RNA was confirmed by Southern blot analysis using PCR positive lines where a single integrated band was observed for transgenic lines when probed with hpRNA-precursor specific sequence (Fig.3.5 C & D). Southern blot data revealed stable integration and inheritance of hp-RNA precursor gene in succeeding transgenic generations (up to T_3), confirming successful hpRNA mediated transgenesis in jute.
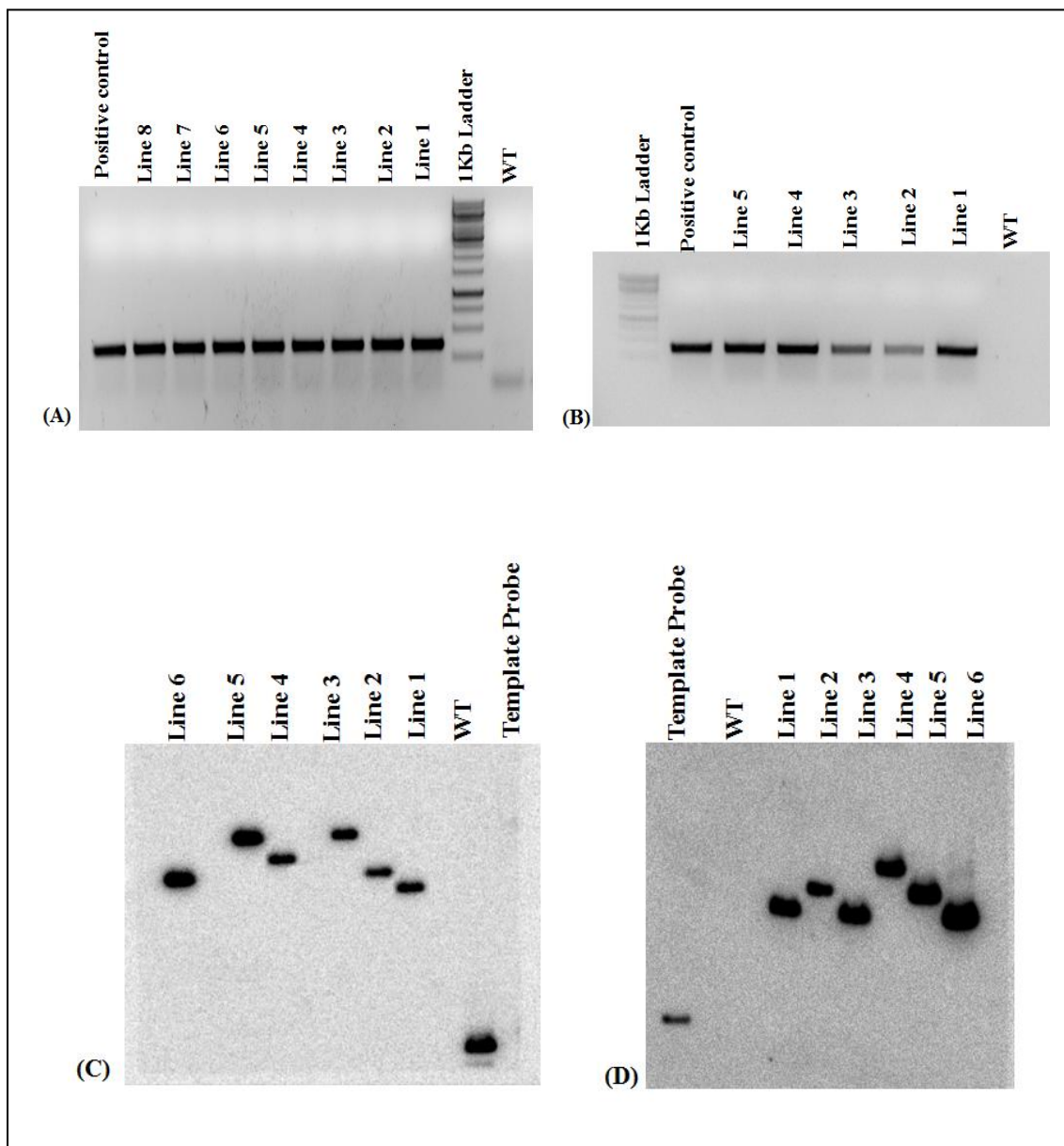
Fig.3.5 Analysis of primary hp-RNAi transformants of jute: Antibiotic screened (400mM Kanamycin) seedlings obtained from each primary transformants were checked for PCR and Southern analysis. Genomic DNA isolated from these seedlings was PCR amplified with NPT (II) primer from T_1 progenies of COMT-hpRNAi (A) and C4H-hpRNAi (B) to confirm the initial transgenesis. The wild type (WT) seedling was used as a negative control while pK7GWIWG (II) plasmid was used as template for positive control. Southern blot: 20 µg of *XhoI* digested DNA from leaves of different plants was blotted and probed with [α ^{32P}] labeled hp-RNAi construct specific region (C) Lanes 1-6, different COMT-hpRNAi transgenic plants; Lane 7, wild type (WT) plant; Lanes 8 template probe (D) Lane 1 template probe, Lane 2 WT, Lane 3-8, different C4H-hpRNAi transgenic plants.

Transgenic analysis:

Expression of target genes was studied to prove functionality of the constructs. Expression was found to drop in the transgenic generations (T_1 to T_3) for both genes when monitored along a time-gradient by semi-quantitative RT-PCR (Fig.3.6 A & B). It is perceptible that, the siRNAs for COMT and C4H are effective in activating the RNAi pathway leading to depleted gene expression, as reported for other plants (Parizotto et. al.2004). The intensity of bands for expressed genes found in the northern blots (for plant lines found positive for Southern) were found to be lower in comparison to non-transgenic plants, further validating our claim (Fig.3.6 C & D). The hp-northern showed a trend opposite to that of transcript level northern, confirming successful and functional hp-siRNA transgenesis (Fig.3.6 E & F). It has been reported that the intron – hairpin structure can enhance stability and efficiency of duplex RNA formation inducing PTGS gene silencing (Smith, Singh et al. 2000; Stoutjesdijk, Singh et al. 2002). Considering the above, it can be claimed that the RNAi machinery in transgenic plants were triggered by the hp-siRNA constructs where the siRNAs for a particular gene had down-regulated transcription of the same.
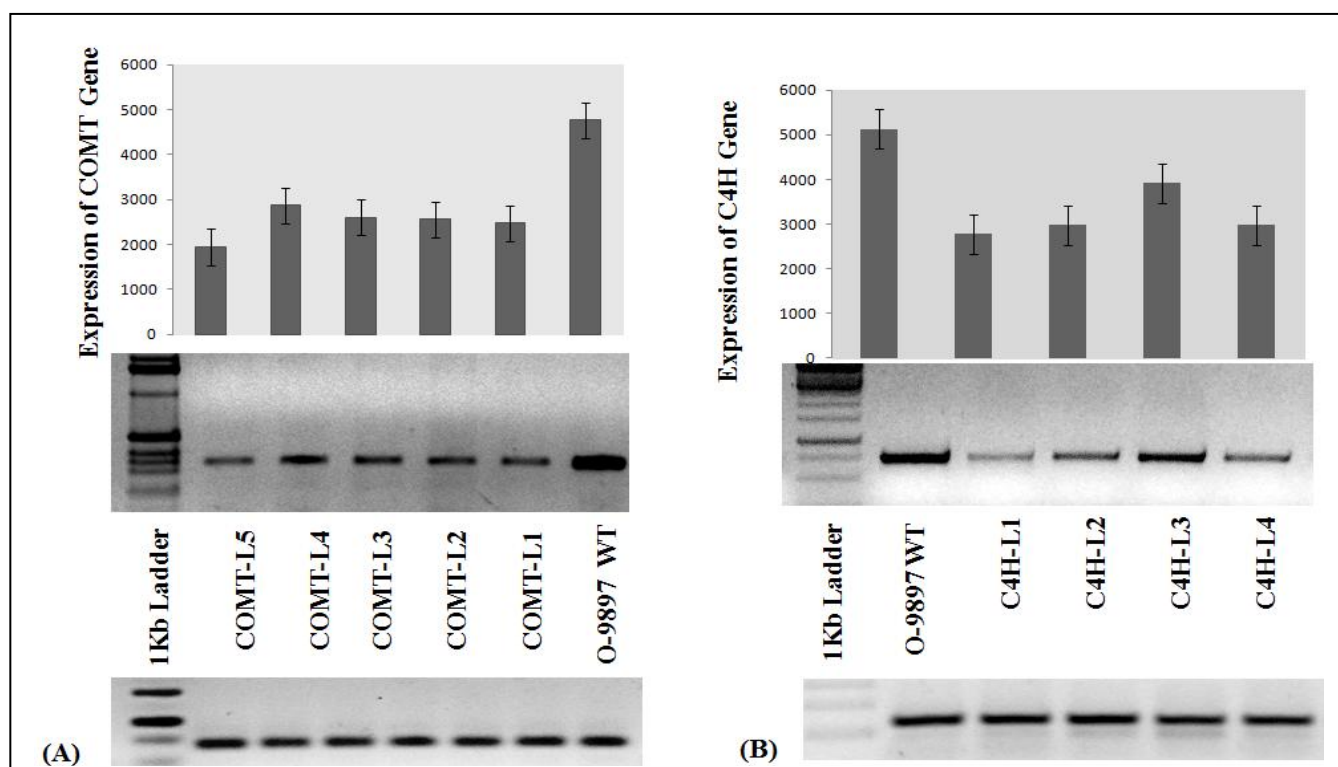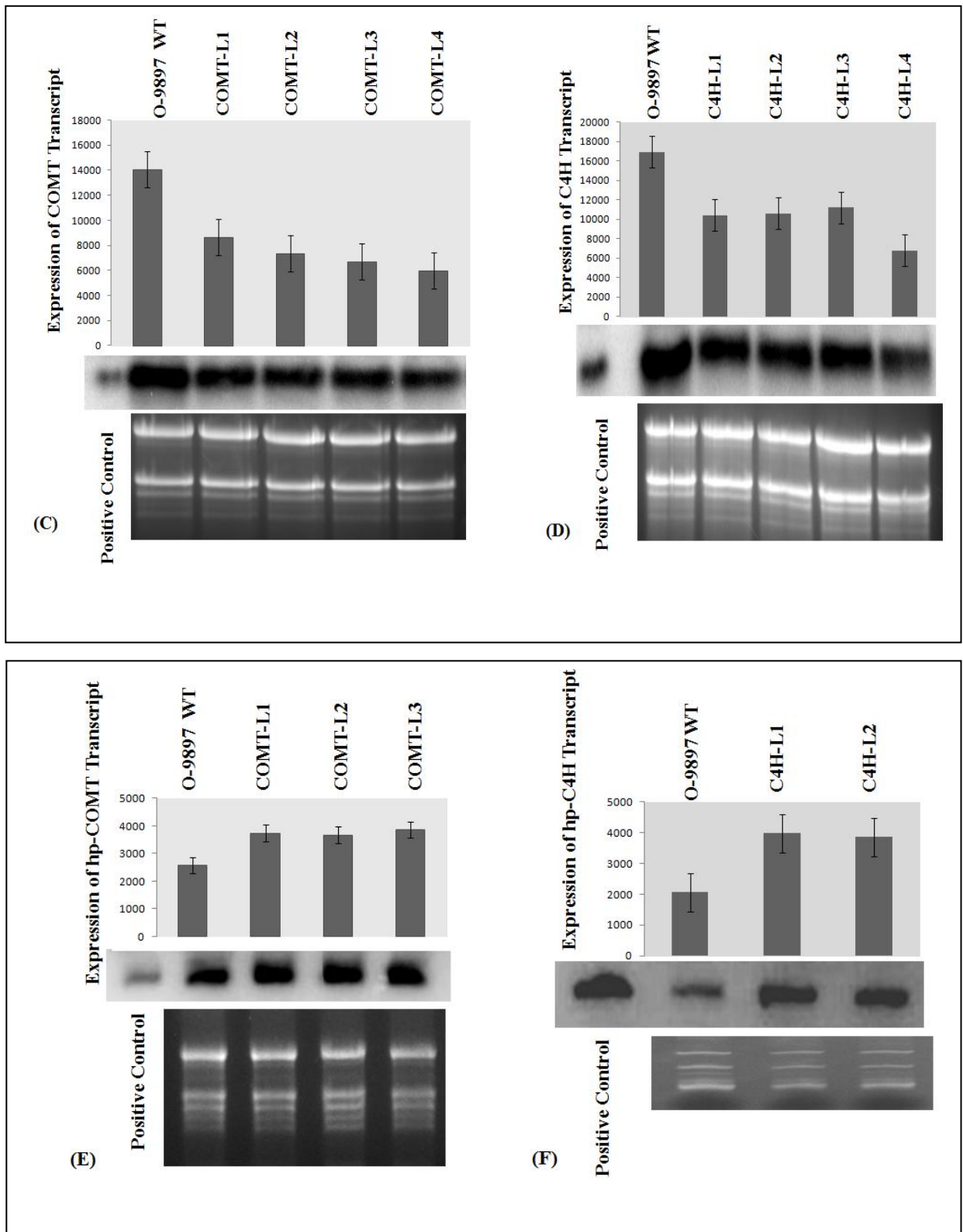
Fig.3.6: Reverse transcriptase polymerase chain reaction (RT-PCR): Total RNA was isolated from seedlings of non-transformed and transformed jute hp-RNAi lines. RT-PCR was performed using the primers amplifying the COMT (A) and C4H (B) gene along with β-ACTIN genes. cDNA was prepared using 50 U of Super-ScriptTM II reverse transcriptase (Invitrogen). The figures above show the intensities of the

band on the blot normalized with respect to β-ACTIN gene. The band intensities were measured by Alpha Imager Imaging System**.**

 Northern blot: 30μg of total RNA prepared from the seedlings was used for hybridizations with [α^{32P}] labeled cDNA probe of COMT gene (C) and C4H gene (D) separately that spanned the entire coding sequence of the corresponding genes.

