AGROBACTERIUM-MEDIATED TRANSFORMATION OF CHILLI (*Capsicum annuum* L.) WITH CRISPR/CAS9 CONSTRUCT TARGETING *CHILLI LEAF CURL VIRUS*

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By

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CERTIFICATE

This is to certify that the thesis entitled **"*AGROBACTERIUM*-MEDIATED TRANSFORMATION OF CHILLI (*Capsicum annuum* L.) WITH CRISPR/CAS9 CONSTRUCT TARGETING *CHILLI LEAF CURL VIRUS*"** submitted by **Miss KAVERI S. DESHMUKH** for the degree of **MASTER OF SCIENCE (AGRICULTURE)** in **MOLECULAR BIOLOGY AND BIOTECHNOLOGY** to the University of Agricultural Sciences, Dharwad is a record of research work carried out by her during the period of her study in this university, under my guidance and supervision, and the thesis has not previously formed the basis of the award of any degree, diploma, associateship, fellowship or other similar titles.

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C O N T E N T S

LIST OF ABBREVIATIONS

Abbreviations	Particulars
%	Per cent
°C	Centigrade
m	Metre/s
cm	Centimetre/s
mm	Millimetre/s
nm	Nanometre/s
g	Gram/s
mg	Milligram/s
ng	Nanogram/s
l	Litre/s
ml	Millilitre/s
µl	Microlitre/s
$T_{10}E_1$	Tris (10 mM) - EDTA (1 mM)
Tm	Melting temperature
pH	Potential of hydrogen
µM	Micromoles
CTAB	Cetyl-trimethyl-ammonium Bromide
°C	Degree Celsius
Viz.,	Videlicet
etc.	ecetera
hrs	Hours
i.e.	That is
ppm	Parts per Million
mins	Minutes
e.g.	Example
ChLCV	Chilli leaf curl virus
CRISPR	Clustered Regularly Interspaced Short Pallindromic Repeat sequences
Cas	CRISPR associated protein
IGR	Intergenic region
PAM	Protospacer Adjacent Motif
RCR	Rolling circle replication
sgRNA	Single guide RNA
TALEN	Transcription Activator Like Effector Nuclease
YEMA	Yeast Extract Mannitol Agar
ZFN	Zinc Finger Nuclease
Rep	Replication-associated protein
syn.	Synonym

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1. INTRODUCTION

Chilli (*Capsicum annuum* L.) is a self-pollinated, dicot plant in the *Solanaceae* family. It has been cultivated since 3500 BC in tropical and sub-tropical regions. Its origins are in the American tropics of Mexico, and Christopher Columbus brought it to the rest of the world (Srivastava and Mangal, 2019). Within the genus *Capsicum*, Sanatombi and Sharma (2007) listed 25 wild and five domesticated species. Chilli is one of the most widely grown spices in India. *Capsicum* is a commercially important crop that is used in a variety of edible forms all over the world because it provides a rich source of pungent flavour (Agarwal *et al.,* 1988). In addition, its powder disinfects the oral gastric mucous membranes and kills pathogenic bacteria in the intestine, making it medicinal.

In terms of international trade, India is the world's leader, accounting for approximately 40 per cent of total global production and exporting 17 per cent of its total output. Malaysia is the largest importer of Indian chilli, with Bangladesh (20%), Sri Lanka (15%), the United States (9%), and the United Arab Emirates (8%) following closely behind (FAO, 2014). The exports include chilli powder, dried chillies, pickled chillies, and chilli oleoresin. Chilli was grown in an area of 1.45 lakh hectares in Karnataka in 2018-19 with a production of 1.95 lakh tonnes and a productivity of 1.93 t/ha. In India, chillies were grown in an area of 7.21 lakh hectares, with a production of 16.89 lakh tonnes and a productivity of 2.34 t/ha (2018-19) (Anon, 2019). In 2018, the world produced 5.1 million tonnes of dry chilli. Telangana (3.4 lakh tonnes), Karnataka (2.6 lakh tonnes), Madhya Pradesh (2.4 lakh tonnes), Odisha (69 thousand tonnes), Gujarat (22 thousand tonnes), and Assam (20 thousand tonnes) were the top chilli-producing states in India in 2018 (Anon, 2021).

In Karnataka, Belagavi, Haveri, Bagalkot, Bellary, Mysore, Kolar, and Gadag districts were the top chilli-producing districts in 2017 (Anon, 2018). The area under chilli production in Dharwad district has fluctuated from 31 to 82 thousand ha over the last few decades, depending on weather conditions and the previous year's chilli price. Guntur Sannam, LC 334, Byadgi, and Pusa Jwala are the most popular chilli varieties in India. Approximately 75 per cent of Byadgi chillies are sold and exported to the United States, Europe, and the Middle East, either directly or indirectly (Kareem, 2017). The key determinants of Indian chilli exports include domestic demand, as well as uneven production due to drought, erratic monsoon, and yield factors.

Cultivation in rainfed areas, as well as diseases such as damping off, anthracnose, bacterial leaf spot, and chilli leaf curl, are the major contributors to low productivity. The tropical climate of India supports year-round intensive crop cultivation and the survival of the vector whitefly. Due to this, a large number of *Begomoviruses* have been reported in India, which cause serious diseases in many crop plants.

The whitefly-transmitted *Geminiviruses* are in the genus *Begomovirus*. The remarkable emergence of *Begomoviruses* has been attributed to the *Bemisia tabaci* vector, especially the polyphagous biotype B that has spread throughout the world (Gilbertson *et al.,* 2015). From among the 45 viruses reported worldwide, the *Chilli leaf curl virus* (ChLCV) is a destructive virus. It is a small, circular, ss DNA virus in the family *Geminiviridae* and genus *Begomovirus* and is transmitted in a persistent manner by whiteflies (*Bemisia tabaci*) from the family *Aleyrodidae* and order Hemiptera.

In Greece in 1889, the whitefly was first described as a tobacco pest known as the tobacco whitefly (*Aleyrodes tabaci*). It is a polyphagous pest that infects over 600 plant species and spreads over 60 plant viruses (Rishi, 2004). ChLCV was successfully transmitted from field samples to 50-100 per cent of test chilli plants, causing typical disease symptoms (Senanayake *et al.,* 2007). Several isolates were transmitted by whitefly in an outbreak of chilli leaf curl disease in Jodhpur (Rajasthan), all of which caused acute leaf curl symptoms in chillies (Senanayake *et al.,* 2012).

A single-stranded DNA molecule (2.7 kb) and a beta satellite (~1361 nucleotides in size) make the ChLCV genome. Leaf curling, puckering, rolling, blistering of inter-veinous areas, thickening and swelling of veins, crowding of leaves, shortening of internodes and petioles, and stunting of whole plants are the common symptoms of ChLCV infection. The leaves may become wrinkled and brittle as they get older. According to Kumar *et al.* (2011a), ChLCV infection resulted in a complete loss of marketable fruit. More than one *Begomovirus* is associated with leaf curl disease in India (Khan *et al.,* 2006). According to Gundannavar *et al.* (2007), the most significant causes of crop loss in chilli were viral diseases and insect pest ravages. Chilli pests are diverse, with over 293 insect and mite species wreaking havoc on the crop both in the field and in storage (Anon, 1987).

The use of resistant cultivars, the introduction of resistance genes (R), RNA interference (RNAi), and pesticides are used to manage DNA and RNA viruses in crops. Insecticidal control of chilli pests in general, and especially in irrigated crops with high pesticide use, has caused residue problems in the fruits over the last two decades (Joia *et al.,* 2001).

Insecticide resistance and the decline of natural enemies (Rao and Ahmed, 1986) had an impact on the domestic consumption and export of chilli. This necessitates the cultivation of high-quality chillies free of pesticides, industrial chemicals, and aflatoxins. On the resistant chilli cultivar, *Capsicum annuum* cv. Kalyanpur Chanchal, resistant to *begomoviruses*, which was developed through conventional breeding, a severe leaf curl disease was observed. The synergistic interaction among *begomoviruses* created a permissive cellular environment in the resistant chilli plants and led to the suppression of host defense-related gene expression and the breakdown of resistance (Singh *et al.,* 2016). When compared to traditional breeding, genetic engineering has a number of advantages. Genome editing allows for deletions and insertions of endogenous plant DNA at specific targets (Barrangou and Doudna, 2016).

ChLCV was found to be associated with the phloem region in the early stages of infection, and later spreads to non-vascular tissues (Kushwaha and Chakraborty, 2017). Several strategies have been used to engineer *Geminivirus* resistance in crops, including viral (coat) protein mediated resistance, non-coat protein mediated resistance, viral RNA mediated resistance (PTGS), and host derived resistance (R genes). However, RNAi and CRISPR/Cas9 technology have been effectively used to manage viruses in the *Geminiviridae* family (Loriato Virgilio *et al.*, 2020). The CRISPR/Cas9 system controls viruses at the DNA level by impairing their replication through impaired transcription and translation, whereas RNA interference (RNAi) controls viruses post-transcriptionally (mRNA). *Bean golden mosaic virus* resistance was developed using an RNAi strategy in a transgenic common bean variety (Bonfim *et al.,* 2007).

CRISPR/Cas9 stands for Clustered Regularly Interspaced Short Palindromic Repeat sequences and Cas9 for CRISPR-associated 9. It is a genome editing tool that is both faster and less expensive than other genome editing techniques. It is a bacterial and archaeal adaptive immune system that protects them from the invasion by foreign nucleic acids such as bacteriophages. Short guide RNAs (20 nucleotides) specific to virus genomes have been used

to control both DNA and RNA plant viruses. CRISPR is an antiviral defence mechanism in which an RNA-guided nuclease (for example, the Cas9 protein) cleaves at specific sites on viral DNA or RNA substrates, causing them to degrade. Base complementarity between the CRISPR RNA and the target DNA or RNA molecules determines cleavage specificity. For example, the RNA-guided endonuclease Cas9 from *Streptococcus pyogenes* (*Sp*Cas9) induces double-stranded breaks in DNA *in-vivo* (Jinek *et al*., 2012).

Baltes *et al.* (2015) reported that by using CRISPR technology, they were able to reduce the number of copies of the virus (ssDNA) and symptoms of Bean yellow dwarf viral disease in *Nicotiana benthamiana* plants when sgRNA targeted *Rep* gene, which are essential for rolling circle virus replication. Tripathi *et al.* (2019) observed that *Banana streak virus* (dsDNA) caused lower infection in *Gonja manjaya* plants by using CRISPR technology to target the genes encoding virion assembly and movement.

Plant transformation has been accomplished using a variety of methods. The *Agrobacterium*-based plant DNA transformation method including *in-planta* approach and the particle bombardment method have been the most successful.

The *Agrobacterium*-based plant DNA transformation offers unique advantages: 1) precise integration of genes with a defined end; 2) transfer of desired DNA along with the marker gene; 3) high frequency of stable and intact gene transfer; and 4) ability to transfer long-stretch of DNA (> 150 kb) with a low rate of transgene silencing (Tzfira and Citovsky, 2002). The genus *Capsicum* is recalcitrant with regard to its *in-vitro* regeneration potential (Liu *et al*., 1990), which obstructs the application of recombinant DNA technologies *via* genetic transformation aimed at genetic improvement of resistance against pests and diseases (Ochoa and Malagon, 2001).

One of the most important factor influencing the organogenesis response of *in-vitro* plant culture is genotype. Strong genotype specificity in the regeneration capacity of different cultivars is a significant limiting factor that necessitates the development of a unique regeneration protocol for each cultivar. The type of explant used, as well as the cultivar or variety used, have a significant impact on the crop's regeneration efficiency. As a result, optimising *in-vitro* propagation protocols for specific cultivars is required (Alejo and Moreno, 1990). In Pusa Sadabahar, regeneration media containing MS medium supplemented with 1

mg/L IAA and 5 mg/L 6-BAP resulted in an 81 per cent shoot regeneration frequency, while in Pusa Jwala, it was 78 per cent (Mahto *et al.,* 2018).

Plant regeneration, particularly elongation of buds produced by cultured explants, appears to be a formidable task despite several studies on establishing systems for regeneration of chilli and bell pepper cultivars (Agrawal *et al.,* 1989). As a result, the bell pepper's recalcitrance and difficult-to-regenerate nature *in-vitro*, is one of the major drawbacks of the *Agrobacterium*-mediated tissue culture dependent transformation (Li *et al.,* 2000).