Hp-Northern Blot: 20 μg of each RNA sample was resolved on a 12% denaturing urea-PAGE gel, blotted and hybridized with [α^{32P}] dCTP labeled cDNAs that covered the respective hp-RNAi amplicon specific sequences of COMT (E) and C4H (F) genes. The figures above show the intensities of the bands on a blot normalized with respect to 28S rRNA. The band intensities were measured by Alpha Imager Imaging System**.**

Using artificial microRNA, a new horizon of gene silencing for reducing the amount of lignin in jute

Homology based gene silencing has emerged as a convenient approach for repressing expression of genes in order to study their functions. For this purpose, several antisense or small interfering RNA based gene silencing techniques have been frequently employed in plant research (Tiwari, Sharma *et al.* 2014). Artificial microRNA (amiRNAs) mediated gene silencing represents one such technique which can be utilized as a potential tool in functional genomics. Similar to microRNAs, amiRNAs are single-stranded, approximately 21 nt long, and it is designed by replacing the mature miRNA sequences of duplex within pre-miRNAs (Alvarez, Eshed *et al.* 2009). These amiRNAs are processed via small RNA biogenesis and silencing machinery and deregulate target expression. Holding to various refinements, amiRNA technology offers several advantages over other gene silencing methods (Tomari and Zamore 2005; Qavi, Kindt et al. 2010; Khatri, Rathi et al. 2012). This robust tool, can be applied to unravel new insight of metabolic pathways and gene functions across various disciplines as well as in translating observations for improving favorable traits in plants (Tiwari, Sharma *et al.* 2014). Like the hp-RNAi method, amiRNA was used to reduce the amount of lignin in jute, by down-regulation of two other monolignoid biosynthetic genes named ferulic acid 5-hydroxylase (F5H) and coumarate 3-hydroxylase (C3H) in order to develop jute with reduced lignin content.

Design of artificial microRNA

Successful C3H-amiRNA and F5H-amiRNA constructs were designed and produced for post-transcriptional silencing (PTGS) for the respective (C3H and F5H) genes, in jute. Transcripts of target genes were searched through sequence homology with *Arabidopsis thaliana*. WMD3 (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi), a web-based tool for designing artificial microRNA, was used to predict potential 21nt sequences while minimizing possible off-target effects to other transcripts. Subsequently, WMD3 primer designer tool was used to generate a set of four oligos for engineering each amiRNA construct. Next, it is crucial to select the pre-amiRNA backbone for further advancement. In details, the secondary structures of the chosen backbones should be simple with a small number of loops of small sizes. For each backbone, the expression profile should be available in the literature with clearly defined plants, tissues, ages and conditions for their expression (Warthmann *et al.*, 2008). The mature sequence length of artificial miRNA in these backbones has to be 21nt. For example, ath-mir-319a was chosen for its well-known expression in arabidopsis (Palatnik *et al.*, 2003: miR-JAW or miR-319a; Xie *et al.*, 2005) and for the wide conservation throughout plant kingdom of mir-319a precursor (Li *et al.*, 2011). Moreover, ath-pre-miRNA319 backbone shows high conservation of pre-miRNA processing across the plant kingdom (Khraiwesh, Ossowski *et al.* 2008). It has thus been used in different plants (Schwab, Palatnik *et al.* 2005) and was expected to be relevant for jute.

To generate pre-miRNA construct containing mature amiRNA sequence, the secondary structures of the natural pre-miRNA were calculated using Mfold (Zuker 2003) software. The output provides images of the secondary structure of the pre-miRNA with nucleotides from which the location and direction of both guiding strands and star strands of the amiRNAs were identified. The guiding strands of the amiRNAs were overwritten by those of the amiRNAs without changing any other nucleotides. The star strands of the amiRNAs were generated based on the characteristics of the base pairing between the natural guiding and passenger strands. Nucleotide sequences of star strands were therefore changed accordingly to allow for proper dicing activity of DCL1 which is supposed to dice out exactly the desired sequences of the amiRNAs. Secondary structure of the amiRNA precursor sequence predicted using Mfold (Zuker 2003) is given in Figure 3.7. Predicted binding sites of

amiRNA to the respective mRNA sequences and amiRNA/amiRNA* duplex are shown in Figure 3.7. Cleavage mapping for the both genes (C3H and F5H) with their corresponding amiRNAs illustrates the probable cleavage site of amiRNAs (Fig. 3.8).
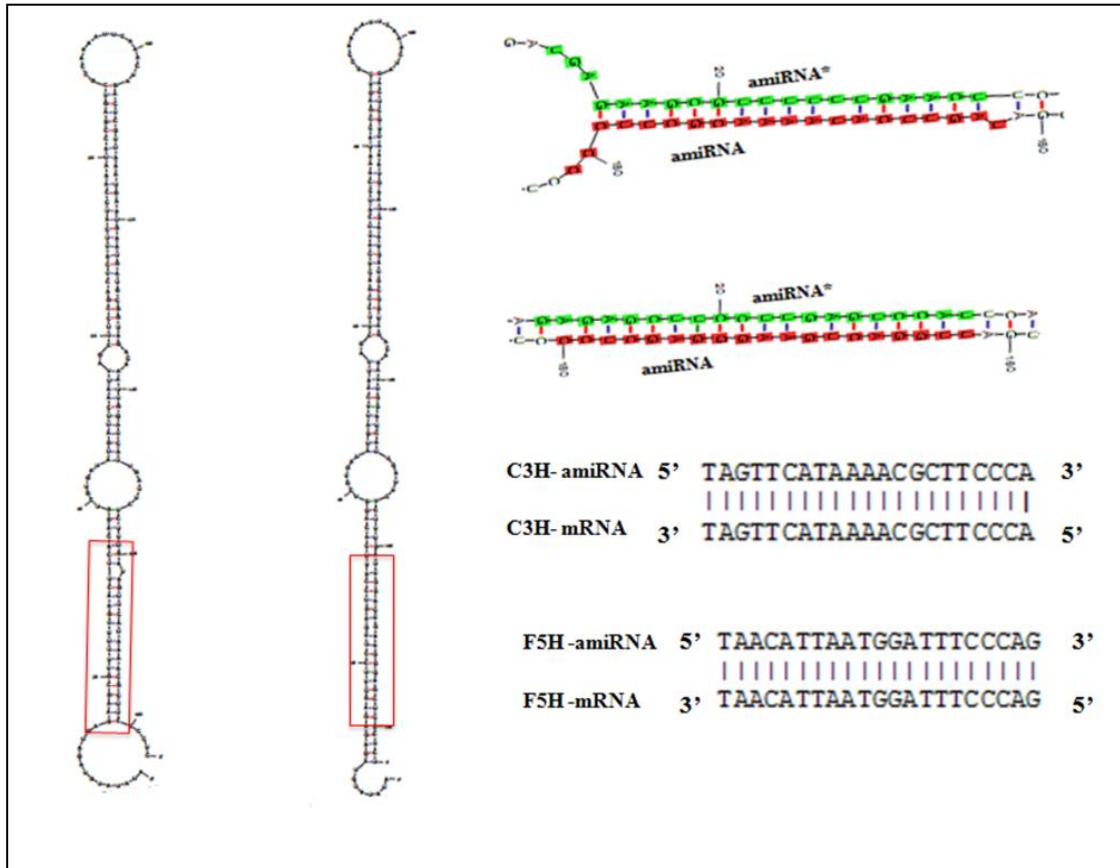

Fig.3.7: Diagrammatic representation of secondary structure of amiRNA precursor predicted by Mfold (Left). The right panel depicts the amiRNA/amiRNA* duplex colored in red and green respectively (top) and the sequence of mRNA corresponding to the respective amiRNA (bottom) to show pairing with the target site.
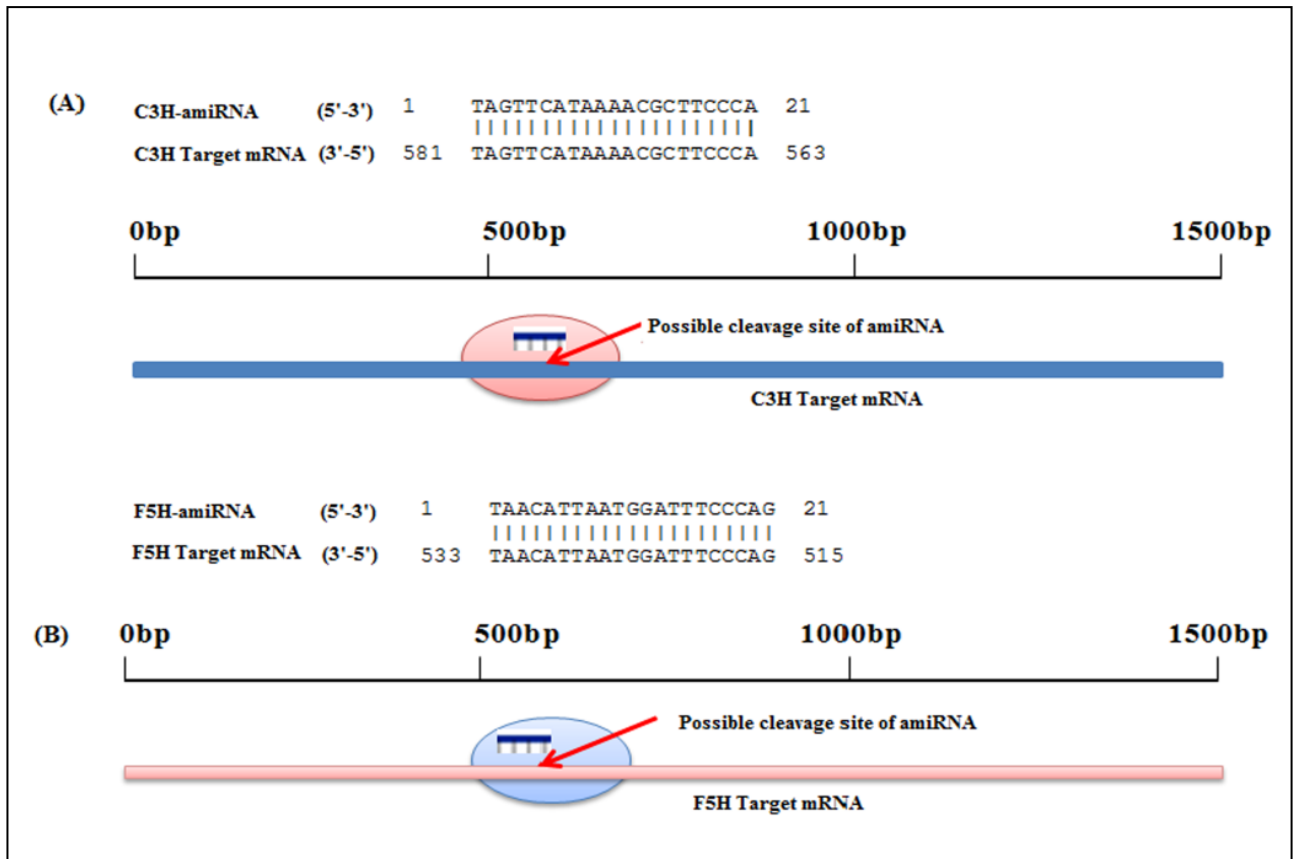
Fig.3.8: Depicts cleavage mapping for C3H (A) and F5H (B) gene by their respective amiRNAs.

In vitro integration of mature amiRNAs into the chosen precursor backbone was done by overlapping PCRs and chemical syntheses. Overlapping PCR was set up with the plasmid, pRS300 (containing ath-pre-miR319a backbone) as a template, using different oligos (provided by WMD3), for replacing the miRNA and its complementary (miRNA*) sequences with jute specific amiRNA duplex (Schwab, Ossowski *et al.* 2006). A diagrammatic representation of the overlapping PCR along with the vector map of pRS300 (containing ath-pre-miR319a backbone) is shown in Figure 3.9. In case of overlapping PCR, initially three fragments viz. fragment a, b and c were amplified separately and finally a fusion PCR was set using the three PCR products (i.e. fragment a, b and c) as templates to amplify fusion D fragment spanning the pre-amiRNA precursor (Fig. 3.10).
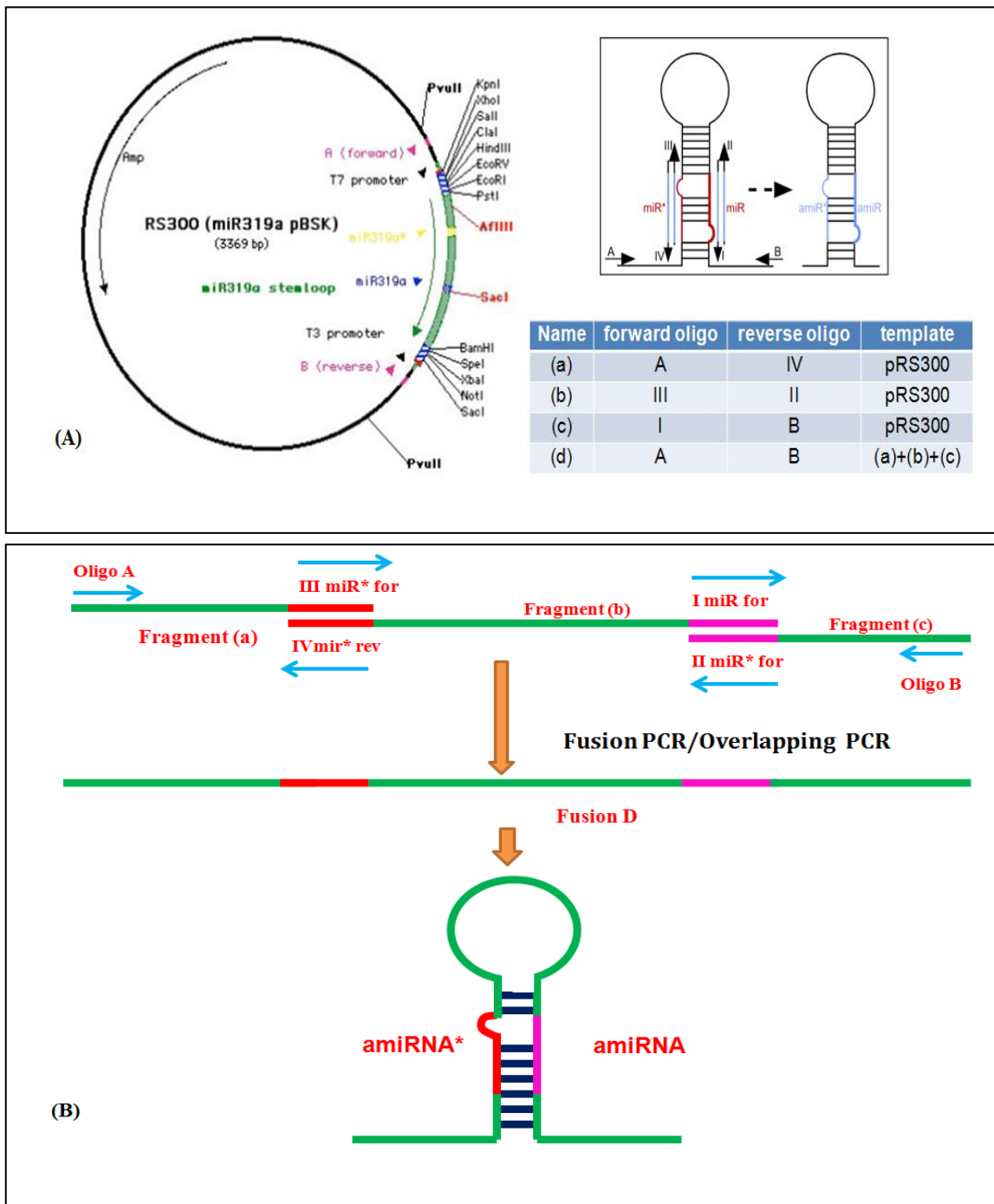
Fig.3.9: **(A)** Map of plasmid pRS300 and PCR strategy using different oligos **(B)** illustrates the steps of overlapping PCR in details.
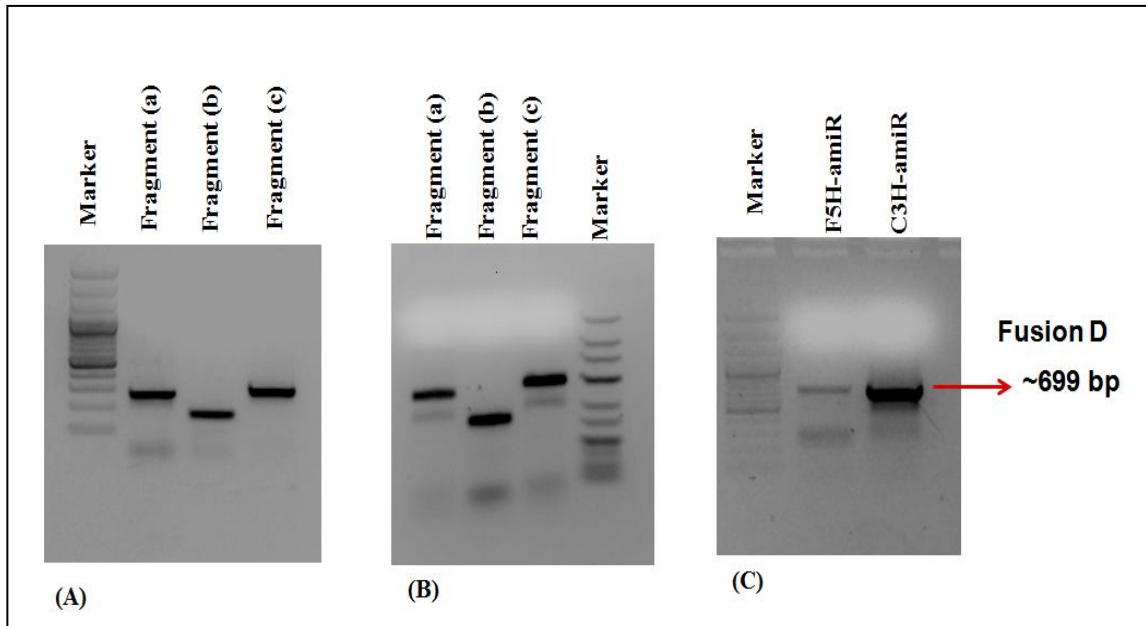
Fig.3.10: Agarose gel electrophoreses depicts the PCR amplified products of fragment a, b and c for C3H-amRNA **(A)** F5H-amiRNA **(B)** and fusion D fragments for both the corresponding amiRNAs **(C).**

These synthetic/artificial miRNA precursor DNAs (i.e. fragment D) were cloned into entry vector pGEMT followed by mobilization into suitable plant vector (pBI121) as destination vector for over-expression in plants. Subsequently, *EcoRV* and *Sacl* restriction enzymes were used to transfer the C3H-amiRNA and F5H-amiRNA precursor cassette into plant expression vector (pBl121) where pBI121 was digested with *Smal* and *Sacl* respectively (Chen, Wang *et al.* 2003). A schematic representation of amiRNA cloning strategy is given in figure 3.11.
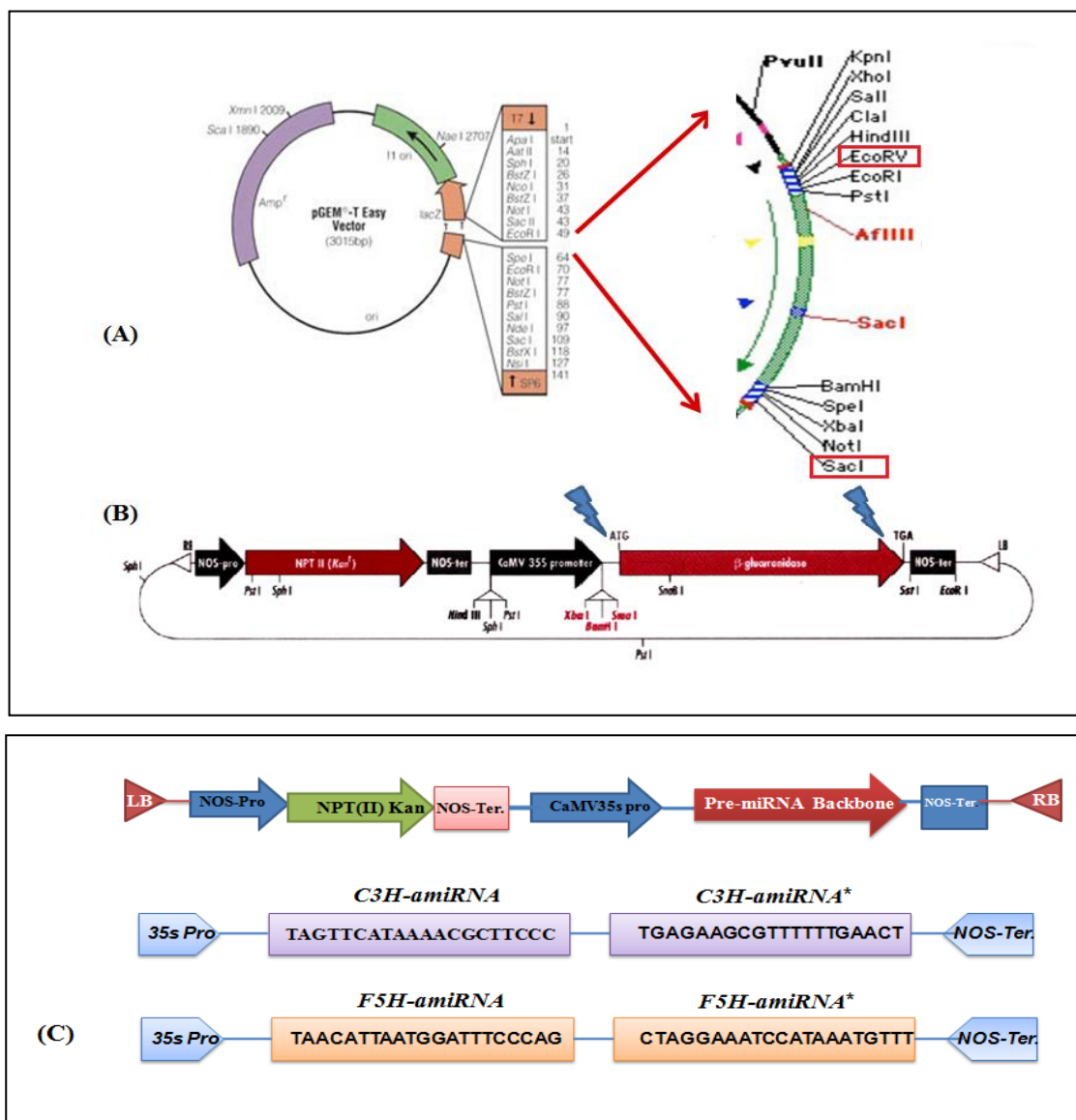
Fig.3.11: A schematic diagram representing the amiRNA cloning strategy (A) Entry vector pGEMT was used to clone pre-amiRNA precursor initially. Restriction sites of *EcoRV* and *SacI* were used to release the entry clone for subsequent mobilization into destination vector. **(B)** Plant expression vector pBI121 was used as the destination vector where *SmaI* and *SacI* restriction digestions were used for insertion of the entry clone. **(C)** Represents the map of destination vector containing pre-artificial microRNAs (C3H-amiR and F5H-amiR) cassette along with the amiRNA and amiRNA* sequences for the corresponding genes.

Validation of the entry clone was done by restriction digestion with *SmaI* and *SacI*. The entry vector cloned products initially screened by restriction digestion, were further sequenced from pGEMT recombinant vector to confirm the incorporation of

jute specific amiRNA and amiRNA* in the mir319 backbone for both C3H and F5H genes. After mobilization to the destination vector pBI121 the constructs were further confirmed by cassette specific PCR and restriction digestion with *SmaI* and *SacI.* (Fig.3.12)
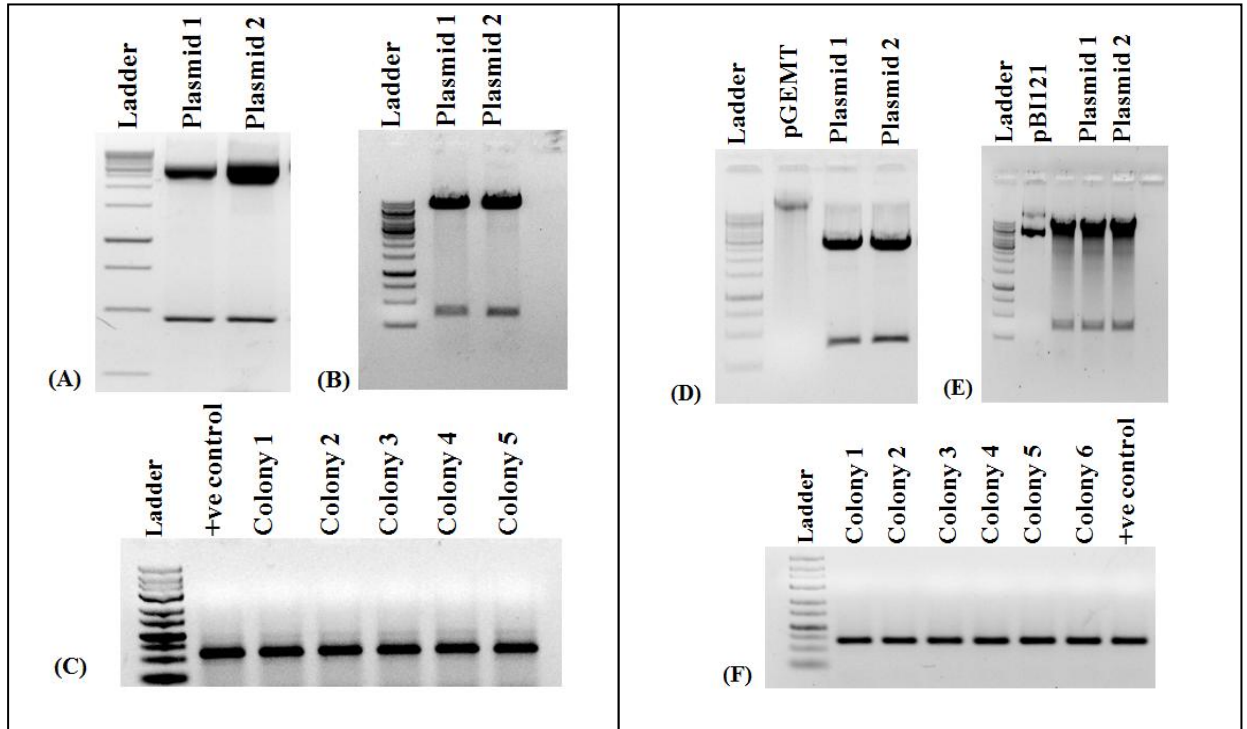

Fig.3.12: Agarose gel electrophoreses shows the pre-amiRNA insert release from pGEMT and pBI121 upon digestion with *SmaI* and *SacI* for C3H-amRNA (A & B) and F5H-amiRNA (C &D) respectively. Colony PCR of pBI121-C3H-amiR (C) and pBI121-F5H-amiR (F) using respective construct specific primers.