Due to recalcitrant nature and high genotype dependence in *Capsicum*, plant transformation procedures are not well defined as compared to other *Solanaceae* members. As a result, the development of simple, dependable, and efficient bell pepper transformation protocols for areis required, particularly for Indian cultivars that have been adapted to local climate conditions. Establishing alternate protocols would be beneficial. Tissue culture steps in *capsicum* improvement are reduced or eliminated as a result of this. The *in-planta* transformation protocol is a non-tissue culture method of transforming cells in or around the apical meristems with *Agrobacterium* T-DNA, which is then allowed to grow into plants (primary transformants) and produce seeds (Birch, 1997). Kumar *et al.* (2009) used an *in-planta* approach in chilli and found 17.8 per cent and 11.4 percent of T_0 plants as chimeric in Arka Gaurav and Arka Mohini, respectively, and 35.7 percent and 29.7 per cent of plants as stable transformants in the T_1 generation, respectively, based on PCR analysis.

CRISPR/Cas9 technology was recently used in the Department of Biotechnology, College of Agriculture, Dharwad, to develop chilli cultivar Byadgi kaddi, resistant to the *Chilli leaf curl virus*. A total of ten guide RNAs were chosen to target common bidirectional promoter and different genes of the *Chilli leaf curl virus*. An *Agrobacterium* binary vector containing the guide RNA scaffold, *Streptococcus pyogenes* endonuclease (*Sp*Cas9), and a neomycin resistance gene (*npt* II) was custom synthesised with three of the selected guide RNAs (two targeting *Rep* genes and one targeting capsid protein gene). One of two constructs targeting the ChLCV promoter region was verified and evaluated for scaffold RNA and Cas9 RNA expression.

In the current study, the CRISPR/Cas9 construct targeting the promoter region of *Rep* gene was used to transform the chilli cultivar Byadgi kaddi through an *in-planta* method (tissue-culture independent) and also through an *Agrobacterium*-mediated tissue culture dependent strategy using the cotyledonary nodal region as an explant.

Objectives of investigation

1) Transformation of chilli with a CRISPR/Cas9 construct targeting *Chilli leaf curl virus.*

2) Analysis and confirmation of putative transformants

2. REVIEW OF LITERATURE

Chilli (*Capsicum* sp.) is a self-pollinated dicot plant in the *Solanaceae* family. The word "*capsicum*" is derived from the Greek word 'kapsimo', which means 'to bite'. *Capsicum annuum* L., *Capsicum frutescens* Mill., *Capsicum chinense*, *Capsicum baccatum* L., and *Capsicum pubescens* are the five species that have been domesticated and cultivated (Kothari *et al.,* 2010).

India is regarded as a secondary centre of chilli diversity (Dhaliwal *et al*., 2014). Among the five cultivated species of the genus *Capsicum*, *C. annuum* is the most widely cultivated species in India, with pungent (chilli, syn. hot pepper) and non-pungent (sweet pepper, syn. *capsicum*, bell pepper) fruits. Cultivation of *C. frutescens*, *C. chinense*, and *C. baccatum* is limited to homestead gardening in various parts of the world (Reddy *et al*., 2014). Onus and Pickersgill (2004) found that most *Capsicum* species are self-compatible, and *C. annuum* is a partially self-pollinating crop (Allard, 1960). It is classified as a facultative cross-pollinating species since out-crossing in the field normally ranges from 7 to 90 per cent (Tanksley 1984; Singh *et al*. 1994).

Chillies are susceptible to a number of pathogens, including viruses, which can lead to significant production losses. So far, 65 viruses have been identified, including *begomoviruses* that cause ChLCVD (Chilli leaf curl viral disease) in chillies all over the world (Nigam *et al.,* 2015), leading to almost hundred per cent losses of marketable fruits in extreme cases (Senanayake *et al.,* 2007; Kumar *et al.,* 2011a, b; Senanayake *et al.,* 2012).

Leaf curling, leaf rolling and puckering, blistering of intravenous areas, thickening and swelling of veins, shortening of internodes and petioles, crowding of leaves, and overall plant stunting are common symptoms of ChLCVD. In India, *Chilli leaf curl virus* (ChLCV), *Tomato leaf curl New Delhi virus* (ToLCNDV), *Tomato leaf curl Joydebpur virus* (ToLCJV), and *Chilli leaf curl Palampur virus* (ChLCPV) have all had their genome sequences characterised in India (Khan *et al*., 2006; Shih *et al*., 2006; Kumar *et al*., 2011a, b). Attempts at evasive measures such as pesticide sprays to control vectors, removal of diseased plants, and agronomic interventions have all had limited success. The use of host plant resistance to manage diseases, particularly those caused by viruses, is an effective, cost-effective, environmentally safe, and long-lasting strategy.

2.1 Genome editing techniques

Genome editing techniques have progressed from being complex techniques that required years of training to being simple techniques that can now be performed in many labs by ordering commercial kits from various scientific research supply companies. Zinc-Finger Nucleases (ZFNs) and Transcription Activator-Like Effector Nucleases (TALENs) were previously used to edit genomes. These engineered nucleases recognise and cleave specific nucleotide sequences in genomes, resulting in mutations induced by the host cell's DNA repair machinery, such as substitutions, deletions, and insertions (Ebina, 2013). Designing, developing, and testing these techniques in living cells is still difficult and time-consuming.

ZFNs are fusion proteins that contain both zinc finger proteins (ZNP) and the Fok I restriction enzyme's nuclease domain (Urnov, 2010). The ZNP enables the ZFN to bind to site-specific sequences *via* an array of Cys2-His2 fingers, each of which recognises roughly 3 nucleotides of DNA (Wolfe, 2000). To cleave DNA, the Fok I catalytic domain must dimerize (Vanamee, E.S., 2001). Two ZFNs must be designed so that one binds to a target site downstream of the desired cute site and the other binds to the complementary DNA strand upstream of the targeted cute site, forming the left and right pair. The use of ZFNs necessitates a different "design" of the ZFP, which makes ZFN production time-consuming and expensive.

The study of the bacterial plant pathogen *Xanthomonas* led to the discovery of TALENs, which function similar to ZFNs (Nemudryi, 2014). The *Xanthomonas* genus secretes an effector protein called Transcription activator-like effectors (TALEs) into the cytoplasm of plant cells, which affects plant cell processes and increases the pathogen's susceptibility. Following further investigation into the mechanism of the effector protein, it was discovered that the protein could bind to DNA and activate the expression of target genes by mimicking eukaryotic transcription factors (Nemudryi, 2014).

TALE proteins have a core DNA-binding domain, a nuclear localization signal, and an acidic activation domain that activates target gene transcription (Morbitzer, 2010). Monomers in the DNA binding domain each bind to one nucleotide in the target sequence (Lamb, 2013). Following the discovery of the code for TALE protein's DNA recognition, researchers looked into creating chimeric TALEN nucleases by inserting TALE's DNA-binding domain into a plasmid vector that had previously been used to make ZFNs (Christian, 2010).

CRISPR (Clustered Regularly Interspaced Short Palindromic repeat) systems, which were first discovered in bacteria and archaea, have recently been used to develop a new method of genome editing (Barrangou *et al.,* 2007). CRISPR systems are adaptive immune systems that bacteria use to defend themselves against foreign nucleic acids (Fineran, 2012).

2.2 CRISPR/Cas system

CRISPR sequences were first discovered in the genomes of *Escherichia coli* bacteria (Ishino *et al*., 1987). It was found that they were DNA fragments made up of a succession of repeat sequences (non-contiguous short DNA repeats) separated by spacer DNA sequences containing random nucleotides (variable sequences). CRISPR-associated (Cas) genes were also eventually discovered near CRISPR loci in CRISPR-containing prokaryotes (Jansen *et al.,* 2002). Bacteriophage genomic fragments were identified to be the source of the variable spacer sections within CRISPRs, revealing that the CRISPR/Cas system's role was virus recognition and suppression (Barrangou *et al.,* 2007). This technology has enabled researchers to edit any of the genomes that have been sequenced and manipulate them according to their needs or the objectives of the study.

2.3 CRISPR/Cas-mediated adaptive bacterial immunity

CRISPR/Cas has three distinct functional phases, according to Makarova *et al.* (2011): adaptation (acquisition), expression and processing (crRNA synthesis), and interference (targeting). A tiny fragment of phage DNA called a "new spacer" integrates into the CRISPR loci during the first phase of virus entry. After transcription, the CRISPR locus produces a long precursor CRISPR RNA (pre-crRNA) that contains both repeat and spacer sequences during the biogenesis phase. Other spacers are transcribed with the new spacer as well. The repeat and spacer sequences are cleaved off of the lengthy precursor, resulting in tiny crRNAs. This same invading phage DNA is targeted and cleaved in the third phase, called interference.

When a bacterial cell is invaded by a bacteriophage with the same sequence as the spacer sequence present in the cell, the spacer of the crRNA, which is present in the cell, identifies the phage's target sequence to be cleaved. A relatively small (3 or 4 nucleotides), conserved nucleotides downstream of the protospacer (target DNA) is known as the Protospacer Adjacent Motif (PAM), which provides the recognition signal and determines protospacer selection (Marraffini and Sontheimer, 2010). The crRNA and Cas protein

combine to form the ribonucleoprotein complex, which cuts the invading phage genome at few nucleotide upstream of the PAM sequence. The position of the PAM sequence determines the location of cut sites as well as the efficiency of Cas9 DNA target cleavage.

2.4 CRISPR/Cas9 gene-editing system

CRISPR/Cas9 is a type II clustered regularly interspaced short palindromic repeats system that cleaves RNA-guided double-stranded DNA (dsDNA) using a single Cas9 protein containing HNH nuclease and RuvC domains (Makarova *et al.,* 2015; Jinek *et al.,* 2013). Both the non-coding RNAs, CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA), are required for Cas9 nuclease target cleavage. Both CrRNA and tracrRNA make up to constitute a single guide RNA molecule (sgRNA), which complementarily basepairs to a specific 20-nucleotide (nt) DNA sequence (spacer). The spacer is immediately followed by a 3' PAM sequence (Jinek *et al.,* 2012). The binding affinity of the crRNA to the target DNA is reduced by mismatches in the seed region *viz.,* PAM-proximal 10-12 nucleotides at the 3' end of the 20-nt spacer sequence (Jinek *et al.,* 2012; Semenova *et al.,* 2011; Cong *et al.,* 2013; Jiang *et al.,* 2013a).

2.5 Mechanism of CRISPR/Cas9-mediated gene editing

Cas9, a gene originating from the soil bacteria *Streptococcus pyogenes*, is the most frequently used endonuclease gene in genome editing experiments. Cas9 (also known as CRISPR-associated protein 9) is a 1368 amino-acid protein. It is a multi-domain and multi-functional protein (Mali *et al.,* 2013). Cas9 cleaves the target double-stranded DNA at 3-bp upstream of the PAM sequence by using two nuclease domains, i.e., the HNH-like and the RuvC-like domains. The HNH-like domain, which is complementary to the guide RNA, cuts the target DNA strand. The non-target complementary DNA strand is cut by the RuvC-like nuclease domain (Gasiunas *et al*., 2012; Chen *et al*., 2014). PAM sequence of Cas9 is NGG, where N is any nucleotide (Hsu *et al*., 2013). Cas9 enzymes are present in cells as nucleases in an inactive state (Jinek *et al*., 2012).

The sgRNA binds to the Cas9 protein, resulting in a ribonucleoprotein complex with a conformational change. For the sgRNA-Cas9 complex to find a potential target DNA, PAM recognition is very critical (Jiang *et al*., 2015; Jinek *et al*., 2014). Cas9 unwinds the target DNA at the PAM-adjacent site after finding a potential target DNA with the appropriate PAM, allowing sgRNA to bind to the target DNA and form an RNA-DNA hybrid through

base pairing. PAM binding identifies a potential target, and complete annealing of sgRNA-DNA causes the HNH domain to change conformation and cut the target. This conformational transition activates the catalytic activity of the RuvC domain (Jiang *et al*., 2016; Sternberg *et al*., 2015).

Non-homologous end joining (NHEJ) or homology-directed repair (HDR) is used to repair the double-stranded break caused by Cas9 (Cong *et al*., 2013). NHEJ ligates the breaks, which can result in mutations such as deletions or insertions (indels). The reading frame is altered by indels, which disrupts translation and subsequent loss of gene function. Gaps are filled by the DNA repair mechanism *via* homologous recombination when a donor/template strand is present (Symington and Gautier, 2011).