Validating the amiRNA expression in Nicotiana tabacum

To authenticate the functionality of the constructs cloned in pBI121 vectors, a rapid and simple transient assay was performed. The assay involves *Agrobacterium*-mediated transient expression of the construct in plant tissues for analyzing the expression of small RNAs including amiRNAs (Qu, Ye et al. 2007; Ai, Zhang et al. 2011; Kung, Lin et al. 2012). The C3H-amiRNA and F5H-amiRNA constructs were agro-infiltrated in tobacco leaves. RNA isolated from the infiltrated zones was subjected to further molecular analysis to assess the potency of amiRNAs.

To estimate the degree of precursor efficacy towards amiRNA formation, infiltrated zone from different dpi (7-21) have been subjected for artificial amiRNA northern blot i.e. artificial microRNAs with an estimated size of 21 nt were separately detected by artificial microRNA northern blot analyses using the reverse complementary sequence of the corresponding amiRNAs as probes which were end labeled with $[\gamma^{32P}]$-ATP (6000 Ci/mmol; Perkin Elmer Life Sciences, USA). Results showed specific signals for C3H-amiR (Fig. 3.12 A) and F5H-amiR (Fig. 3.12 C), in contrast, no significant variations were found in amiRNA accumulation over the period of 7-21 dpi (Fig. 3.12 A & C). Usually the level of amiRNA is supposed to increase with time during the post infiltration period for transiently expressed amiRNA constructs (Qu, Ye et al. 2007; Tang, Wang et al. 2010; Ai, Zhang et al. 2011; Kung, Lin et al. 2012). However these are variable indicators as the amount of amiRNA depends on the amount of infiltrated *Agrobacterium* and their expression.

To corroborate the amiRNA functionality upon particular gene expression, another transient assay strategy, followed by subsequent reverse transcriptase PCR was carried out. In this case, tobacco leaf (*Nicotiana tabacum* L. cv Xanthi) was infiltrated in a quadrant fashion where four different *Agrobacterium* suspensions contained different amiR-constructs, full length gene construct, mixture of gene construct and amiR construct and MES respectively. Agro-infiltration of T-DNA vectors of each construct is expected to result in the expression of amiRNAs, initiating the RNA-guided transcript silencing, which eventually limits the activity of the target gene (Johansen and Carrington 2001; Voinnet, Rivas et al. 2003). RNA isolated from tobacco infiltrated zone after a fixed dpi (21) when subjected to RT-PCR gave a significant reduction in the level of C3H (Fig. 3.13 B) and F5H transcripts (Fig. 3.13 D). There were no bands of full length transcript for infiltrated zone of amiR construct and MES trial, when subjected to PCR with transcript based primers (Fig. 3.13 B& D). This allowed initial confirmation of effectual C3H-amiRNA and F5H-amiRNA constructs.
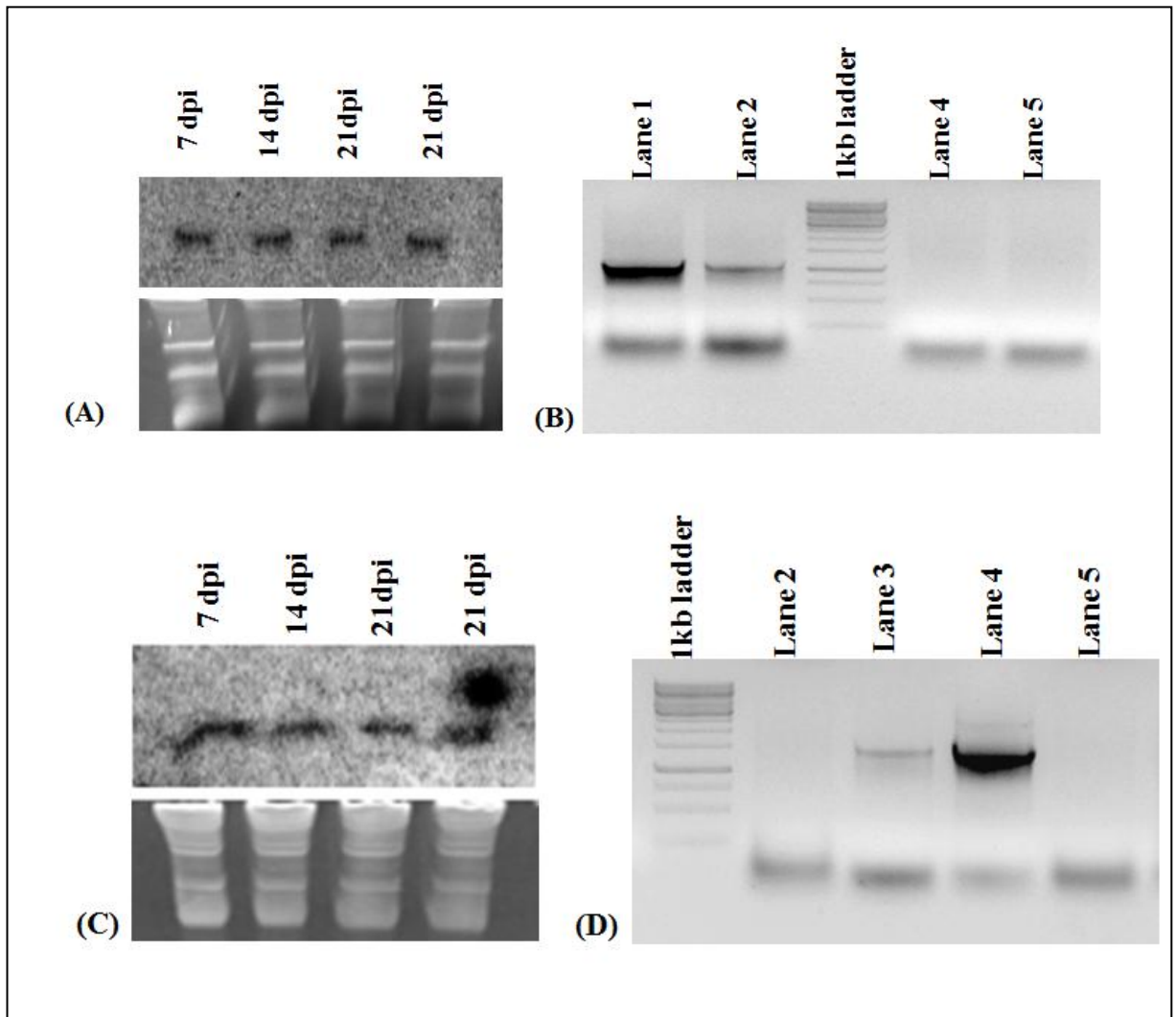
Fig.3.13: amiRNA accumulation in *Agrobacterium* infiltrated zone at 7 -21 dpi for (A) C3H-amiR and (C) F5H-amiR. Right panel **(B)** represents RT-PCR amplified cDNA of C3H gene from leaf tissue of tobacco infiltrated zone; Lane 1 shows band for C3H gene; Lane 2 shows reduced expression of C3H gene from tobacco leaf tissue infiltrated with a combination of amiR-C3H and full length C3H gene construct; Lane 3 - 1Kb marker; Lane 4 shows no band for C3H gene where only amiR-C3H construct was infiltrated; lane 4 shows no signal for MES (used as a mock). Right panel of **(D)** depicts RT-PCR amplified cDNA of F5H gene from leaf tissue of tobacco infiltrated zone; Lane 1-1Kb marker; Lane 2 shows no signal when only amiR-F5H construct was infiltrated; Lane 3 shows low expression of F5H gene for the zone infiltrated with a mixture of full length F5H gene and F5H-amiR construct; Lane 4 shows strong signal for full length F5H gene; Lane 5 shows no band when MES alone was used.

Generation of amiRNA transgenic lines through in planta transformation

A tissue culture independent *Agrobacterium tumefaciens* mediated gene transformation protocol (Sajib, Islam *et al.* 2008) was used to introduce the C3H-amiRNA and F5H-amiRNA based constructs into *C. olitorius* var 0-9897 as previously described in the hp-RNAi section. Screening of primary transformants was carried out by growing the seeds on antibiotic selection media containing 400 mM kanamycin. Seedlings tested positive were subjected to molecular analysis and plants used in these analyses were randomly selected to rule out screening biasness.

Molecular analysis of the amiRNA jute transformants

The pre-amiRNA gene integration and expression was checked by genomic PCR, Southern blotting, northern blotting and RT-PCR for both C3H-amiRNA and F5H-amiRNA lines.

T_1 progenies of both C3H-amiRNA and F5H-amiRNA lines were grown in the presence of kanamycin (400mM), using seeds from different independent transformants. DNA was isolated from 1 month old T_1 seedlings. Initial transgenesis of the amiRNA constructs in the transgenic T_1 progenies were confirmed by genomic PCR for the amplification of the antibiotic gene present in the construct. They showed ubiquitous signal for NPT (II) gene whereas WT plants were PCR negative which confirmed stable inheritance of the transgene in the T_1 progenies of C3H-amiRNA (Fig. 3.14 A) and F5H-amiRNA (Fig. 3.14 B). Integration of pre-amiRNA gene was validated by Southern blot analysis using PCR positive lines where *BamHI/HindIII* digested DNA was probed with amiRNA-precursor specific sequence. A single integrated band was observed for transgenic lines i.e. single copy integration events were obtained in amiRNA transformants (C3H and F5H) only whereas the parental line (used as negative control) showed no band (Fig. 3.14 C & D). Stable integration and inheritance of amiRNA precursor gene in the succeeding transgenic generation (T_2) as revealed by the Southern blot data confirmed successful amiRNA mediated transgenesis in jute.
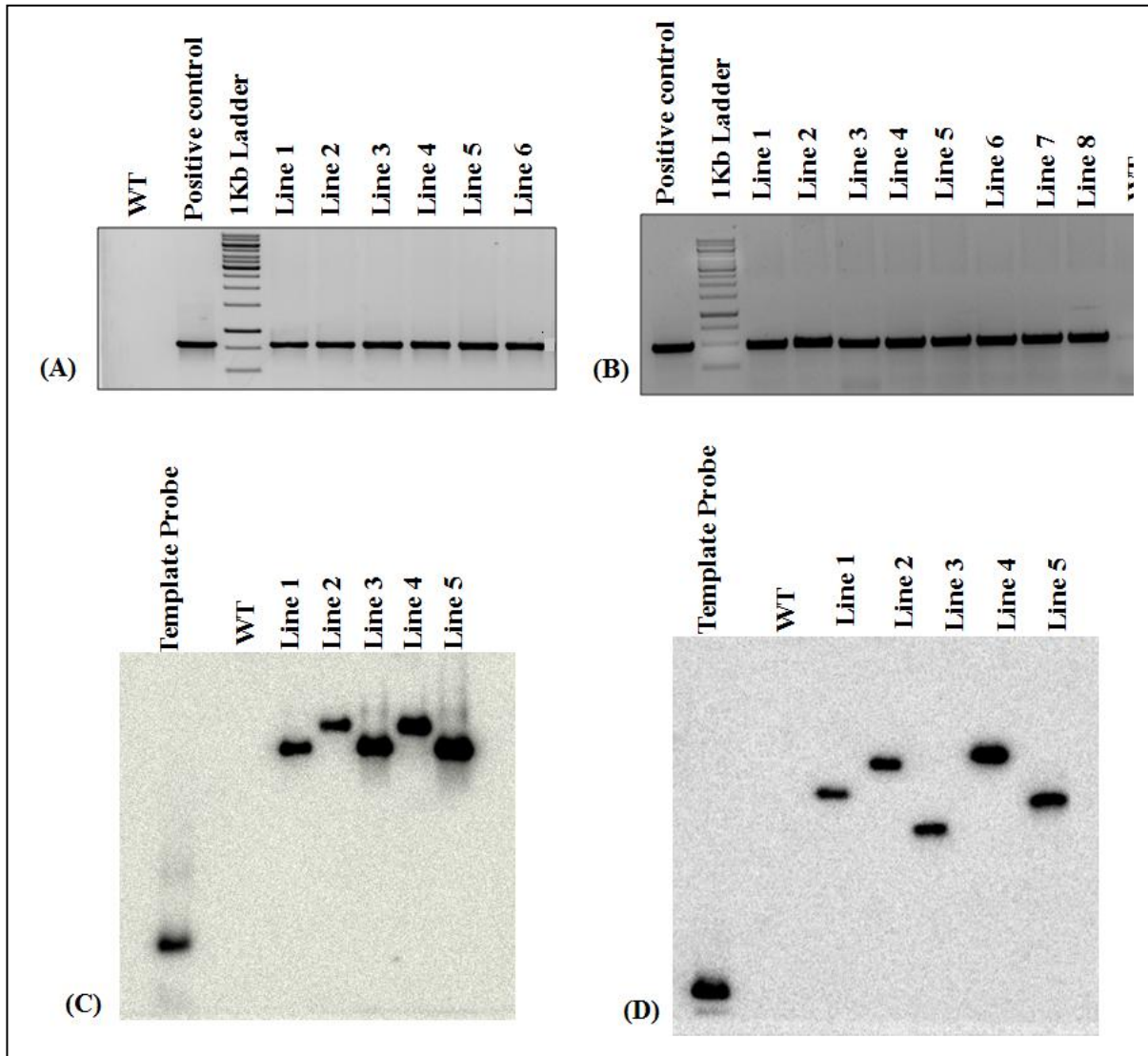
Fig.3.14: Analysis of primary hp-RNAi transformants of jute: Antibiotic screened (400mM Kanamycin) seedlings obtained from each primary transformants were checked for PCR and Southern analysis. (A) Agarose gel electrophoreses of PCR amplified product using reporter gene NPT (II) primer as a proof of transformation for C3H-amiR (Lanes 4-9); Lane 1 shows no band in WT control, Lane 2: positive control and Lane 3: size marker. **(B)** PCR amplification of transgene NPT (II) from F5H-amiR jute transgenic lines; (Lanes 3-10); Lane 1: positive control, Lane 2: size marker and Lane 11 shows no signal for WT control. **Southern blot analysis**: **(C)** Lane 1: template probe used as a control; Lane: 2 WT plants; Lanes 3–7: independently transformed jute C3H-amiR lines. **(D)** Southern blot analysis for F5H-amiR T$_1$ lines (Right) shows stable integration of F5H-amiR precursor in jute. Lane 1: template probe used as a control; Lane: 2 WT plants; Lanes: 3–7: independently transformed jute F5H-amiR lines.

Functionality of the amiRNA transgenic lines

To assess the functional potency of the amiR jute transformants, RNA was isolated from antibiotic screened seedlings from both C3H-amiRNA and F5H-amiRNA lines and subsequently, subjected to northern analysis (for both the transcripts and artificial microRNA) and reverse transcription PCR (RT-PCR).

Northern for artificial miRNA revealed the accumulation of C3H-amiR and F5H-amiR in transgenic lines in contrast to WT where no signal was found (Fig. 3.15 A & B). Transgenic lines for both T_1 and T_2 generations of C3H and F5H genes showed a decrease in their expression when monitored by northern blots of their transcripts (Fig. 3.15 B & C) and semi-quantitative RT-PCR (Fig. 3.15 E & F). These findings support correct processing and functionality of amiR-C3H and amiR-F5H in the transgenic lines. Our results validate the effectiveness of RNAi in reducing lignin through gene silencing in jute (Bhagwat, Chi *et al.* 2013). A positive correlation is known to exist between amiRNA expression and the strength of target gene silencing but the degree of silencing is found to vary between the different transgenic plants (Schwab, Ossowski et al. 2006; Qu, Ye et al. 2007; Ai, Zhang et al. 2011; Kung, Lin et al. 2012). However in our case no variation in the degree of silencing was observed.
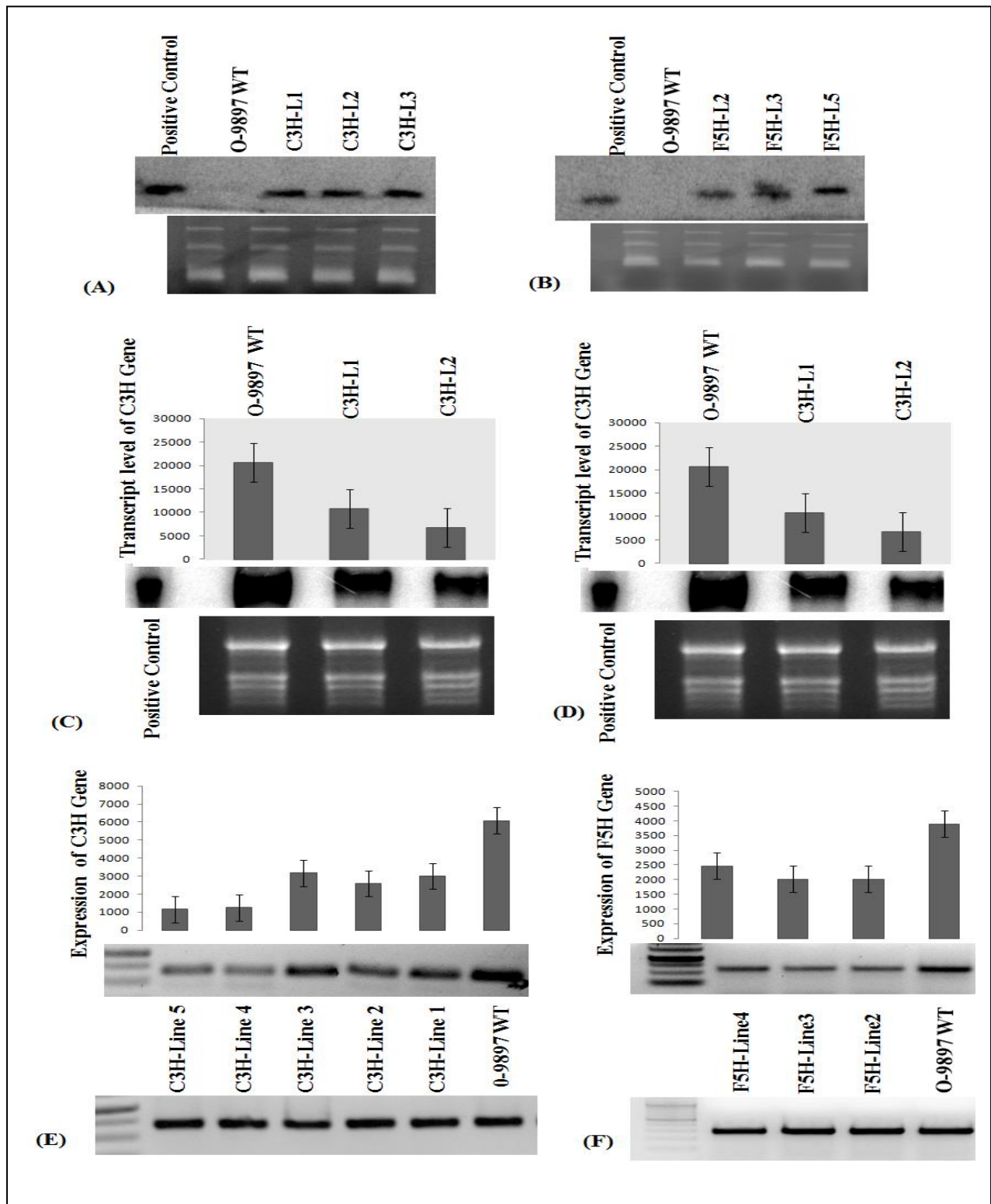
Fig. 3.15: amiRNA northern blot: Represents abundance of amiRNA in transgenic (T$_1$) lines for C3H-amiR (A) and F5H-amiR (B). 20 µg of each RNA sample was resolved on a 12% denaturing urea-PAGE gel, blotted and hybridized with end labeled antisense amiRNAs for corresponding gene using 6000 Ci/mmol [γ^{32P}] ATP (PerkinElmer Life Sciences, USA). **Northern blot:** 30µg of total RNA prepared from

the seedlings was used for hybridizations with [α^{32P}] labeled cDNA probe of C3H gene (C) and F5H gene (D) separately that spanned the entire coding sequence of the corresponding genes. The figures above show the intensities of the band on the blot normalized with respect to 28S rRNA. The band intensities were measured by Alpha Imager Imaging System.

Reverse transcriptase polymerase chain reaction (RT-PCR): Total RNA was isolated from seedlings of non-transformed and transformed jute amiRNA lines. Semi quantitative RT-PCR was performed using the primers amplifying the C3H (E) and F5H (F) gene along with β-ACTIN genes. cDNA was prepared using 50 U of Super-ScriptTM II reverse transcriptase (Invitrogen). The figures above show the intensities of the band on the blot normalized with respect to β-ACTIN gene. The band intensities were measured by Alpha Imager Imaging System.

An abundance of reports illustrate the effectiveness of amiRNA-strategy for gene silencing (Alvarez, Pekker et al. 2006; Khraiwesh, Ossowski et al. 2008; Molnar, Bassett et al. 2009; Shi, Yang et al. 2010; Toppino, Kooiker et al. 2011; Warthmann, Ossowski et al. 2013; Cantó-Pastor, Mollá-Morales et al. 2015), but non-specificity resulting in morphological deformities cannot be ruled out.

Morphological study

The RNAi-transgenic generations (hp-RNAi and amiRNAi) were screened morphologically throughout the growing period. For a relative assessment of plant health, different growth parameters such as plant height, plant width, pod number and average pod length of all plants grown under field condition were measured and compared (Table 3.1). The data found from the table gives a clear indication to the overall similar performance of WT and transgenic plants which have been morphologically analyzed (Fig. 3.16). As transgenic plants showed no significant dissimilarities compared to the WT plants, one parameter related to photosynthesis i.e. chlorophyll estimation were further analyzed to authenticate the previous claim. Photosynthetic machinery is composed of different parts and its functionality is dependent on various cellular components. One of them is chlorophyll, the major pigment of chloroplast. Transgenic plants found to produce same amount of chlorophyll compared to the WT (Fig. 3.17) supported the ability of the transgenic plants to maintain identical photosynthetic capacity as the WT plants.

Table: 3.1: Measurement of various growth and yield parameters from WT and Transgenic lines

Parameters	WT	COMT-hpRNAi			C4H-hpRNAi			C3H-amiRNA			F5H-amiRNA		
		T₁	T₂	T₃	T₁	T₂	T₃	T₁	T₂	T₃	T₁	T₂	T₃
Plant Height	335.24 ±0.25*	334.84 ±0.32*	334.7± 0.49*	335.52 ±0.32*	334.8 ±0.31*	335.5± 0.32*	335.4± 0.4*	335.3± 0.32*	335.1± 0.31*	334.9± 0.37*	335.3± 0.3*	334.9± 0.4*	334.9 ±0.3*
Plant width	1.118 ±0.02*	1.18± 0.003*	1.18±0. 0003*	1.19±0. 004*	1.22±0. 008*	1.19±0. 008*	1.20±0 .005*	1.19±0. 005*	1.19±0. 005*	1.21±0. 004*	1.19±0 .004*	1.21±0 .004*	1.18±0 .003*
Pod Number	61.8± 1.06*	58± 0.44*	60± 0.44*	59± 0.31*	62± 0.31*	60± 0.31*	58± 0.31*	58.6± 0.31*	56.6± 0.4*	60.6± 0.6*	61± 0.44*	63± 0.44*	61.8± 0.58*
Pod Length	5.00± 0.007*	4.9± 0.005*	5.00± 0.003*	4.9± 0.003*	4.9± 0.005*	4.9± 0.005*	5.00± 0.004*	4.9± 0.004*	4.9± 0.008*	5.03± 0.006*	4.9± 0.006*	5.00± 0.006*	4.97± 0.006*