2.6 Steps of CRISPR/Cas9-mediated genome editing in plants

The first steps in CRISPR/Cas9-based genome editing are selecting the target gene, identifying the PAM sequence in the target sequence, and designing the sgRNA. Designing a gene-specific sgRNA can be done manually or with the help of online tools such as CGAT, CRISPRdirect, CRISPRseek, CHOPCHOP, Crispr-P (Park *et al*., 2015; Labun *et al*., 2019; Brazelton *et al*., 2015; Naito *et al*., 2015). The next step is to clone Cas9 and sgRNA into a vector that will allow for single or multiplex editing. The transcriptional promoter is required for both elements (Cas9 and sgRNA) to be transcribed *in-vivo* for genome editing in plants.

Cas9 is usually expressed under the control of an RNA polymerase II (pol II) promoter. Cas9 gene expression in plants is regulated by constitutively active promoters such as the Cauliflower mosaic virus 35S promoter (CaMV-35S) (Nekrasov *et al*., 2013), *Arabidopsis* ubiquitin (AtUbi) (Mao *et al*., 2013), maize ubiquitin (ZmUbi) (Wang *et al*., 2014), or rice ubiquitin (OsUbi) (Zhang *et al*., 2014). The small nuclear RNA promoters (U6 and U3) express sgRNA, which is transcribed by RNA polymerase III (Pol III) (Shan *et al*., 2013). The CRISPR/Cas9 vector expression system is primarily based on the co-expression of a Cas9-carrying plasmid under the promoter of RNA Pol II and a plasmid expressing sgRNA under the promoter of RNA Pol III (Nekrasov *et al.,* 2013). In the same construct, Cas9 nuclease and sgRNA can be used together, or they can be used separately (Mao *et al*., 2013; Upadhyay *et al*., 2013).

For multiplex genome editing, multiple sgRNA expression cassettes (each sgRNA cassette with an RNA promoter) can be piled into a single construct (Ma *et al.,* 2015). Cas9

and sgRNA have been expressed in *Arabidopsis* using a dual RNA pol II promoter system (Gao *et al.,* 2015b). In rice genome editing, Tang *et al.* (2016) has used a single promoter-driven CRISPR/Cas9 system (single transcriptional unit, STU). Cas9 and the sgRNA(s) were separated by ribozyme (RZ) cleavage motifs, and after transcription, a single transcript was processed by a hammerhead ribozyme (RZ), allowing the Cas9 mRNA to be translated and form the functional complex with the sgRNA(s) (Tang *et al*., 2016). Based on the Csy4 ribonuclease system and the endogenous tRNA-processing system (Xie *et al*., 2015), many sgRNAs can be produced in a cell from a single transcript (Kurata *et al*., 2018; Liu *et al*., 2019).

The delivery of CRISPR/Cas9-mediated editing reagents into plant cells is the next step. As a protein or as a DNA or RNA expression cassette incorporated into plant cells, these reagents are delivered directly to the nuclei. The two main delivery methods for stable expression of CRISPR/Cas DNA into plants are particle bombardment (Hamada *et al.,* 2018) and *Agrobacterium*-mediated transfer DNA (T-DNA) transformation (Gao *et al.,* 2015a; Ma *et al.,* 2015), combined with a selection gene (e.g. antibiotic or herbicide resistance), allowing integration and expression of DNA into the genome of a plant in order to bring about genetic segregation at T_1 and successive generations by selfing and crossing such as to result in transgene-free plants.

CRISPR/Cas9 reagents delivered and expressed transiently offer an alternative method for achieving transgene-free editing. Transient CRISPR/Cas9 expression in plants can be achieved using *Agrobacterium*-mediated transformation (Chen *et al*., 2018), protoplast transfection (Andersson *et al*., 2017), or particle bombardment (Zhang *et al*., 2016). The DNA is naturally degraded after it has been expressed. As a result, edited plants are regenerated without the need for foreign DNA integration or selection. To achieve DNA-free genome editing *via* CRISPR/Cas9, a pre-assembled ribonucleoprotein (RNPs) or a mixture of *in vitro* Cas9 and sgRNAs (Zhang *et al.,* 2016) can be delivered (Liang *et al.,* 2018; Liang *et al.,* 2017; Svitashev *et al.,* 2016; Woo *et al.,* 2015). Viruses were used to deliver sgRNA to Cas9-overexpressed transgenic plants (Ali *et al*., 2015a; Yin *et al*., 2015). Viruses have not been employed as vehicles for Cas9 in non-transgenic plants because the Cas9 gene is too large (> 4 kb) for them to transport. The considerations of the effects of virus infection on the host

plant and the need for insect-proof containment glasshouses are two potential drawbacks of using viruses in this way.

The final step in CRISPR/Cas9 editing experiments is to identify and confirm mutations. PCR amplification and sequencing of target gene amplicons are usually used to accomplish this (Zhang *et al.,* 2014). The restriction enzyme (RE) site loss assay (Shan *et al.,* 2013), surveyor assay (Mao *et al.,* 2013), restriction fragment length polymorphism (RFLP) analysis (Feng *et al.,* 2013), T7 endonuclease I (T7E1) assay (Wang *et al.,* 2014), and polyacrylamide gel electrophoresis (PAGE) (Guo *et al.,* 2018) have been developed as methods for screening edited events. Whole-genome sequencing is very effective for identifying mutants, despite the fact that it is expensive and time-consuming (Braatz *et al*., 2017). Depending on the function of the target genes, biochemical and physiological assays could be used to identify specific mutants. When the phytoene desaturase (PDS) gene is disrupted, an albino phenotype is produced, which can be used to identify mutants (Chen *et al*., 2018).

2.7 Applications of CRISPR/Cas9 in plant genome editing

In August 2013, Nature Biotechnology published the first three short studies on the CRISPR/Cas9 system (Li *et al.,* 2013; Nekrasov *et al.,* 2013; Shan *et al.,* 2013). Targeted mutagenesis has been demonstrated in *Arabidopsis thaliana* (Li *et al.,* 2013), *Nicotiana benthamiana* (Li *et al.,* 2013; Nekrasov *et al.,* 2013), rice (Shah *et al.,* 2013) and wheat (Shah *et al.,* 2013). Several studies on CRISPR/Cas9 applications to various crop species like rice, wheat, potato, cotton, sweet orange, Duncan grapefruit, chilli, apple, and banana, were published a short time thereafter (Feng *et al.,* 2013; Jiang *et al.,* 2013b; Miao *et al.,* 2013; Xie and Yang, 2013).

By modifying yield-related genes (Li *et al.,* 2016b), as well as the regulatory sequences and promoters of significant yield genes, the CRISPR/Cas9 system has been used to address yield and yield-related aspects. It's also been utilised to create loss-of-function mutations in rice yield-determining genes such as *GW5* (grain weight), *Gn1a* (grain number), *GS3* (grain size), *DEP1* (panicle size) and *OsAAP5* (tiller number) (Li *et al.,* 2016b; Liu *et al.,* 2017; Wang *et al.,* 2019).

Plants with better quality traits such as nutritional value, starch content, and crop storage quality were created using CRISPR/Cas9. The *granule-bound starch synthase* (*GBSS*)

gene, which is responsible for the formation of amylose in potato plants, was successfully knocked out using CRISPR/Cas9, leading in a change in starch quality (Andersson *et al.,* 2017). The *Fatty Acid Desaturase 2* (*FAD2*) gene was targeted using the CRISPR/Cas9 technology to improve the fatty acid content of *Camelina sativa* (Jiang and Doudna*,* 2017). The CRISPR/Cas9 technique was used to mutate the *FAD2* genes in peanuts (Yuan *et al*., 2019). CRISPR/Cas9 was used to mutate seed storage protein genes in soybeans (Li *et al*., 2019).

In a variety of plants, CRISPR/Cas9 could modify genes, pathways, and regulatory networks involved in abiotic stress tolerance. In *A. thaliana*, the CRISPR/Cas9 system was used to mutate the *Open Stomata 2* (*OST2*) gene, which confers abiotic stress tolerance by increasing stomatal closing responses to environmental conditions (Osakabe *et al*., 2016). *SlMAPK3* (the mitogen-activated protein kinase) gene knockout mutants were developed using the CRISPR/Cas9 technology, which altered the drought tolerance regulation mechanism in tomatoes (Wang *et al.,* 2017). Using CRISPR/Cas9 to precisely alter the *ARGOS8* gene, drought tolerance in maize has now been achieved and verified under field drought condition (Shi *et al.,* 2017). Rice *OsPQT3* deletion mutants demonstrated superior oxidative and salt stress resistance, as well as greater grain yield in the field (Alfatih *et al.,* 2020).

The development of herbicide-resistant crops has been accelerated, thanks to recent advances in gene editing systems. The TIPS (T102I + P106S) amino acid substitutions were engineered into the rice *5-enolpyruvylshikimate-3-phosphate synthase* (*EPSPS*) gene by an intron-targeting gene replacement and insertion approach, conferring glyphosate resistance *via* the non-homologous end-joining route (Li *et al.,* 2016a). CRISPR/Cas9-mediated point mutations in the rice *Acetolactate Synthase 1* (*ALS1*) gene resulted in resistance to the herbicide bispyribac sodium (Sun *et al.,* 2016). Herbicide-resistant watermelon and oilseed rape were obtained using a CRISPR/Cas9-mediated base-editing technology (Tian *et al.,* 2018; Wu *et al.,* 2020).

The CRISPR/Cas9 approach has also been utilised to confer resistance to major diseases by directly targeting the pathogen genome or host-related genes in pathogen sensitivity (Ali *et al.,* 2015b; Wang *et al.,* 2014).

Another method for improving pathogen resistance is to reduce the activity of a host susceptible (S) gene that promotes pathogen colonisation and has an impact on plant immunology, biosynthesis, and metabolite transport. The powdery mildew susceptibility gene, *mlo* (*Mildew Resistant Locus O*), has been effectively targeted using the CRISPR/Cas9 system in wheat, tomato, and grapevine to boost powdery mildew resistance (Malnoy *et al.,* 2016; Nekrasov *et al.,* 2017; Wang *et al.,* 2014). Mishra *et al.,* 2021 reported reduced spore count and fungal growth of *Colletotrichum truncatum*, causing anthracnose on chilli (Arka Lohit), when the *CaERF28* (*Capsicum annuum* Ethylene Responsive Factor) gene was altered by CRISPR/Cas9 technology. In subsequent generations, T-DNA free homozygous mutant lines were developed. CRISPR/Cas9 was used to insert mutations in the SWEET gene promoters to generate resistance to bacterial blight caused by *Xanthomonas oryzae* pv. *oryzae* (Oliva *et al.,* 2019). CRISPR/Cas9 inactivated the *downy mildew resistance 6* (*dmr6*) gene in tomato, increasing broad-spectrum resistance to bacterial infections such as *Pseudomonas syringae* and *Xanthomonas* spp., as well as the oomycete pathogen *Pseudomonas capsici* (De *et al.,* 2021).

2.7.1. CRISPR/Cas9-mediated immunity against viral diseases

In the *Beet severe curly top virus* (BSCTV), when its gene encoding for virus replication and coat protein synthesis and the intergenic region (IGR), was targeted by sgRNA, Ji *et al.* (2015) found a reduced copy number of the virus and its infestation on *Arabidopsis thaliana* plants. Baltes *et al.* (2015) reported a lower copy number of the virus, *Bean yellow dwarf virus* (ssDNA) and its lowered infection on *Nicotiana benthamiana* plants when its gene encoding for virus replication was targeted by sgRNA. Lowered infection of *Tomato yellow leaf curl virus* (TYLCV) on *Nicotiana benthamiana* plants (Ali *et al.* 2015b) and *Banana streak virus* (dsDNA) on *Gonja manjaya* plants (Tripathi *et al.* 2019) was also detected with reduced or no symptoms though use of CRISPR/Cas9 approach. Multiple guide RNAs targeted multiple parts of the same viral genome at the same time, resulting in a reduction in viral load (Ali *et al.,* 2015b; Kis *et al.,* 2019; Roy *et al.,* 2019). In a transient study in *N. benthamiana*, multiple sgRNAs targeted multiple regions of the *Chilli leaf curl virus* (ChLCV) genome to create a high level of ChLCV resistance (Roy *et al.,* 2019).

The CRISPR/Cas9 technology was utilised to target the TYLCV genome for interference and cleavage. In TYLCV, the Intergenic region (IGR) was targeted, which

resulted in a considerable reduction in TYLCV buildup and disease symptoms. Because CRISPR/Cas9-mediated interference is virus strain-specific, it can be utilised to combat a variety of viruses (Ali *et al.,* 2016). Because both *CP* (Coat protein) gene and *Rep* gene encode proteins, a tiny amount of protein may be enough for TYLCV to complete its cycle and generate symptoms, targeting CP and rolling circle replication II (RCRII) motif of the Rep ORF decreased symptoms less than targeting the IGR. Furthermore, because the IGR contains the TYLCV IGR's stem-loop invariant sequence (TAATATTAC), Cas9's alteration of this region renders it inaccessible to *Rep* and/or other viral replication-associated proteins (Ali *et al.,* 2016). The same IGR within which the bidirectional promoter, i.e., the promoter of the *Rep* gene, was targeted in the current research.