*Indicates statistical analysis determined by (P\0.05, ANOVA and Duncan test)
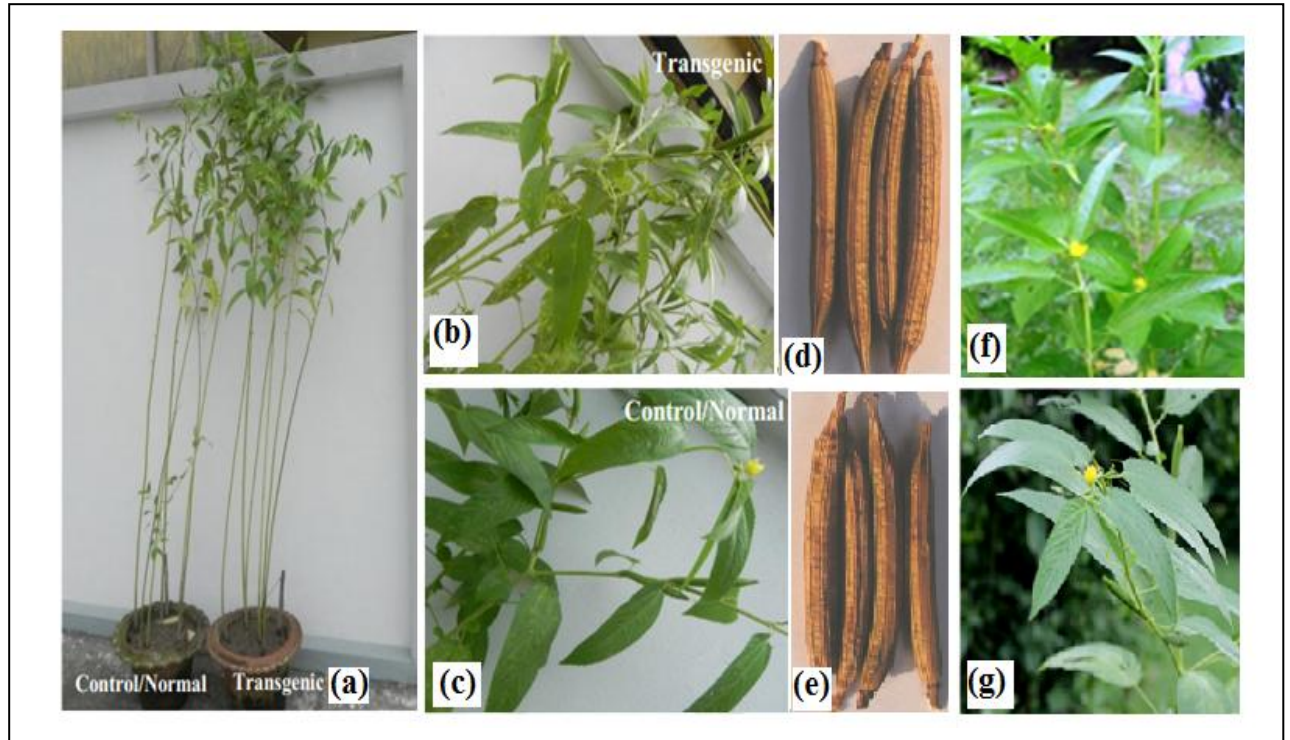
Fig.3.16: Different developmental stages of transgenic plants along with control plant showing no significant morphological differences. Vegetative and reproductive growth is depicted in the photographs (a), (b) and (c) both for control and transgenic plants in pot conditions. In contrast, photographs (d), (e), (f) and (g) were taken in field condition.
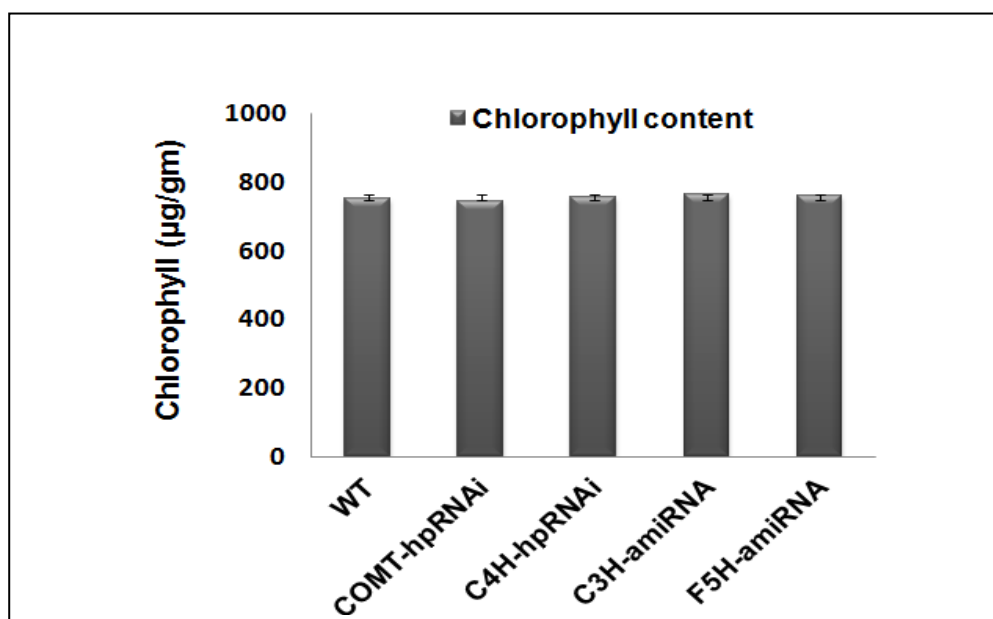
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Fig.3.17: Graphical representation of average chlorophyll content showing no significant variations between WT and transgenic plants suggesting that transgenic plants are able to maintain same photosynthetic apparatus as the WT.

By comparing all the parameters studied here (plant height and width, pod number, pod length, chlorophyll) between the WT and transgenic (average of five lines) plants, no significant morphological differences s (statistical differences were measured by ANOVA and Duncan test, P\0.05). Vegetative and reproductive growth characteristics of the transformed jute lines resembled the control plants, indicating the reduction in the lignin content did not lead to any major growth deformity.

Klason Lignin Determination

Lignin composition is highly variable in plants and it can vary from 15% to 40% (Sarkanen and Ludwig 1971). There are two main types of subunits in lignin viz. guaiacyl (G) and syringyl (S) units. The G units are derived from coniferyl alcohol monomers results in lignin, which is atypical of softwoods. The S units are derived from polymerization of the sinapyl alcohol monomers (Sarkanen and Ludwig 1971). The ratio of S:G units varies greatly between hard and softwoods (Day, Ruel et al. 2005; Esteves, Marques et al. 2007; Kline, Hayes et al. 2010). Chemically, jute fiber

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has about 59-61% of cellulose while lignin content hovers from 11 to 13.5% (Islam and Sarkanen 1993; Templeton and Ehrman 1995; Giwa and Akwu 2007; Del Rio, Rencoret et al. 2009). However, till date, not much is known about the lignin content of either the jute stem or the whole plant. Chemical lignin estimation method optimized for jute (Tanmoy, Alam et al. 2015) was used for estimating the stem and fiber lignin content of both transgenic and non-transgenic lines. The control plants on an average were found to have ~29.50% (Table 3.2) of lignin for the whole stem whereas the lignin amount of retted, dried fiber was ~13.50 % (Table 3.3). Reduction of acid insoluble lignin was observed for both the whole stem and the fiber of engineered plants in comparison to the wild type. Tweaking with the expression of COMT, C4H, F5H and C3H genes in jute resulted in about ~22-25% (Table 3.2) reduction in the lignin content of the whole stem and ~11-13% (Table 3.3) for the fiber, as estimated in some randomly selected transgenic (T_1-T_3) generation plants of hp-RNAi and amiR- transgenic jute lines. Reports claim that more than 40% of lignin reduction can be detrimental to the physiology of a plant and at this point they become susceptible to diseases (Franke, McMichael et al. 2000; Reddy, Chen et al. 2005).

However, the percent reduction did not exceed more than 25 % (Table 3.2) and 15 % (Table 3.3) for whole stem and fiber respectively. Klason lignin data for some randomly selected transgenic (T_1-T_3) generations of both hp-RNA and amiRNA transgenic jute lines were statistically analyzed by ANOVA and Duncan test and categorized into groups (a, b, c and d) on the basis of reduction intensity. Graphical illustrations for whole stem (Fig.3.18) and fiber (Fig.3.19) show that almost all transgenic lines for the four genes have a significant reduction in the amount of lignin in comparison to the corresponding WT lines. In addition, reduction trend was also stable into the succeeding generations (T_1-T_3). Therefore, alteration in the expression of COMT, C4H, F5H and C3H in jute did not reduce lignin to the extent at which the plants are put in danger. The reduction observed in our transgenic jute lines can therefore be expected to be free from any deleterious effects.

Table: 3.2 Klason lignin estimation value for whole jute stem

Parameters	WT	COMT-hpRNAi			C4H-hpRNAi			C3H-amiRNA			F5H-amiRNA		
		T_1	T_2	T_3	T_1	T_2	T_3	T_1	T_2	T_3	T_1	T_2	T_3
% Klason lignin (average of replicates)	29.484 ±0.44*	25.61± 0.12*	24.29± 0.23*	23.86± 0.15*	22.89± 0.15*	22.83± 0.18*	22.78± 0.08*	22.09± 0.24*	22.07± 0.21*	22.05± 0.15*	21.95± 0.28*	21.86± 0.22*	22.03± 0.25*
% Klason lignin (average of samples)	29.50±0 .044*	24.58±0.21*			22.83±0.15*			22.07±0.1*			21.94±0.24*		
% Lignin Reduction		16.33*			22.06*			25.08*			25.46*		

*Indicates statistical differences were determined by (P\0.05, ANOVA and Duncan test)

Table: 3.3 Klason lignin estimation value for fiber

Parameters	WT	COMT-hpRNAi			C4H-hpRNAi			C3H-amiRNA			F5H-amiRNA		
		T1	T2	T3	T1	T2	T3	T1	T2	T3	T1	T2	T3
% Klason lignin (average of replicates)	13.45 ±0.016*	11.55± 0.12*	11.74± 0.15*	11.58± 0.20*	11.55± 0.18*	11.66± 0.20*	12.00± 0.06*	11.55± 0.11*	11.89± 0.22*	11.97± 0.23*	11.70± 0.16*	11.62± 0.09*	11.41± 0.16*
% Klason lignin (average of samples)	13.45 ±0.016*	11.63±0.12*			11.73±0.15*			11.80±0.20*			11.57±0.16*		
% Lignin Reduction		13.53*			14.12*			12.16*			15.25*		

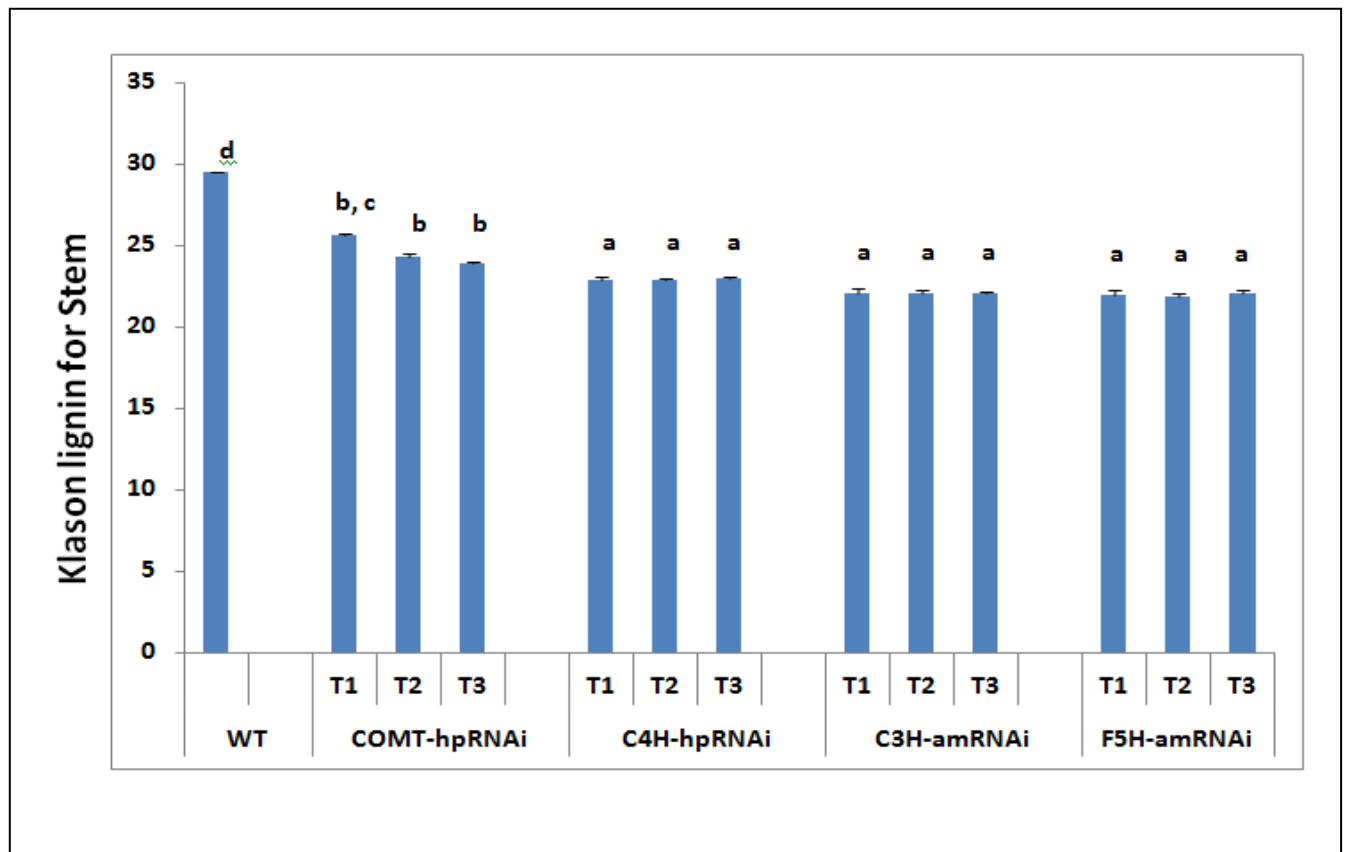*Indicates statistical differences were determined by (P\0.05, ANOVA and Duncan test)