Reducing the activation of a host susceptibility (S) gene, which inhibits pathogen colonisation, is another technique for increasing pathogen resistance. CRISPR/Cas9 was used to alter the host susceptible gene, *eIF4E* (eukaryotic translation initiation factor 4E) in cucumber plants to make them resistant to *Potyviruses* (Chandrasekaran *et al.,* 2016). The similar approach was used in *A. thaliana* to produce mutations in the *eIF4E* gene, resulting in resistance to *Turnip mosaic virus* (TuMV) (Pyott *et al.,* 2016).

Virus interference in plants mediated by CRISPR/Cas9 offers a number of unique advantages, including: 1) the ability to target multiple DNA viruses simultaneously using a single sgRNA; 2) the ability for multiplexed editing of single or multiple viruses using one conserved sgRNA or multiple sgRNAs; 3) the potential to overcome resistance by targeting newly evolved viral revertants with new sgRNAs; and 4) applicability to all plant virions (Ali *et al.,* 2016).

2.8 *Agrobacterium*-mediated plant transformation

Agrobacterium tumefaciens is a gram-negative bacterium that is usually associated with plant roots. It is a soil-borne, rod-shaped bacterium (Kado and Hooykaas, 1991) and parasitize a variety of plants mostly dicots, causing crown gall, a neoplastic tumour disease that is economically important (Kado and Hooykaas, 1991; Kado, 2002). Nester *et al*. (1984) demonstrated that the tumour is caused by the transfer of a DNA fragment from the bacterial Ti (for tumour-inducing) plasmid, known as "transferred DNA," or T-DNA, to the host plant cell chromosomes, where it is integrated into the plant cell genome (Kado, 2002) and expresses onco-genes found on the T-DNA (Scandalios, 2007).

Later research indicated that the T-DNA within the Ti plasmid is strongly defined by the presence of two 25 bp surrounding borders as direct repeats, referred to as the left and right T-DNA borders (Yadav *et al*., 1982). These discoveries paved the way for plant genetic engineering. Any foreign DNA inserted between the T-DNA borders can indeed be transported to plant cells, and the earliest vector systems for plant transformation were built on this early model (Hamilton, 1997). The Ti plasmid also contains the transfer (*tra*), opine catabolism, and virulence (*vir*) genes, all of which are critical in the transformation process (Zhu *et al.,* 2000).

The border repeats that delimit the T-DNA, the virulence genes (*vir*) that code for the trans-acting type IV secretion system, and different bacterial chromosomal genes are the three required genetic elements for T-DNA transfer and integration into the plant genome (Lee and Gelvin, 2008). Several bacterial, plant, and environmental factors influence the frequency of *Agrobacterium*-mediated plant transformation (Tzfira *et al*., 2002). It has been observed that the type of plant tissue employed is a significant element in the effective *Agrobacterium*-mediated transformation of cereals at the plant level (Orczyk *et al*., 2000b). On the bacterial side, the density of the bacterial culture (Cheng *et al*., 2004; Opabode, 2006) and the strain's ability to adhere and transmit its T-DNA to the host cells (Cheng *et al*., 2004) have been shown to influence the frequency of transformation. Depending on the transformed plant or crop, different *Agrobacterium* strains have been widely reported to influence transformation frequency.

2.9 *Agrobacterium tumefaciens*-mediated gene transfer in chilli

Agrobacterium tumefaciens is utilised to efficiently transform most plants. The most extensively used approach for genetic transformation of various plants is the *Agrobacterium*-mediated transformation system (Cardoza and Stewart, 2004). For a long time, most significant *solanaceous* plants were difficult to genetically engineer, owing to their resistance to *in-vitro* regeneration and *Agrobacterium* infection (Ke *et al.,* 2001; Repellin *et al.,* 2001; Sahrawat *et al.,* 2003).

Liu *et al*. (1990) investigated *Agrobacterium*-based *in-vitro* regeneration and transformation systems in bell pepper and reported that transformed shoots and leaf-like structures exhibited beta-glucuronidase activity (GUS) in the vascular tissue without bacterial contamination. Wang (1991) employed cotyledon, hypocotyl, and leaf explants for

transformation and regeneration, and the transformants showed temporary GUS activity. Zhu *et al*. (1996) used *Agrobacterium* strain GV311-SE expressing the *Cucumber mosaic cucumovirus coat protein* (*cms-cp*) gene to turn sweet pepper into a transgenic sweet pepper. Siregar and Sudarsano (1997) used non-disarmed *Agrobacterium* isolates to induce shoot regeneration from hot pepper hypocotyl segments.

Manoharan *et al*. (1998) developed a methodology for cotyledonary leaf regeneration and *Agrobacterium*-mediated genetic transformation in hot chilli (*C. annuum* cv Pusa Jwala). Lim *et al*. (1999) had effectively employed cotyledon segments of hot peppers for regeneration and *Agrobacterium*-mediated transformation. Kim *et al.* (1997) studied RNA-mediated resistance to *Cucumber mosaic virus* in the progeny of hot pepper transgenic plants expressing RNA.

Although efforts are being made to achieve stable transformation of *Capsicum* by *Agrobacterium tumefaciens*, additional approaches such as particle gun bombardment, electroporation, floral dip, and sonication-assisted *Agrobacterium-mediated* transformation are also being used. All of these transformation approaches are emerging as the preferred methods for the introduction of agronomically important genes for quality improvement, secondary metabolite regulation, and engineering molecular pharming and improvement of *Capsicum* spp. for maximum usage in pharmaceutical, nutraceutical, and food industries.

2.10 *In planta* transformation

In 1986, Graves and Goldman introduced the concept of the "*in-planta* transformation approach". However, the transgenic plant was chimeric. Feldmann and David Marks (1987) later generated a stable transformed non-chimeric plant. Several studies have been undertaken since then to improve the technique. Various elements influence *in-planta* transformation methods, including plant parts (explants), stage, and methodology employed, ionic detergents used, vacuum or negative pressure, and treatment duration, among others.

In-planta transformation of rice for sheath blight resistance was accomplished by piercing the apical meristem region of imbibed seed twice with a needle dipped in the *A. tumefaciens* (EHA-101) inoculums harbouring the vector: pAJ21-CaMV35-tlpD34. PCR for 710 bp of the *tlp* gene was used to confirm potential transformants. The observed transformation efficiency (T.E.) was 24 percent (Naseri *et al*., 2016).

Similarly, *in-planta* transformation was performed in *Gossypium hirsutum* cv. LRK-516 Anjali to improve resistance to Alternaria leaf blight. The seedling was utilized as an explant, and a vertical incision was made along the length of the seedling's shoot apex at the junction of cotyledonary leaves, and *Agrobacterium* inoculums (strain EHA-105 harboring vector pBinAR with At-NPR1 gene) were dropped at the cut end. The T.E. was 6.8 per cent (Kalbande and Patil, 2016).

Mayavan *et al*. (2013) developed an effective, reproducible *in-planta* transformation system in sugarcane. Surface sterilised seeds were pre-cultured, sonicated, vacuum infiltrated in *Agrobacterium* inoculation media (strain EHA-105 harbouring vector pCAMBIA with 1304-bar gene), co-cultivated, germinated, transferred, and screened for CoC671. GFP assay, PCR analysis, and Southern blotting were used to confirm the results, with T.E. of 45.4 per cent.

Sujipuli *et al*. (2014) conducted an experiment with the floral dip method in rice (cv. RD41) florets. The tops of selected rice spikelets were cut off and inoculated with *Agrobacterium* strain AGL1 containing the *gus A* gene for one minute. A plastic bag was placed over each immersed flower. Anthers were the major targets in the floral dip method of plant transformation, with transformation effectiveness of 89.16 per cent but ovary transformation is uncommon (7.23 %). The GUS assay and PCR were used to confirm the results.

The *in-planta* transformation through Agroinfiltration of fruit method was carried out in tomatoes (*Lycopersicon esculentum* Mill.). Inoculation with *A. tumefaciens* (strain EHA-10 harbouring vector pROKII GUSint AP1) culture by using 1 ml sterile syringe was carried out. Later, the fruits were incubated at 28 °C to 48 hrs. Transformation efficiency was found to be 68 per cent. The report concluded that the method needs to be further refined for its application as a novel method in the field of *Agrobacterium*-mediated transformation (Hasan *et al*., 2008).

2.11 Parameters affecting *In planta* transformation

Agrobacterium suspension and its optical density (OD), infection period, co-cultivation period, and selection pressure are the most relevant parameters. According to the studies by Paramesh *et al*. (2010), numerous aspects were important in developing an efficient transformation technique. Similarly, Hasan *et al*. (2008) demonstrated the impact of bacterial

density on tomato fruit infiltration-based *in-planta* transformation. Yasmeen *et al*. (2009) discovered that 48-hour incubation was the most successful. Pre-culture has been shown to be beneficial in both tissue culture-dependent and tissue culture-independent transformation techniques (Paramesh *et al*., 2010). Khoudi *et al*. (2009) demonstrated the importance of explant type in tissue culture-dependent *Agrobacterium*-mediated transformation. The main leaves of tomatoes were shown to be the optimum explant for *Agrobacterium* infection in the study.

Although acetosyringone was not used in the transformation trials in Bangladesh, it had positive impact on overall transformation efficiency (Raj *et al*., 2005; Yarra *et al*., 2012; Das *et al*., 2015). They discovered that utilising an *Agrobacterium* suspension with an OD 600 of 0.8 for 15 minutes of infection and a 3-day co-cultivation gave the highest transformation rates in tomato variety (BARI Tomato 8). In the similar study conducted five years ago with three Bangladesh varieties (BINA Tomato 3, BINA Tomato 5, and BAHAR) and one Indian variety (Pusa Ruby), similar results were observed (Islam *et al*., 2010). Both studies emphasised the advantages of preculture.

Table 1: Present status of chilli genetic transformation

Scientists and year	Crop (chilli), Variety	Explant	Vector, *Agrobacterium* strain	Regeneration media	Co-cultivation media	Shoot induction media	Root induction media	Confirmation analysis	Transformation efficiency
Manoharan *et al*., 1998	*Capsicum annuum* L. var. *Pusa Jwala*	Three week old cotyledonary leaf	pBI 121, strain EHA-105	MS media + 0.5 mg/L TDZ	MS media + 0.5 mg/L TDZ For 48 hr	MS media + 0.5 mg/L TDZ +50 mg/L kanamycin + 400 mg/L cefotaxime	Half MS media + 0.5 mg/L IAA	PCR, Southern analysis, GUS analysis	0.02 per cent
Shivegowda *et al*., 2002	*Pusa Jwala* and G4	Cotyledonary leaves	pGV 1040, strain C58	MS media + 9.08 µM TDZ	MS media + 9.08 µM TDZ For 24 hr	MS media +13.68 µM zeatin +2.89 µM GA_3 + 500 mg/L cefotaxime	MS media + 4.90 µM IBA + 500 mg/L cefotaxime + 75 mg/L kanamycin	GUS, PCR and Southern hybridization analysis of *npt* II gene.	0.05 per cent
Ashajyothi , 2004	Byadgi Dabbi and Sankeshwar local	Whole cotyledon, shoot tip and hypocotyl portion	pBinBta , strain EHA-105	MS media + 10 mg/L BAP	MS media +2 mg/L BAP (selection media of 300 mg/L cefotaxime) For 2 days	MS media + 0.1 mg/L NAA + 0.2 mg/L BAP +2 mg/L GA_3	MS media + 2 mg/L IBA	PCR, GUS expression, Southern hybridization analyses of *npt* II gene	0.50 per cent
Channappa goudar, 2007	Byadgi kaddi	Hypocotyls, cotyledonary nodal, cotyledon	pBINBt3, strain EHA-105	MS media + 0.5 mg/L TDZ	MS media +0.5 mg/L TDZ +50 mg/L kanamycin + 400 mg/L cefotaxime For 72 hr	MS media + 0.5 mg/L TDZ +1 mg/L GA_3	MS media + 0.5 mg/L IAA + 0.1 mg/L BA	RT-PCR	0.50 per cent

Table 1: Contd...

Scientists and year	Crop (chilli), Variety	Explant	Vector, *Agrobacterium* strain	Regeneration media	Co-cultivation media	Shoot induction media	Root induction media	Confirmation analysis	Efficiency
Subramanyam *et al*., 2011	*Capsicum annum* L. var. Aiswarya 2103	Four week old leaf	pBINASCOSM, strain EHA-105	MS media	MS media + BA(5 mg/L) +IAA (1 mg/L)	MS media + BA (5 mg/L) + IAA (1 mg/L) + kanamycin (50 mg/L) + kanamycin (50 mg/L) + cefotaxime (200 mg/L	MS media + IBA (0.5 mg/L) + kanamycin (50 mg/L)	PCR, southern blot analysis, Western blotting	---
Kumar *et al*., 2012	ACA-10, Kashi Anmol, LCA-235, PBC-535, Pusa Jwala and Supper	Hypocotyls, cotyledonary leaves and leaf discs	pBinARβC1, strain EHA-105	MS media + BAP (1-4 mg/L)	MS media + 2 mg/L BAP + 100 µM Acetosyringone For 48 hr	MS media + 1 mg/L GA_3 + kanamycin + cefotaxime (500 mg/L)	MS media + 0.5 mg/L NAA	Kanamycin PCR and Southern hybridization analysis.	0.12 per cent
Verma *et al*., 2013a	*Capsicum annuum* L. cv. California Wonder	Hypocotyl, cotyledon	pBI121, strain LBA-4404	MS media + 6 mg/L BAP + 0.3 mg/L IAA	For 72 hr	MS media + 6 mg/L BA + 0.3 mg/L IAA + 500 mg/L cefotaxime + 50 mg/L kanamycin	Half MS media + 0.25 mg/L IAA +500 mg/L cefotaxime + 50 mg/L kanamycin	PCR, GUS assay.	---
Maligeppagol *et al*., 2016	G4 and LCA334	Cotyledonary leaf, hypocotyls	pCAMBIA 2301, strain LBA-4404	MS media + 0.25 mg/L zeatin + 2 mg/L PAA	MS media + 3 g/L gelrite + 0.25 mg/L zeatin + 2 mg/L PAA	MS media +3 g/L gelrite + 0.25 mg/L zeatin + 2 mg/L PAA + 100 mg/L kanamycin + 300 mg/L augumentin	Half MS media + 1.5 g /L gelrite + 0.5 mg/L IBA + 300 mg/L augumentin	PCR and Southern blotting	---

Table 1: Contd...

Scientists and year	Crop (chilli), Variety	Explant	Vector, *Agrobacterium* strain	Regeneration media	Co-cultivation media	Shoot induction media	Root induction media	Confirmation analysis	Efficiency
Bhutia *et al*., 2016	Dhalle Khursani	Cotyledon, shoot tip, hypocotyl	---	MS media + 4 mg/L TDZ	--	MS media + 2 mg/L GA_3 + 0.5 mg/L IAA (shoot elongation media)	MS media + 2 mg/L GA_3 + 0.5 mg/L IAA	---	----
Hegde *et al*., 2017	Bharat and Indra	Cotyledonary leaves	---	MS media + 7.5 mg/L kinetin or 5 mg/L TDZ or 10 mg/L BAP with 2 mg/L GA_3	--	Half-strength MS media + 0.5 mg/L IBA.	Half-strength MS media + 0.5 mg/L IBA.	---	---
Mahto *et al*., 2018	Pusa Sadabahar and Pusa Jwala	Hypocotyl and cotyledon explants	pCAMBIA2301, strain LBA4404	MS media + 5 mg/L BAP + 1 mg/L IAA	MS media + 5 mg/L BAP + 1 mg/L IAA For 72 hr	MS media+ 5 mg/L BAP + 1 mg/L IAA+ kanamycin (30 mg/L) + augmentin (300 mg/L)	Half-strength MS + IAA (1mg/L) + Augmentin (300 mg/L)	GUS, PCR, Southern and RT-PCR analysis	---

Abbreviations:
IAA: Indole 3-acetic acid
IBA: Indole 3-butyric acid
BA or BAP: Benzyl amino purine
TDZ: Thidiazuron
PAA: Phenylacetic acid
NAA: Naphthaleneacetic acid
PCR: Polymerase Chain Reaction
GUS: Beta-glucuronidase
GA_3: Gibberellic acid

3. MATERIAL AND METHODS

Ten guide RNAs were designed to target key genomic regions of the *Chilli leaf curl virus*. The guide RNA targeting the promoter region within the intergenic region (IGR) was selected in the previous study to construct a CRISPR/Cas9 vector, which was tested using agroinfiltration and transient expression of Cas9 and sgRNA. The goal of this study was to use the same CRISPR/Cas9 construct to create transgenic chilli plants for further evaluation of its resistance to the *Chilli leaf curl virus* and to confirm the integration of CRISPR/Cas9 construct introduced into the chilli genome through PCR.

The present study was aimed at the transformation of chilli with the CRISPR/Cas9 construct targeting the promoter region of the *Rep* gene of the *Chilli leaf curl virus* and confirmation of putative transformants. The research work was carried out at the Department of Biotechnology, College of Agriculture, Dharwad, and University of Agricultural Sciences, Dharwad during 2019-2021.

3.1 Materials

3.1.1 Plant material

Byadgi kaddi (*Capsicum annuum* L.), a popular cultivar in Dharwad used for this study, was grown and maintained in a greenhouse in pots at the Department of Biotechnology, College of Agriculture, Dharwad, UAS, Dharwad.

3.1.2 Sources of explants

Cotyledonary nodal segments (6-8 mm long) of the plantlets of *C. annuum* L. cv. were excised and used as explants.

3.1.3 Plant growth regulators

The following plant growth regulators were used in different concentrations in different media prepared and used in the studies.

Auxins : Indole Acetic Acid (IAA)
: Naphthalene Acetic Acid (NAA)
: Zeatin

Cytokinins : Benzyl aminopurine (BAP)
: Thidiazuron (TDZ)

: Kinetin (KIN)

Gibberellins : Gibberellic acid (GA_3)

3.1.4 Medium

In this experiment, Murashige and Skoog (1962) basal media supplemented with different plant growth regulators (PGRs). The media and its composition are given in Table 2.

Table 2: Concentrations and combinations of plant nutrients used in this study

Constituents	Components	Medium composition (mg/L)
Macronutrients	NH_4NO_3	1650.00
	KNO_3	1900.00
	$MgSO_4.7H_2O$	370.00
	KH_2PO_4	170.00
Micronutrients I	$MnSO_4.4H_2O$	22.30
	$ZnSO_4.7H_2O$	8.60
	H_3BO_3	6.03
	KI	0.83
	Na_2MoO_4	0.25
Micronutrients II	$CuSO_4.5H_2O$	0.025
	$CoCl_2.6H_2O$	0.025
Fe.EDTA	Na_2.EDTA	60.00
	$FeSO_4.7H_2O$	27.80
$CaCl_2$	$CaCl_2$	440.0
Organics/vitamins	Thiamine HCl	10.00
	Nicotinic acid	1.00
	Pyridoxine HCl	1.00
	Biotin	0.50
	Glycine	10.00

Ready-made MS packets from Sigma-Aldrich contain all of the aforementioned chemicals and were used to prepare MS media. The media was referred to as "full-strength MS media" if a full MS packet powder was mixed in 1000 ml of distilled water. However, it was referred to as "half-strength MS media" if half of the packet of MS was dissolved in 1000 ml of distilled water.

3.2 Methodology

3.2.1 Surface sterilization

After soaking for a day in autoclaved distilled water, the seeds were surface sterilised with 0.1 percent mercuric chloride ($HgCl_2$) for three minutes with continuous swirling before being rinsed three times with sterile distilled water. The duration of surface sterilisation was important. If it was too short, the culture would easily get contaminated. If it was too long, the immature zygotic embryo explant would die. Sterilized seeds were then sown on half-strength MS basal media without any growth regulators for germination. After 21 days of germination, the cotyledonary nodal region was excised from the seedlings and used for culturing.

3.2.2 Physical factors

The culture room was maintained at 25 ± 2 ^{o}C with uniform light (1000 lux) provided by fluorescent tubes (7200 ^{o}K) over a light/dark cycle of 16/8 hours. The *in-vitro* culture studies were carried out aseptically in the laminar airflow. Glass jar bottles, Petri plates, and molecular biology grade chemicals were used.

3.2.3 Preparation of media

A full-strength MS basal culture media containing macronutrients, micronutrients, and vitamins, Fe.EDTA, as well as $CaCl_2$, were used in this study. Table 2 shows the different concentrations and combinations of chemicals that were added to the basal media. Half-strength MS media containing calcium chloride at 440 mg/L and sucrose at 30 g/L was prepared as germination media. The pH was adjusted to 5.6-5.8 by using HCl or NaOH. After adjusting the pH, agar (8 g/L) was added and made up the volume to one litre with distilled water, autoclaved at 121°C and 15 psi for 1 hour and 15 min. About 15 ml of media was poured into each glass jar bottle. The heat-sensitive compounds like growth regulators, if required, are added once after sterilisation and cooling of the media.

3.3 Transformation protocol in chilli

In the present study, *Agrobacterium*-mediated *in-vitro* plant transformation and *in-planta* transformation methodology was used for transfer of CRISPR/Cas9 construct.

3.3.1 *Agrobacterium* strain and plasmid vector

The disarmed *Agrobacterium* strain EHA-105 was used during the study. Electroporation was used to insert the CRISPR/Cas9 construct into the *Agrobacterium*, which was confirmed by agro-inoculation of the chilli plant. RNA was isolated from agro-inoculated leaves, and RT-PCR confirmation of the construct was done uing cDNA.

The CRISPR/Cas9 (p54) construct with a 20 nt guide RNA sequence (5'-AATTCAAATTCCGACAAAAG-3') was custom synthesised by Sigma Aldrich, USA and was obtained from IIHR, Bangalore under a material transfer agreement. Genomic region from the intergenic region (common region) of the *Chilli leaf curl virus*, was selected for designing guide RNAs. Since Cas9 protein was used in the present study, a PAM sequence of 5' NGG 3' (AGG) was considered in the virus genome.

The p54 plasmid (CRISPR/Cas9 construct) used contained three cassettes within the T-DNA region (Fig. 1).

1. The neomycin phosphotransferase (*npt* II) gene, which is a Kanamycin resistance gene acting as a selectable marker and governed by the CaMV-35S promoter
2. sgRNA composed of two parts: a target binding region and a sccafold RNA with the *AtU6* promoter.
3. *At*Cas9 (*Streptococcus pyogenes* Cas9 gene codon optimised for expression in *Arabidopsis thaliana,* a dicot host) had a 35S promoter and T7 promoter for *in-vitro* transcription.
4. The pVS Ori is the origin of the replication of *Agrobacterium* and the pUC Ori is the origin of the replication of *E. coli*. For choosing transformed *Agrobacterium*, a kanamycin resistance gene outside the T-DNA region is used, and another kanamycin resistance gene, *npt* II is used as a selection pressure for selecting putatively transformed plants.

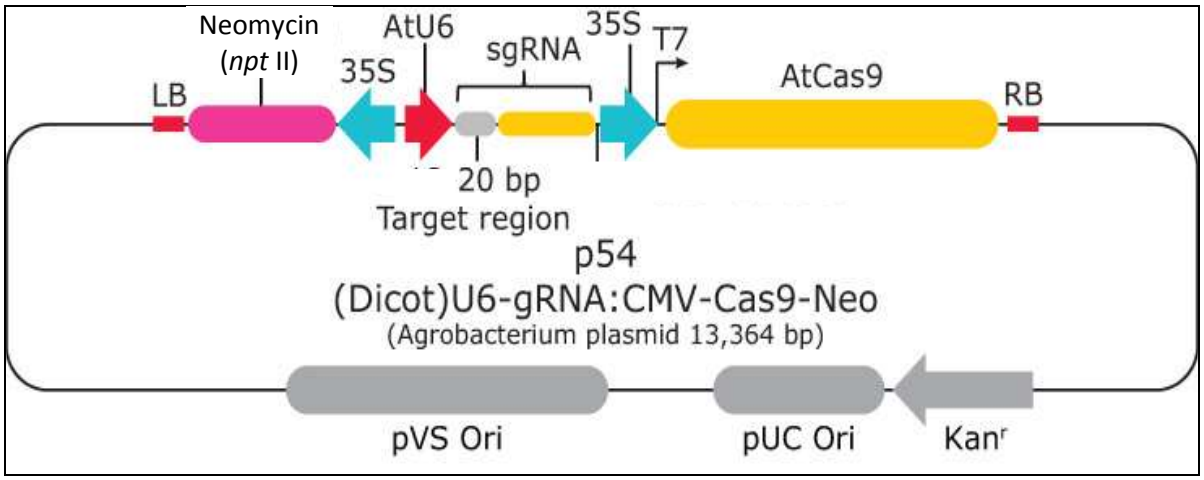


Figure 1: CRISPR/Cas9 construct harbouring Cas9 gene, sgRNA gene, selection marker gene (*npt* II gene), origin of replication pVS ori (*Agrobacterium* ori), pUC ori (*E. coli* ori) and kanamycin resistance gene

3.3.2 Maintenance and growing of *Agrobacterium*

The *Agrobacterium* strain EHA-105 containing the CRISPR/Cas9 construct was maintained on Yeast Extract Mannitol Agar (YEMA) media containing 100 mg/L kanamycin. Subculturing was done on fresh media. For transformation analysis studies, a single *Agrobacterium* colony was selected and inoculated in 100 ml of Yeast Extract Mannitol Broth (YEMB) containing a selective antibiotic (kanamycin) and was incubated on a rotatory shaker for 48 hrs at 28 °C. *In-vitro* transformation was selected for a culture reading of 0.8 O.D. at 600 nm.