Fig.3.18: Graphical representation of Klason lignin of whole stem from WT and RNAi transgenic lines. Each bar represents the mean ±SE (n=5). Different letters (a, b, c and d) indicate significant differences (P\0.05, ANOVA and Duncan test).
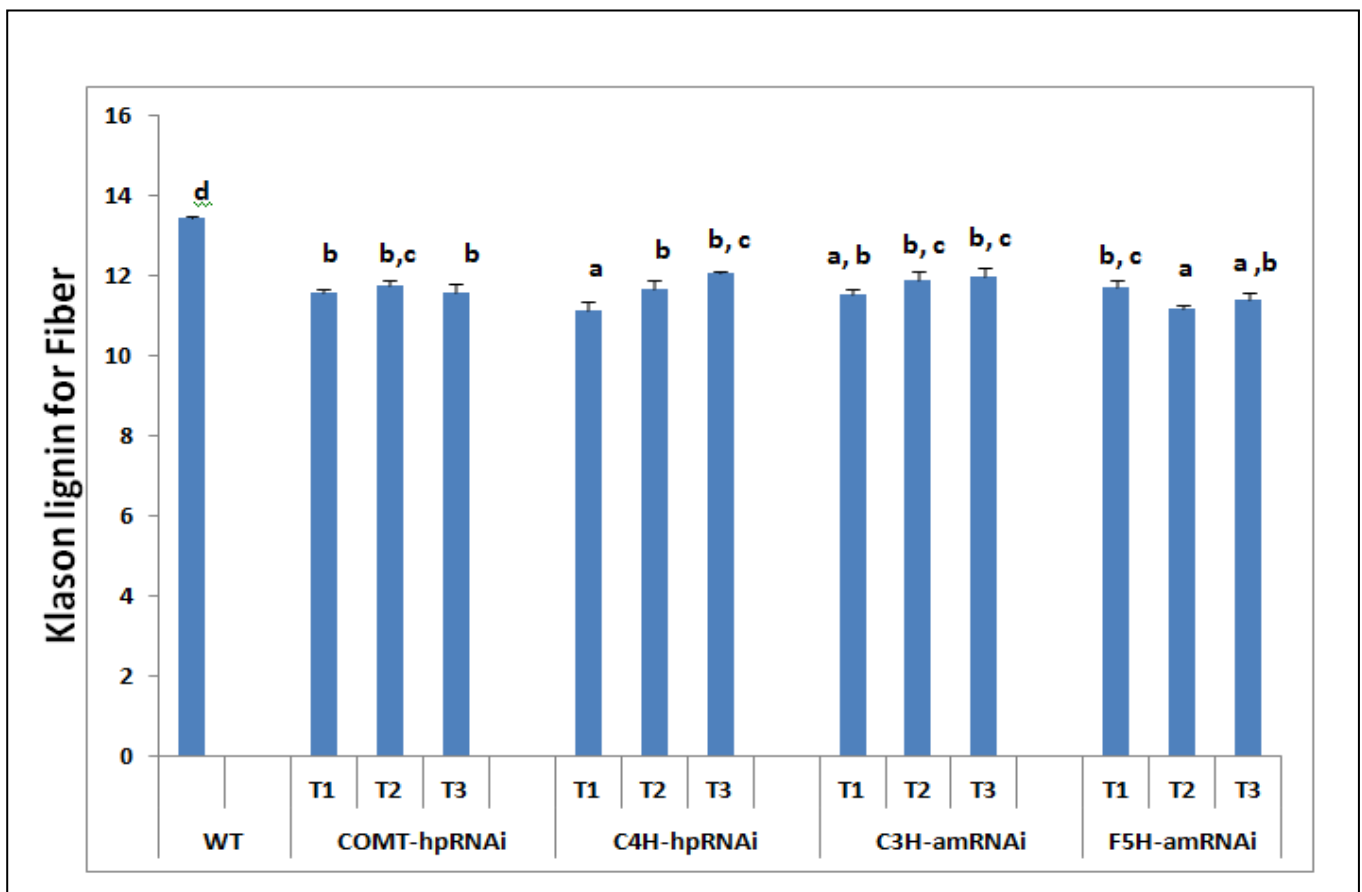
Fig.3.19: Graphical representation of Klason lignin of fiber from WT and RNAi transgenic lines. Each bar represents the mean ±SE (n=5). Different letters (a, b, c and d) indicate significant differences (P\0.05, ANOVA and Duncan test).

Discussion

Ever since the harmful effect of synthetic fiber on the environment has been understood, search for suitable natural fibers is gaining more and more attention. Jute, one of the best natural bast fibers is acquiring importance because of an increasing demand of bio-polymers. It's applications in the manufacture of paper-pulp, bio-plastics, automobile, electronics, construction and packaging goods (Li *et al.* 2007) is highly positive. An elevated amount of lignin distinguishes jute fiber from other non-wood bast fibers (flax, hemp, ramie and kenaf), which makes it the strongest of them all (Del Rio, Rencoret et al. 2009; Stevens and Müssig 2010; Sarkar, Kundu et al. 2011). Being the second most abundant biopolymer of vascular plants, the deposition of lignin confers rigidity to the cell wall for structural support, provides impermeability for water and nutrients transport and contributes in the mechanisms of plant defense against both biotic and abiotic stresses (Boerjan, Ralph et al. 2003; Ralph, Lundquist et al. 2004; Ralph, Brunow et al. 2007). Intrinsic properties of lignin polymers have been indispensable for growth and development of plants, but are also crucial in determining the value of plants as raw materials (Boerjan, Ralph et al. 2003; Leple, Dauwe et al. 2007). Since lignin is the most recalcitrant component of a plant cell wall (Angelidaki and Ahring 2000), its higher proportion imparts a detrimental effect in textile, pulp and paper industry (Peter, White *et al.* 2007) and is considered as a core limiting factor in bioethanol production and fodder digestibility (Chen and Dixon 2007). Therefore the downstream processing of jute would benefit from a reduced amount of this polymer.

While a large amount of lignin alteration and an impact of the same has been studied in several plant species through *in vitro* experiments and genomic strategies (Ralph, Akiyama *et al.* 2012; Chapple et. al., 2007; Ralph, J., et al, 2006; Boerjan et. al., 2003), lignin manipulation in jute is still to be initiated. The most effective of such approaches is to down-regulate the enzymes involved in lignin biosynthesis, in other words silencing the expression of such genes (Ralph, Akiyama et al. 2006; Chapple, Ladisch et al. 2007; Chen and Dixon 2007).

The monolignol biosynthetic pathway has been evaluated through a combination of forward and reverse genetics and also characterized by *in vitro* experiments at every metabolic phase for different plants (Anterola, Jeon et al. 2002; Franke, Hemm et al.

2002; Franke, Humphreys et al. 2002; Hemm, Rider et al. 2004; Nair, Bastress et al. 2004).

Here, we report, the down-regulation of four lignin biosynthetic genes, caffeic acid O-methyltransferase (COMT), cinnamate 4-hydroxylase (C4H), ferulic acid 5-hydroxylase (F5H) and coumarate 3-hydroxylase (C3H) of jute by the technique of RNAi (Wesley, Helliwell *et al.* 2001). These genes are found not to overlap with the defense mechanism of a plant (Boerjan, Ralph *et al.* 2003; Ralph, Akiyama *et al.* 2006; Chapple, Ladisch *et al.* 2007; Li, Weng *et al.* 2008).

The reason behind using RNAi technique is simple. RNA interference (RNAi) is based on homology sequence dependent degradation of cognate RNA that turns out the formation of double-stranded RNA (dsRNA) followed by transcript depletion of homologous target gene (Baulcombe 2004; Brodersen, Sakvarelidze-Achard et al. 2008). With the advent of functional genomics, RNAi vector mediated gene silencing has emerged as an efficient approach for genetic manipulation in plants because of its ease of application and the possibilities for genome-wide reverse genetics overcoming the drawbacks of insertional mutagenesis or knockout method (Travella, Klimm *et al.* 2006). hpRNAi (gene constructs encoding intron spliced RNA with a self complementary hairpin structure) have been found to show post-transcriptional gene silencing with almost 100% efficiency for endogenous genes and transgenes (Smith, Singh et al. 2000; Travella, Klimm et al. 2006; Travella and Keller 2009). Like hpRNA, the approach of artificial microRNA appends a new dimension in the gene silencing epoch. Owing to certain attributes like exceptionality, efficacy and precision, gene silencing through amiRNAs is considered a second generation RNAi technology, and has been conveniently applied in many studies (Warthmann, Chen *et al.* 2008).

Stable transgenesis is a critical issue while genetic manipulation is being carried out. Our transgenic plants showed in each successive lines (T_0 to T_3), stable incorporation of hp-RNA/amiRNA constructs. This was examined by genomic PCR and Southern blot analyses which confirmed successful RNAi transgenesis in jute. In case of hp-RNAi strategy, it was expected that, the hpRNAs for COMT and C4H would be effective in activating the RNAi pathway leading to depleted gene expression, as has been reported for other plants (Parizotto et. al.2004). Expression of both COMT and C4H gene was found to decrease in the transgenic generations (T_1 to T_3) when

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evaluated by RT-PCR as well as by northern analyses. Concomitantly high expression was found for hp-RNA transcripts. This confirmed successful and functional hp-RNA transgenesis in jute. It has been reported that intron–hairpin structures enhance stability and efficiency of duplex RNA formation inducing PTGS gene silencing (Smith *et al.*, 2000). Considering the above, it can be assumed that the RNAi machinery in transgenic jute plants has have been triggered by the hp-RNA constructs where the siRNAs for the particular genes down-regulated transcription of the same. On the other hand, integration of amiRNAs for C3H and F5H gene was validated by Southern blot analysis using PCR positive lines. Southern blot data revealed stable integration and inheritance of amiRNA precursor gene in the succeeding transgenic generation (T_2) which confirmed successful amiRNA arbitrated transgenesis in jute. Our artificial microRNA northern blot analysis for jute revealed the abundance of amiRNAs for both C3H and F5H gene and a decrease in expression was observed in transgenic lines by northern of transcript blots and semi-quantitative RT-PCR. These findings support correct processing and functionality of amiR-C3H and amiR-F5H in the transgenic lines. Our findings corroborate the prediction that RNAi will be effective in reducing lignin through gene silencing in jute (Bhagwat, Chi *et al.* 2013) because of its success in other plants (Wang, Luo et al. 2008; Liu, Zhang et al. 2010). This strategy has been found to be useful in several documentations (Sablok, Pérez-Quintero et al. 2011; Belide, Petrie et al. 2012; Li, Chung et al. 2013). An abundance of reports illustrates the effectiveness of amiRNA-strategy for gene silencing (Alvarez, Pekker et al. 2006; Khraiwesh, Ossowski et al. 2008; Molnar, Bassett et al. 2009; Shi, Yang et al. 2010; Toppino, Kooiker et al. 2011; Warthmann, Ossowski et al. 2013; Cantó-Pastor, Mollá-Morales et al. 2015), but non-specificity resulting in morphological deformities cannot be ruled out. The transformed jute lines (hp-RNA and amiRNA) were found to resemble morphologically the control plants indicating that over-expression of the amiRNA/hp-RNA had not led to any major growth deformity.

A chemical method to estimate the acid insoluble lignin i.e. klason lignin, optimized for jute (Tanmoy, Alam *et al.* 2015) was used for estimating the klason lignin content of both transgenic and non-transgenic lines. Silencing of COMT and C4H genes using hp-RNAi technology; and   C3H and F5H genes by amiRNA methodology in *C. olitorius* species, resulted in variable levels of lignin reduction. Down-regulation of

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the four lignin biosynthetic genes exhibited normal growth and reduced lignifications. Data showed that the percent reduction did not exceed 25% (Table 3.1) and 15% (Table 3.2) for whole stem and fiber respectively for some randomly selected generations (T_1-T_3) of hpRNA transgenic jute lines. Maximum impact was ~25% reduction of lignin in COMT-hpRNA transformed generations (up to T_3) in comparison to control plants. C4H-hpRNA transformed generations (up to T_3) was found to have ~22% less lignin. Down-regulation of COMT to low activity levels have reduced lignin content in alfalfa, maize, and poplar by 30%, 30% and 17% respectively (Chabbert, Tollier et al. 1994; Jouanin, Goujon et al. 2000; Guo D 2001). COMT-RNAi suppression in sugarcane has been successfully attempted which resulted in 6-12% reduction in klason lignin (Jung, Vermerris *et al.* 2013). Efforts with C4H gene have shown more satisfactory outcomes in lignin reduction for arabidopsis, tobacco, poplar and alfalfa (Sewalt, Ni et al. 1997; Franke, McMichael et al. 2000; Shadle, Chen et al. 2007; Li, Weng et al. 2008); (Franke, McMichael *et al.* 2000).

Reduction of acid insoluble lignin was also observed for amiRNA transgenic lines for both the whole stem and the fiber of engineered jute plants in comparison to the wild type. Tweaking with the expression of F5H and C3H in jute resulted in about 25% (Table 3.1) reduction in the lignin content of the whole stem and 13% (Table 3.2) for the fiber, as estimated in some randomly selected T_1-T_3 generation plants of amiR-transgenic jute lines. Klason analysis of the RNAi-C3H down-regulated poplar lines revealed significant decrease in lignin content (20-40%), consistent with the earlier work of Franke *et al.* in arabidopsis (Franke, Humphreys *et al.* 2002) and alfalfa (Reddy, Chen *et al.* 2005). A 25–35% reduction in Klason lignin content was observed in F5H-overexpressing arabidopsis (Humphreys and Chapple 2002), poplar (Stewart, Akiyama *et al.* 2009) and tobacco (Franke, Humphreys *et al.* 2002).

Lignin plays a crucial role in the mechanical support and plant defense, thus lowering lignin always imposes a risk on plant physiology. It has been reported that more than 40% of lignin reduction is detrimental for plant physiology and the plants become susceptible to diseases (Franke, McMichael *et al.* 2000; Reddy, Chen *et al.* 2005). However, alteration of lignin content and its monolignol composition (S/G/H ratio) in plant biomass via genetic engineering has been shown to significantly impact delignification efficiency for different industrial uses (Pu, Chen *et al.* 2009).