3.3.3 *In-vitro* transformation

3.3.3.1 Plant material

In the present study, the cultivar Byadgi kaddi was selected for *in-vitro* transformation studies. The cotyledonary nodal region of the shoot was used as an explant source.

3.3.3.2 Optimisation of plant regeneration protocol for the chilli c.v. Byadgi kaddi

Table 3: The plant regeneration protocols of chilli followed in different cultivars.

Scientists	Cultivar	Shoot induction	Shoot elongation and Rooting
Mathew, 2002	Byadgi Dabbi, Arka Lohit	MS + 3 mg/L BAP + 2 mg/L kinetin	MS + 0.5 mg/L IBA
Hailu *et al.,* 2015	Mareko Fana	a)MS + 4.5 mg/L BAP + 0.5 mg/L zeatin b)MS + 8 mg/L zeatin	MS + 0.5 mg/L IBA
Bhutia *et al.,* 2016	Dalle Khursani	a)MS + 8 mg/L BAP b)MS + 4-6 mg/L TDZ	MS + 2 mg/L GA_3+ 0.5 mg/L IAA

3.3.3.3 Methodology

Direct regeneration: Cotyledonary nodal region explants were excised from the germinated seedlings.

Pre-culture: The explants were pre-cultured for 2 days on media: MS + 8 mg/L zeatin

Co-cultivation: The pre-cultured explants were co-cultivated on co-cultivation media (discussed in brief in the latter part of the thesis).

Shooting: The shooting media used was MS media + 8 mg/L zeatin.

Shoot elongation media: The shoot elongation media used was MS media + 7 mg/L zeatin+ 2 mg/L GA_3+ 50 mg/ L kanamycin were used as shoot selection media.

Rooting media: The rooting media used was MS media + 2 mg/L NAA

Rooted shoots were hardened in peat: soil (1:1) mixture for establishment.

3.3.3.4 Acclimatization and hardened plantlets

Rooted shoots were carefully removed from the medium and washed thoroughly with sterile distilled water and planted in a plastic cup containing autoclaved soil: manure (1:1).

3.3.3.5 Co-cultivation of explants with *Agrobacterium*

The cotyledonary nodal explants obtained from 15-21 days old *in vitro* grown seedlings were pre-cultured for 2 days. Later, explants were transferred on to co-cultivation media (MS media + 8 mg/L zeatin) from pre-culture media.

The overnight grown *Agrobacterium* culture was centrifuged at 4930 x g for 10 minutes at 22 °C, the supernatant was discarded and the bacterial pellet was suspended in MS media. Acetosyringone (100 µM) was added to *Agrobacterium* suspension cells and left for at least two hours for *vir* gene induction. A drop of (10 µl) *Agrobacterium* suspension cells (containing acetosyringone @ 100 µM) was pipetted at wounded cotyledonary nodal meristematic region of explant without touching the media.

After two days of co-cultivation, explants were placed on shoot induction media of MS media with 8 mg/L zeatin.

3.3.4 *In-planta* transformation method

In the present study, Kalbande and Anita's (2016) protocol for the *in-planta* method of plant transformation was followed. The four weeks old (two to four-leaf stage) chilli seedlings were selected for transformation. The cotyledonary nodal region of seedling were excised with a 'V'-shaped cut, removing the meristematic cells and a drop of *Agrobacterium* suspension cells (containing acetosyringone @ 100 µM) was inoculated with a sterile pipette tip. The inoculated plant were then kept in a tissue culture room (dark) for 2 days for co-

cultivation and later transferred to the greenhouse for further growth of seedlings into transformed plants.

3.4 Confirmation of transformants

After the establishment of the plant, confirmation of the transgene (CRISPR/Cas9) gene introduced into treated plants of both *in-planta* and tissue culture-dependent transformed plants was done using PCR.

The isolation of genomic DNA was conducted using the CTAB method

1. Young, healthy leaves of chilli plants (90 to 100 mg) were collected from the pots in 2 ml Eppendorf tubes.
2. The extraction buffer was preheated to 65 ^{o}C by keeping it in the water bath.
3. Samples were crushed into a fine powder in pestle and mortar using liquid nitrogen.
4. Crushed samples were then put into 2 mL Eppendorf tubes. 1 mL of extraction buffer was added to it and mixed properly by inverting the tubes.
5. Samples were incubated for at least 30 to 45 minutes in a 65 ^{o}C water bath and intermittently mixed after every 5 to 10 minutes.
6. The samples were centrifuged at 10,000 rpm for 10 min and the supernatant was later transferred into another 2 mL Eppendorf tube.
7. An equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) solution was added. The solution was mixed gently by inverting the tubes till a milky white emulsion was formed.
8. The tubes were centrifuged at 10,000 rpm for 15 minutes and the aqueous phase was transferred into a new tube.
9. The aqueous phase was transferred to a fresh 1.5 ml tube and an equal volume of chilled isopropanol was added.
10. The sample was incubated at -20 ^{o}C for 30 minutes.
11. The samples were then centrifuged at 10,000 rpm for 10 min at 4 ^{o}C and the aqueous phase was discarded.
12. The pellet was then washed in 100 µl of 70 per cent ethanol by centrifuging at 10,000 rpm for 2 min.

13. The pellet was dried at 37 ^{o}C for 20 min till the ethanol evaporated completely.
14. The pellet was again resuspended in 50 µl of $T_{10}E_1$ buffer.
15. The 3 µl RNase A was added (10 µg/ml) to the DNA and incubated at 37 ^{o}C in a water bath for half an hour and at 37 ^{o}C for 15 minutes and stored at -20 ^{o}C.

3.4.1 PCR amplification

The genomic DNA of plants obtained from putative transformed plants and control plants were used as a template for PCR confirmation of the targeted CRISPR/Cas9 construct specific regions. Gene-specific primers were used since this is a part of CRISPR/Cas9 gene cassette within T-DNA region.

Components	**Amount added (µl)**
DNA template	1.0 (100 ng)
Forward primer	0.5 (5 pmol/µl)
Reverse primer	0.5 (5 pmol/ µl)
Takara Master mix (2X)	5.0
Nuclease free water	3.0
Total	**10.0 µl**

3.4.2 Polymerase chain reaction conditions

Based on the annealing temperatures of the Cas9 forward and reverse primers, reactions were set up for the amplification of the Cas9 containing CRISPR/Cas9 vector using the following profile:

Steps	**Temperature (^{o}C)**	**Time (min)**
Initial denaturation	94	5
Denaturation	94	1
Annealing	54	1
Extension	72	1.30
Final extension	72	10
Hold	22	∞

30 cycles

Based on the annealing temperature of the *At*U6-1 promoter forward and scaffold reverse primer, a separate PCR reaction was carried out to amplify the IGR-sgRNA containing region of the CRISPR/Cas9 vector $p54^{-IGR}$. After completion, the samples were stored at 4 ^{o}C and their contents were loaded onto the agarose gel for electrophoresis.

PCR analysis for detection of *Agrobacterium*

The primers specific to the Vir D_2 region of *Agrobacterium* were used in the present study for the detection of *Agrobacterium* (Table 5).

3.4.3 Gel electrophoresis of PCR amplicons on 2.0 per cent agarose gel

Two grams of agarose was weighed and mixed in 98 ml double distilled water with 2 ml of 50X TAE buffer. It was melted in the microwave for 1-3 min until the agarose was completely dissolved. The agarose solution was cooled down to about 50 ^{o}C. The ethidium bromide (EtBr) was added to a final concentration of 0.5 µg/ml.

The newly poured gel was left to set at room temperature for 20-30 minutes until it was completely solidified.

3.4.4 Loading samples and resolving in an agarose gel

The loading buffer was added to each of the DNA samples. Once solidified, the agarose gel was placed into the electrophoresis unit. The gel box was filled with 1X TAE buffer until the gel was covered. Molecular weight DNA ladder was loaded into the first lane of the gel and, the samples were loaded into the additional wells of the gel. The gel was run at 65 V until the dye line was approximately 75-80 per cent of the way down the gel. The power was turned off, the electrodes were disconnected from the power source, and then the gel box was carefully removed from the gel. The DNA fragments were visualised under the Gel Documentation unit/UV transilluminator. Those plant samples which showed amplification with the desired gene size were considered as putative transformants.

Table 4: Primers used in the confirmation of the CRISPR vector p54^{-IGR} confirmation

Primer	Start	End	Length	Tm (°C)	Seq 5' to 3'	Expected amplicon size excluding primer
Cas9-F	9711	9730	20	60	TGTTCATCCAGCTCGTGCAG	445 bp
Cas9-R	10175	10,197	23	60	TTCTCTGGGAGCTGCTGTCTAAC	
***AtU*6—1-F**	7916	7937	22	60	ATCCGTAGAAACGAGACGGTCA	335 bp
Scaffold-R	8272	8289	18	62	GCACCGACTCGGTGCCAC	

Table 5: Primers used for detection of *Agrobacterium*

Primer	Start	End	Length	Tm (°C)	Seq 5' to 3'	Expected amplicon size
VirD$_2$-F	1	20	20	48	ATGCCCGATCGAGCTCAAGT	338 bp
VirD$_2$-R	338	312	26	50	CCTGACCCAAACATCTCGGCTGCCCA	

4. EXPERIMENTAL RESULTS

There are several plant transformation methods available, the most well-known among them is the *Agrobacterium*-mediated plant transformation. Other methods, such as floral injection, plumule-stage seedling infection, and seed imbibing culture, were tried in the present study, but they did not produce satisfactory results, and their transformation efficiency was found to be very low.

4.1 Optimisation of *in vitro* direct regeneration protocol

The five different media compositions were checked for chilli explant regeneration using cv. Byadgi kaddi. Among them, MS + 8 mg/L zeatin has worked best with multiple (6 Numbers) shoots. The second best working media was MS + 6 mg/L TDZ, where 4 shoots were found (Plate 1).

Six shoots/explant emerged from MS + 8 mg/L zeatin, four shoots/explant emerged from MS + 6 mg/L TDZ, three shoots/explant emerged from MS + 3 mg/L BAP + 2 mg/L kinetin, one shoot/explant emerged from MS + 3 mg/L BAP + 2 mg/L kinetin, and one shoot/explant emerged from MS + 4.5 mg/L BAP + 0.5 mg/L IAA (Fig. 2).

The experiment was repeated two times, and similar results were found with MS + 8 mg/L zeatin showing the highest multiple shoots among all the combinations used.

4.2 Genetic transformation

4.2.1 *Agrobacterium*-mediated tissue culture dependent transformation

The CRISPR/Cas9 (p54) construct (Fig. 1) was custom synthesised (Sigma-Aldrich, USA) with a 20-nt guide RNA sequence (5'-AATTCAAATTCCGACAAAAG-3') targeting the promoter region of ChLCV located in the inter-genic region. sgRNA complementarity base pairs to a specific 20-nucleotide (nt) DNA sequence and the Cas9 protein cleaves the virus genome at a few nucleotides upstream of the PAM sequence (AGG). The neomycin phosphotransferase (*npt* II) gene, which is a Kanamycin resistance gene governed by the CaMV-35S promoter and located within the T-DNA region, acts as a selection marker for selecting transformed plants, while the other Kanamycin resistance gene, which is located outside the T-DNA region, acts as a selection marker gene for selecting transformed *Agrobacterium* harbouring the CRISPR construct (Fig. 1).

The same construct in *Agrobacterium* was employed for both tissue culture dependent and the *in-planta* transformation studies.

4.2.1.1 Chilli seed germination on MS media:

The cotyledonary nodal region (explant) of the Byadgi kaddi chilli cultivar was taken from a 21-day old *in-vitro* seedling grown aseptically, as shown in Plate 2(a). Among the 40 seeds sown, 25 germinated.

4.2.1.2 Pre-induction media:

MS media supplemented with 8 mg/L zeatin was chosen as the pre-induction medium. Twenty-one explants from 25 seedlings were pre-cultured. Pre-culture duration was for 2 days [Plate 2(b)].

4.2.1.3 Co-cultivation media:

MS media containing 8 mg/L zeatin was chosen as the co-cultivation media. All of the twenty-one explants survived and were transferred from pre-culture to co-cultivation media after infecting them with *Agrobacterium* at nodal region. Co-cultivation duration was for two days [Plate 2(c)].

4.2.1.4 Adventitious shoot induction/multiple shoot induction

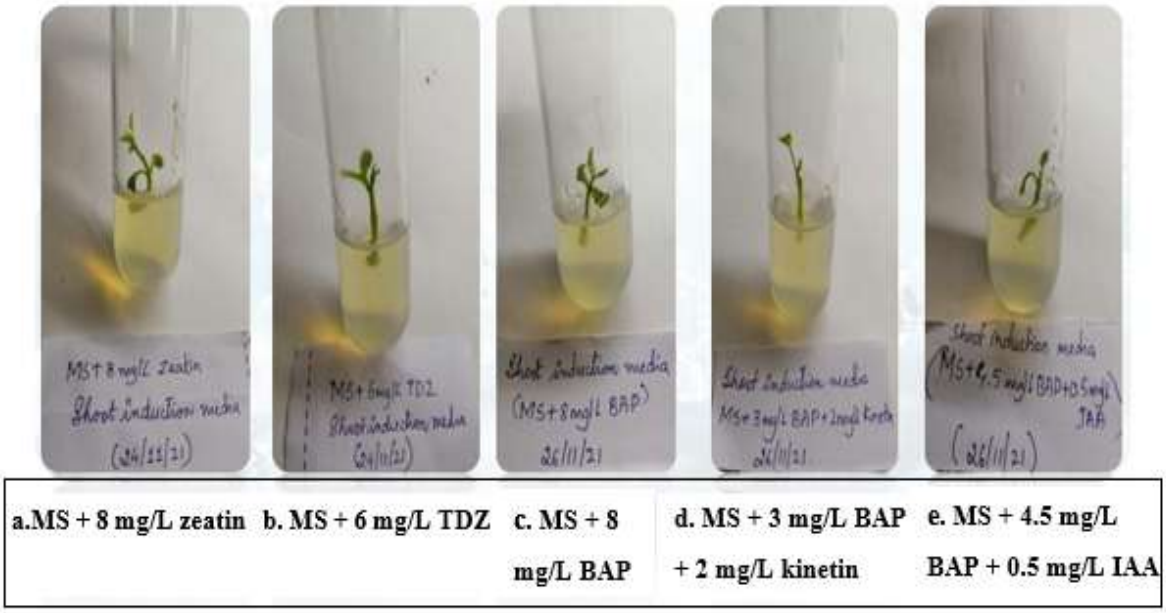
Cotyledonary nodal region explants showed multiple shoot induction (six shoots/explant) on zeatin at 8 mg/L. After two days of co-cultivation in dark conditions, twenty-one explants were placed on shoot induction media containing MS with 8 mg/L zeatin under light [Plate 2(d)].

4.2.1.5 Elongation of shoots:

All twenty-one explants were placed on MS media with 8 mg/L zeatin, 2 mg/L GA_3 and 50 mg/L kanamycin for selective shoot elongation. Ten explants survived out of twenty-one [Plate 2(e)].

4.2.1.6 Rooting and hardening of regenerants:

Roots were induced in the media on half-strength MS with 2 mg/L NAA. Due to the small number (four) of plants and the leaf drop at this stage, the experiment was not continued [Plate 2(f)].



a.MS + 8 mg/L zeatin b. MS + 6 mg/L TDZ c. MS + 8 mg/L BAP d. MS + 3 mg/L BAP + 2 mg/L kinetin e. MS + 4.5 mg/L BAP + 0.5 mg/L IAA

Plate 1 : Diagram showing cotyledonary explants on different shoot induction media

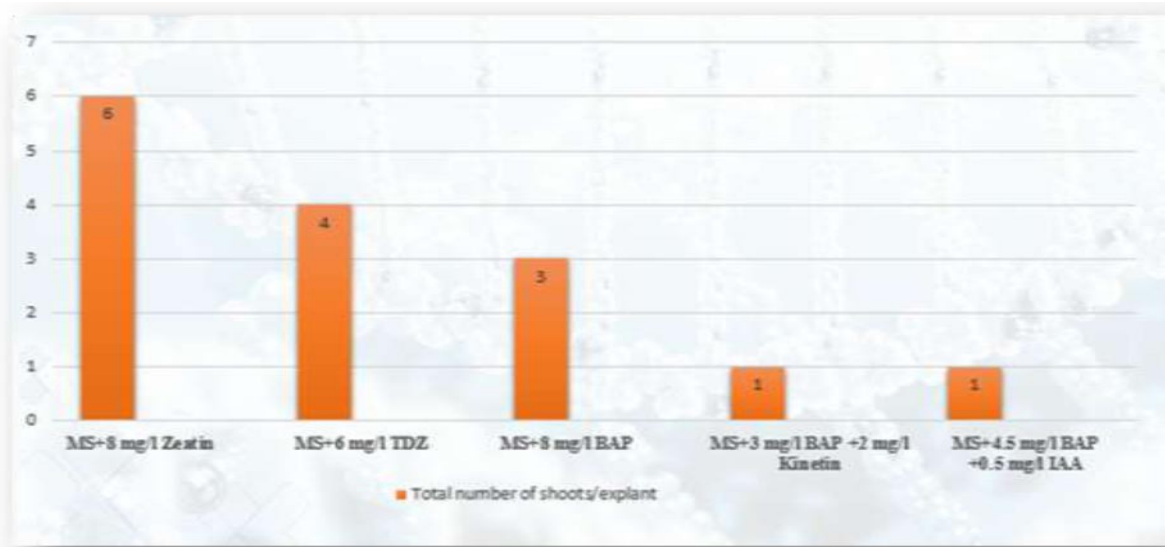


Fig. 2: Effect of different MS media combinations on direct *in vitro* shoot regeneration from cotyledonary explant

Plate 2(a): Seed germination on half MS media

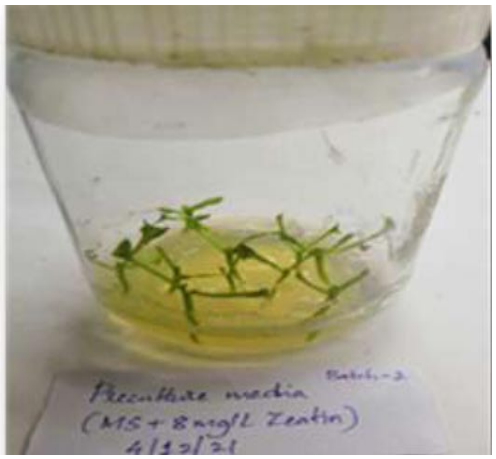


Plate 2(b): Explants on preculture media for 2 days

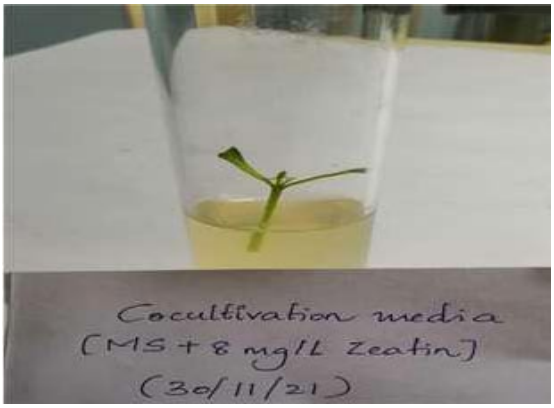


Plate 2(c): Explants on co-cultivation media for 2 days

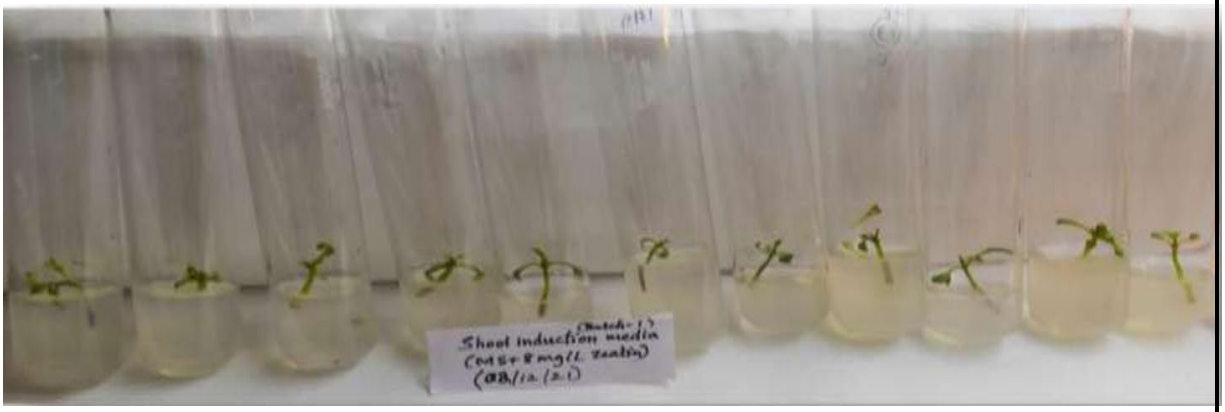


Plate 2(d): Explants on shoot induction media for 3 weeks

Plate 2: *Agrobacterium* mediated plant transformation methodology (tissue culture dependent)

Plate 2(e): Explants on shoot elongation media for 3 weeks

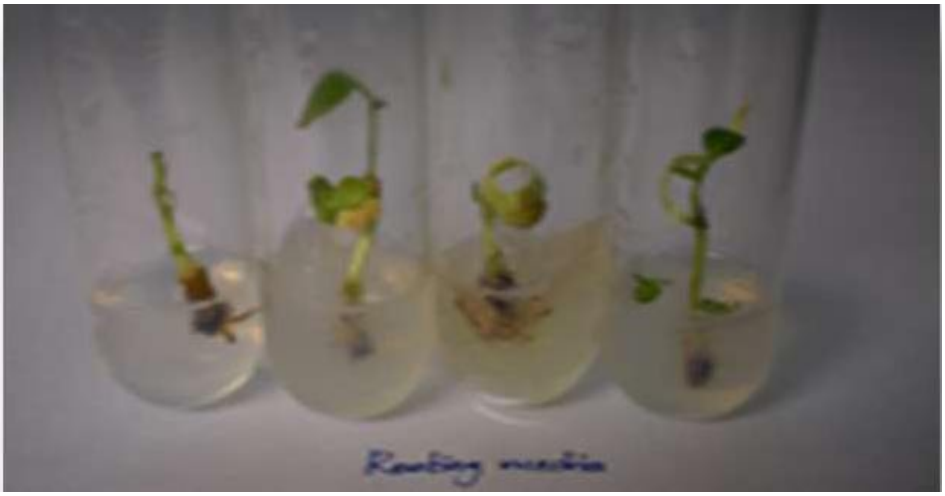


Plate 2(f): Explants on rooting media for 3 weeks

Plate 2: (Contd…..) *Agrobacterium* mediated plant transformation methodology (tissue culture dependent)

The experiment was repeated three times for a total of three batches.

During the co-cultivation stage, when the whole explant was dipped in an *Agrobacterium* suspension culture for fifteen minutes, *Agrobacterium* (EHA-105) proved difficult to get rid off, even after repeated washings with cefotaxime (400 mg/L). The two batches of experiments with twenty explants each, in the process were lost due to excessive *Agrobacterium* growth on the explants cultured. As a result, a pippete was used to inoculate a drop of *Agrobacterium* suspension culture in the cotyledonary nodal region of the explant. Explants were placed on co-cultivation media and were inoculated with *Agrobacterium* culture. Another challenge for getting transgenic plants through the tissue-culture process is contamination, which is observed during each step of the transformation process. It is labour-intensive approach.

4.2.2 *In-planta* transformation methodology

In-planta transformation was found to be less time-consuming and less tedious than tissue-cultue-based transformation of chilli. Kalbande and Anita (2016) employed a transformation technique on cotton to achieve *in-planta* transformation in cotton. The same protocol was followed for *in-planta* transformation of chilli in the present study. This *in-planta* transformation protocol proved to be genotype-independent.