Researchers have found that suppression of COMT did not affect the overall physiology of tobacco, alfalfa, maize and switchgrass under greenhouse conditions, and poplar under field conditions (Chen and Dixon, 2007; Fu *et al.*, 2011; Pilate *et al.*, 2002; Pincon *et al.*, 2001; Piquemal *et al.*, 2002); but down-regulation of C4H in many plant species resulted in a dwarf phenotype (Stevens and Müssig 2010). Down-regulation of the gene encoding 4-coumarate 3-hydroxylase (C3H) in alfalfa massively increased the proportion of p-hydroxyphenyl (P) units relative to the normally dominant guaiacyl (G) and syringyl (S) units (Ralph, Akiyama *et al.* 2006). However, the impact of the C3H perturbation on lignin monomer composition varies substantially among different species i.e. arabidopsis, alfalfa and poplar (Coleman, Park *et al.* 2008). Another hydroxylase enzyme, ferulate 5-hydroxylase (F5H) whose actions are irreversible appears to be the truly S-lignin–specific biosynthetic enzyme (Li, Cheng et al. 2001; Ralph, Akiyama et al. 2006). In arabidopsis, F5H transcripts were found to be most strongly down regulated with a decrease in S lignin consistent with reduced F5H enzymatic activity (Humphreys, Hemm *et al.* 1999). Lignin with exceedingly high syringyl monomer levels are produced by an over-expression of the ferulate 5-hydroxylase (F5H) gene (Stewart, Akiyama *et al.* 2009) as evident from studies on other plants like alfalfa and arabidopsis (Reddy, Chen *et al.* 2005). Such down regulation of C3H and F5H genes is expected to be productive for jute with altered S/G ratio, and is also anticipated to possess improved characteristics for commercial usage while revealing a deep insight of this gene regulatory network (Meyer, Shirley et al. 1998; Franke, McMichael et al. 2000; Hisano, Nandakumar et al. 2009).

Moreover, morphological studies of transgenic plants in this study when compared to wild type showed no significant change in the pattern of plant growth, anatomy and physiology indicating that the reduction in the lignin did not lead to any major growth deformities. Also when subjected to fungal (*Macrophoemina phaseolina*) infestation the transgenic jute lines did not show any increase in susceptibility to infection. Thus, manipulating the expression of COMT, C4H, C3H and F5H genes in jute does not appear to reduce lignin to the extent at which the plants are put at risk.

Jute is considered as one of the potential lingo-cellulosic sources of bio-fuel production (Ioelovich 2015). It has gained much importance because of its high quality to produce improved biomass with properties specifically tailored to various

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industrial applications. Economic and environmental factors favor the adoption of lingo-cellulosic bio-energy crops for production of liquid transportation fuels (Dixon, Reddy *et al.* 2014). But lignin is an impediment in this conversion process. Pretreatment for removing lignin is the most expensive step in the production of lingo-cellulosic biofuels (Poovaiah, Nageswar-Rao *et al.* 2014). Lignin occludes cellulose by physically shielding it from microbial or enzymatic decomposition (Berlin *et al.*, 2006; Vanholme *et al.*, 2010a). On the other hand the process of lignin degradation can affect microorganisms used in bio-processing (Agbor, Cicek *et al.* 2011). Moreover, lingo-cellulosic biomass is recalcitrant to saccharification (sugar release from cell walls), and this is, at least in part, due to the presence of lignin (Lewis and Yamamoto 1990; Vanholme, Demedts et al. 2010). Improved saccharification efficiencies has been successfully implied in a number of lignin mutant crops including transgenic arabidopsis (Stewart, Akiyama et al. 2009; Ciesielski 2014), switchgrass (Fu *et al.*, 2011; Saathoff *et al.*, 2011; Xu*et al.*, 2011), sugarcane (Jung *et al.*, 2012) depicting the utility of lignin engineering for enhanced biofuel production for specialized commercial applications (Chen and Dixon,2007; Dien *et al.*, 2009; Saballos *et al.*, 2008). Therefore, one of the most promising solutions to the lignin problem is to decrease its biosynthesis in the plant itself, as this approach might be more efficient and cost effective than removing it at the bio-refinery (Poovaiah, Nageswara- Rao *et al.* 2014).

The genetic manipulation of lignin biosynthesis pathway enzymes particularly the four genes of this study has been specifically reported to reduce the need for pretreatment processes for better industrial purposes. Independent down-regulation of genes encoding C4H (cinnamate 4-hydroxylase), C3H (4-hydroxycinnamate 3-hydroxylase), F5H (ferulate 5-hydroxylase) and COMT (caffeate O-methyltransferase) have been shown to reduce the recalcitrance of alfalfa and thereby improve the release of fermentable sugars during enzymatic hydrolysis eliminating the prior chemical pretreatment in the production of fermentable sugars (Williams, Templer et al. 2006; Chapple, Ladisch et al. 2007; Chen and Dixon 2007; Sticklen 2007; Pu, Chen et al. 2009).

In addition, down-regulation of COMT gene has been made in many plants, resulting in a reduction in S lignin and low S/G ratio, in order to study the specific effects. For example, in switchgrass, which is used as bio-energy feedstock, lowering the

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expression of COMT gene by RNAi reduced lignin content notably with an increase in ethanol yield by up to 38% (Fu, Mielenz *et al.* 2011). In soyabean, it was shown that reducing COMT using RNAi construct helps to improve optimal utilization of feed with no effect on oil production (Oraby and Ramadan 2014). In sugarcane, down-regulation of COMT transcript which resulted in reduction of total lignin by 6%, improved saccharification efficiency by 19%−23% with no significant difference in biomass yield and other features (Jung, Vermerris *et al.* 2013). Down-regulation of COMT to low activity levels reduced lignin content in alfalfa, maize, and poplar by 30%, 30% and 17% respectively (Chabbert, Tollier et al. 1994; Jouanin, Goujon et al. 2000; Guo D 2001). Such activity has also been shown in maize bm3 mutants with altered lignin composition and digestibility (Zuber MS 1977; Vignols, Rigau et al. 1995). Similar results were also found for sorghum brown midrib (bmr) mutants generated via chemical mutagenesis (Porter, Axtell et al. 1978; Bout and Vermerris 2003). Down-regulation of caffeic acid 3-O-methyltransferase (COMT), were shown to have normal growth behavior and exhibited reduced recalcitrance for saccharification and fermentation to ethanol of switch grass plants (Fu, Mielenz *et al.* 2011).

Lignin genetic modification is of interest for other applications, such as increased pulping efficiency and forage digestibility (Lee and Steinbnchel 2005; Reddy, Chen et al. 2005; Ralph, Akiyama et al. 2006; Pu, Zhang et al. 2008). Reduction of 4-coumarate 3-hydroxylase (C3H) in alfalfa (*Medicago sativa*) resulted in a dramatic shift in the lignin profile and consequent altered lignin structure (Reddy, Chen *et al.* 2005), causing improved digestibility of C3H-deficient alfalfa lines (Ralph, Akiyama *et al.* 2006). Suppression of COMT in tobacco (*Nicotiana tabacum*) resulted in increased biomass production without decreasing the overall lignin content (Blaschke, Legrand *et al.* 2004). Huntley *et al.* demonstrated that increased syringyl lignin in transgenic poplars, by perturbing ferulate 5-hydroxylase, resulted in an increase in chemical pulping by 60% (Huntley, Ellis *et al.* 2003). C3H down-regulation has been successfully implied to improve the quality of feedstocks and kraft pulping properties in poplar (Pilate, Guiney et al. 2002; Coleman, Park et al. 2008). In arabidopsis, Schoch *et al.* (Schoch, Goepfert *et al.* 2001) demonstrated that the expression of C3H gene correlates with the onset of lignification, and at the same time plants (arabidopsis and alfalfa) deficient in C3H activity have been shown to have significant decrease in

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lignin content with an concomitant increase in improved digestibility and pulping performance (Franke, Humphreys et al. 2002; Abdulrazzak, Pollet et al. 2006; Ralph, Akiyama et al. 2006).

Reduction of C4H gene expression has also been of great interest in the context of lignin manipulation in plants. C4H down-regulation lowers the S/G ratio. C4H down-regulation in alfalfa have led to reduced lignin content in stem tissue, with less effect on soluble phenolic compounds (Reddy, Chen *et al.* 2005). The reduction of C4H activity through RNAi resulted in reduced levels of Klason lignin, accompanied by a decreased syringyl/guaiacyl monomer ratio in tobacco (Sewalt, Ni *et al.* 1997). At the same time it has been reported that, down-regulation of C4H gene by antisense-RNA constructs induced substantial reductions in the lignin content of transgenic tobacco without causing any visible abnormalities in the growth and development (Blount, Korth *et al.* 2000).

By using RNAi (hp-RNA and amiRNA) strategy, this study can be regarded as the first successful attempt on jute to reduce its lignin content without any negative effects on the morphological features of the plant. With the results obtained, we can expect to develop a jute variety with low lignin content in near future, which will certainly boost its commercial usability and have an impact on the economic acceleration of Bangladesh.

CHAPTER 4

SUMMARY AND CONCLUSION

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With the advancement of science, the perceptive of the lignin biosynthetic pathway alternation is relentlessly and rapidly pursued. Lignin engineering can improve the fiber quality (Vanholme, Morreel et al. 2012) of jute and improved fiber is expected to have an impact on the enhancement of its profitable use. The permutation of classical biochemical approaches; allied with the use of transgenic plants to investigate the pathway *in vivo* has greatly contributed in this research area. RNAi technology, a means of reducing lignin-related problems which embraces a promising horizon for the genetic improvement of lignified crop like jute is an imperative strategy to accelerate commercial usability and monetary enhancement.

This study reports the first ever use of RNAi-based gene silencing strategy, which successfully reduced the amount of lignin without apparently compromising with the growth and defense mechanism of jute. The down-regulation of four lignin biosynthetic genes, caffeic acid O-methyltransferase (COMT), cinnamate 4-hydroxylase (C4H), ferulic acid 5-hydroxylase (F5H) and coumarate 3-hydroxylase (C3H) by the technique of RNAi (Wesley, Helliwell et al. 2001), are expected to be useful for jute, as these genes do not appear to overlap with the plant defense machinery (Boerjan, Ralph et al. 2003; Ralph, Akiyama et al. 2006; Chapple, Ladisch et al. 2007; Li, Weng et al. 2008). At the same time, work on other plants revealed that change in the lignin content by silencing of these four genes did not appear to cause any physiological abnormalities (Ralph, J., et al, 2006; Huntley et al. 2003; H et. al., 2009; Vanhomole et. al., 2012).

A method for lowering lignin content can be viewed as an attempt that would improve the jute fiber quality. This would allow the use of jute in paper industry, will have potential to be used as a source of bio-fuel and also as a source of finer thread for the garment factories. Measurement of S:G ratios of these jute lines will determine the usefulness of the transgenic lines as modification of monomeric composition (S:G ratio) of lignin can also modify lignocellulose recalcitrance resulting in improved polysaccharide accessibility (Cesarino, Araújo et al. 2012). This is evident in transgenic plants which show efficient pulping or improved digestibility (Baucher, Halpin et al. 2003; Huntley, Ellis et al. 2003; Halpin 2004; Zhu, O'Dwyer et al. 2008;

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Bonawitz and Chapple 2010). It can be claimed that the down-regulation of the four genes used in this study, is going to be highly productive and at the same time will shed light on the gene regulatory network of lignin biosynthesis of this important fiber plant.

In conclusion, this study has dealt with jute lignomics, which provides an overview of lignin biosynthesis and structure, emphasizing the development of low lignin containing transgenic jute plants. Experimental approach and the subsequent outcome have paved the gateway to develop transgenic jute variety with modified lignin content. A major objective for the future will be to learn how to optimize lignin profiles for particular commercial uses.
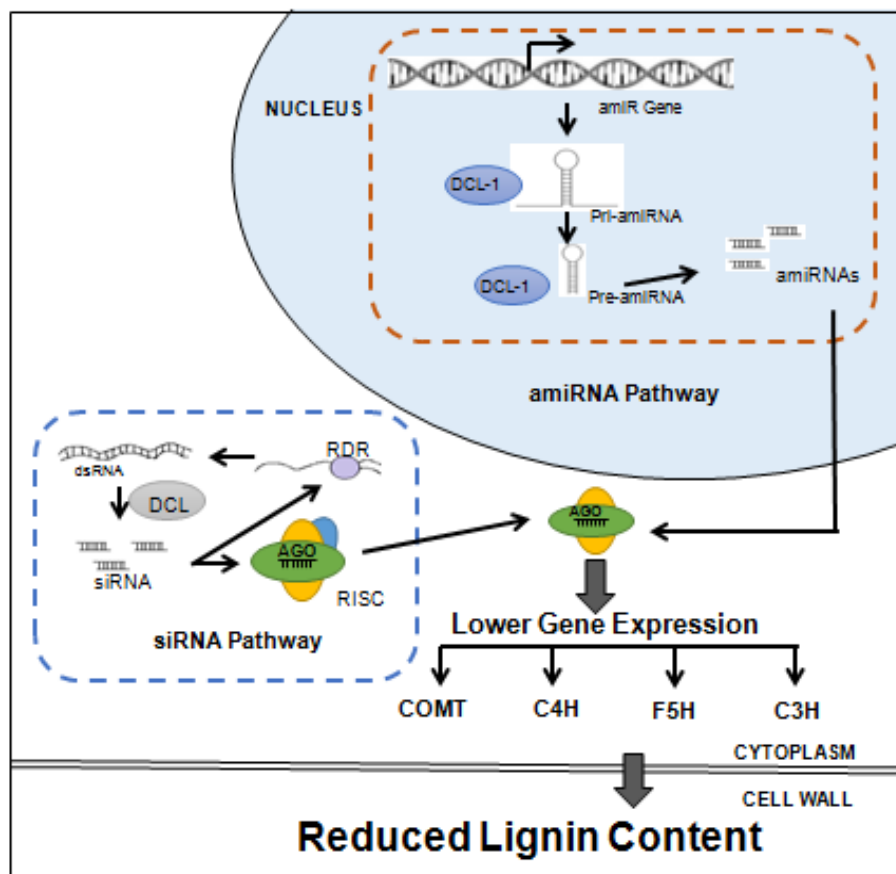

Fig.4.1: Model for RNAi action in jute

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