4.2.2.1 Preparation of seedlings

Chilli seeds of Byadgi kaddi cultivar were sown in germination potrays. To obtain 100 seedlings, 168 seeds were sown because the germination percentage was found to be 60 per cent. Chilli seeds germinated and set their two-leaf stage of growth after two weeks. Seedlings were transferred into cups and hardened for 6 days [Plate 3(a)].

4.2.2.2 *Agrobacterium* inoculation of seedlings

Seedlings with two leaves were chosen. In the meristematic region, a ‘V’-shaped cut was made and *Agrobacterium* was inoculated into 100 seedlings (kept for 2 days in the dark). After inoculation, the seedlings were covered in plastic to retain the moisture within the cups (kept in light, it took 1 month to set its new growth). New leaves emerged from the *Agrobacterium*-inoculated cotyledonary nodal region [Plate 3(b)]. After inoculation with *Agrobacterium*, 40 seedlings survived out of 100.

4.2.2.3 Hardening of putative transformants

The putative transformants were kept in the shade for a month after the emergence of new leaves for hardening before transferring to the greenhouse. After 4 months, fully grown *in-planta* plants were obtained [Plate 3(c)]. Of the 40 seedlings that survived after inoculation, 23 survived after hardening.

4.2.2.4 Flowering and fruiting in *in-planta* plants

Fully grown *in-planta* plants set their flowers and fruits after 4 months of their growth [Plate 3(d), (e)]. The chilli c.v. Byadgi kaddi is an alternate bearing, with 5-15 fruits per plant in each picking. Pickings were done thrice, with a two-week interval in between. Each fruit had 60-150 seeds.

4.2.2.5 T_1 generation plants

Seeds from T_0 generation plants were sown to produce T_1 generation plants [Plate 3(f)]. Twenty seeds from each of the 74th, 76th, 78th, and 48th T_0 plants were chosen at random and sown. The seeds from the 48th plant did not germinate. A total of twenty-two seedlings were obtained. DNA was extracted from leaves, and were analysed using PCR.

4.3 Analysis and confirmation of *in-planta* putative transformants

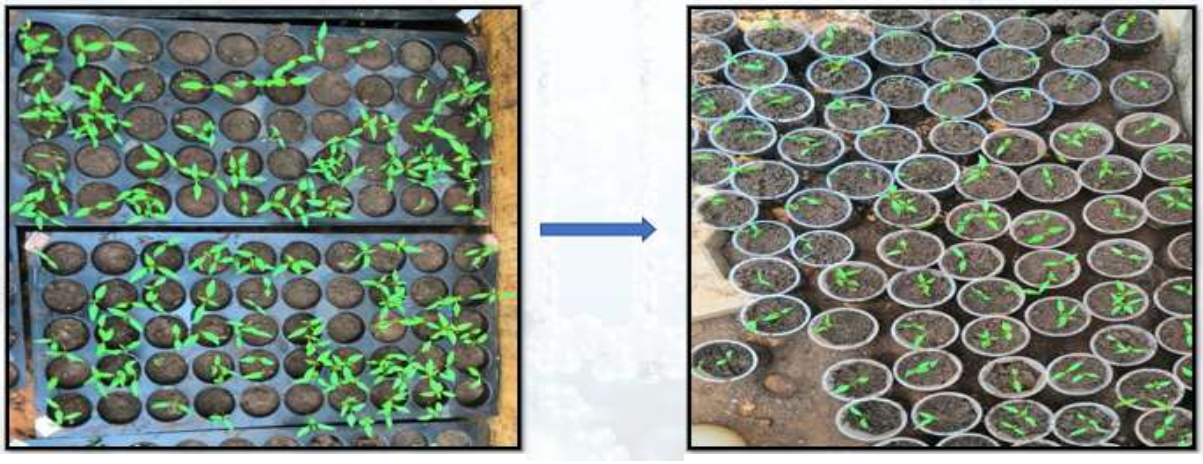
4.3.1 Confirmation of CRISPR/Cas9 construct in T_0 generation chilli plants from upper leaves

DNA was extracted from the topmost leaves of twenty-three transformed *in-planta* plants and were subjected to PCR for both Cas9 and sgRNA, in which twenty plants were PCR positive and three plants were PCR negative for Cas9 (Plate 4) and sgRNA (Plate 5).

4.3.2 Confirmation of CRISPR/Cas9 construct in T_0 generation chilli plants from lower leaves

To find out the chimeras in the T_0 generation DNA was extracted from the lowest leaf of the same plants and were subjected to PCR for both Cas9 and sgRNA, in which twenty plants were PCR positive and three plants were PCR negative for Cas9 (Plate 6) and sgRNA (Plate 7).

It was found that the plant samples 1, 12, 45, 52, and 75 were chimeras. The 46^{th} was PCR negative plant (non-transformed plant). A total of twenty-two plants were PCR-positive.



Chilli seedlings were germinated in portrays, which took 2 weeks to

Seedlings were transferred to cups (kept for 6 days)

Plate 3(a): Preparation of seedlings

A V-shaped cut was made in the meristematic region and inoculated with *Agrobacterium* (Kept for 2 days in dark).

Seedlings were covered in plastic, after inoculation to retain moisture within the cups (Keeping in light, which took 1 month to set its new growth).

New leaves emerged from inoculated cotyledonary nodal region with *Agrobacterium*

Plate 3(b): *Agrobacterium* inoculation of seedlings

Kept in shade for hardening for a month

Fully grown *in-planta* plants obtained after 4 months

Plate 3(c): Hardening of putative transformants

Plate 3*: In-planta* transformation methodology

Plate 3(d): Flowering and fruiting in *in-planta* plants

Plate 3(e): Fruits from T_0 generation plants

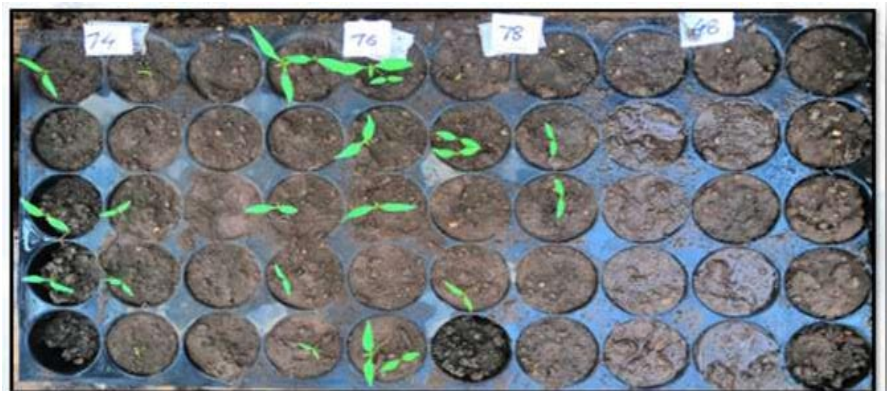
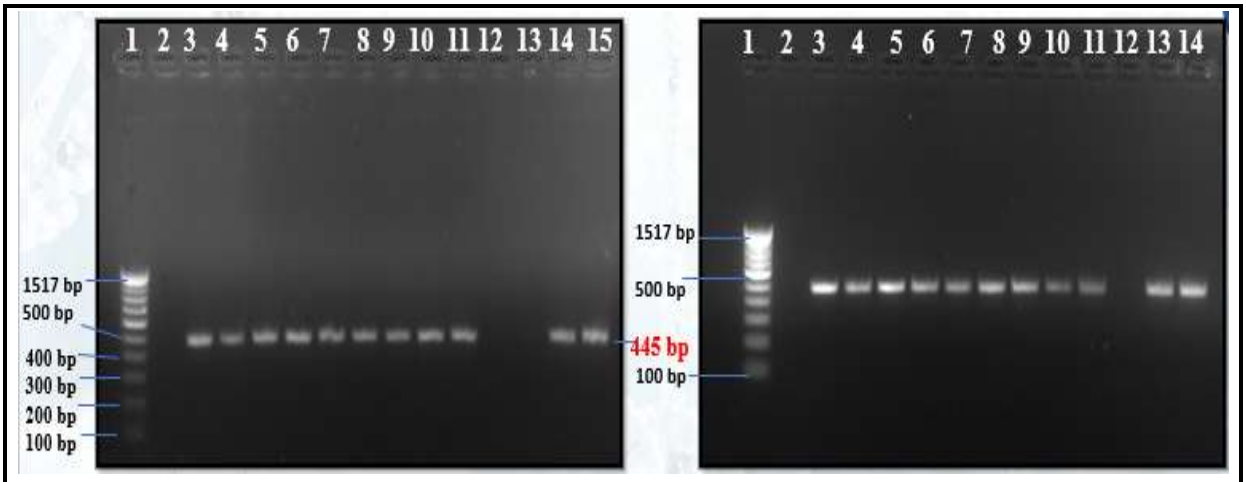


Plate 3(f): T_1 generation plants

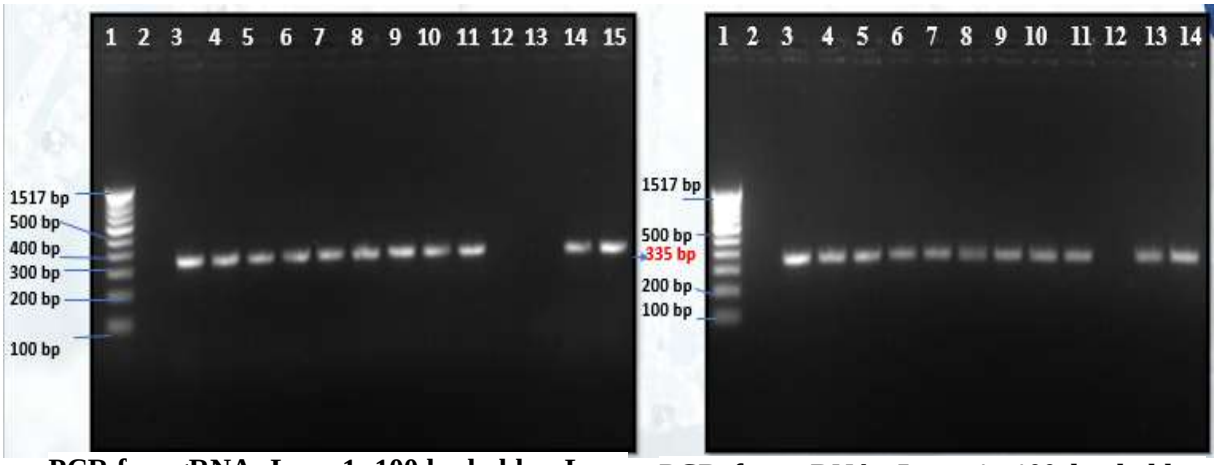
Plate 3*:* (Contd…..) *In-planta* transformation methodology



PCR for Cas9: Lane 1: 100 bp ladder, Lane 2 : Negative control of chilli, Lane 3: Colony PCR product of *Agrobacterium*, Lane 4-15: PCR product of transformed chilli plant samples (78, 80, 76, 75, 49, 48, 55, 53, 1, 46, 85, 77 respectively)

PCR for Cas9: Lane 1: 100 bp ladder, Lane 2: Negative control of chilli, Lane 3: Colony PCR product of *Agrobacterium*, Lane 4-14: PCR product of transformed chilli plant samples (2, 8, 12, 41, 42, 45, 46, 47, 52, 69, 74 respectively)

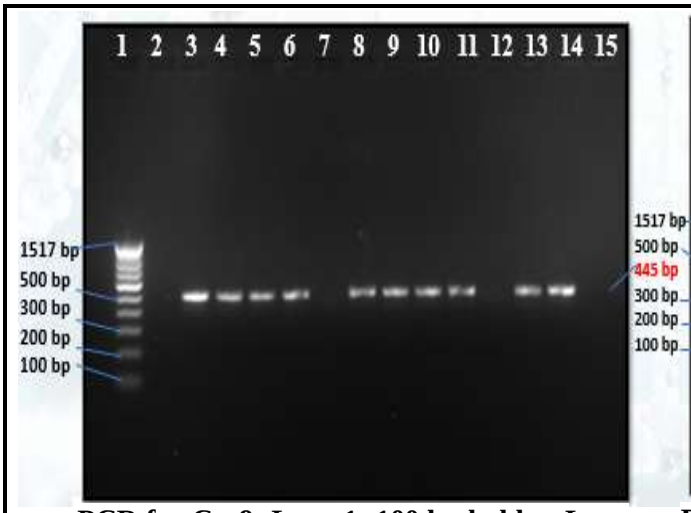
Plate 4: PCR for Cas9 from upper leaves of T_0 generation chilli plants



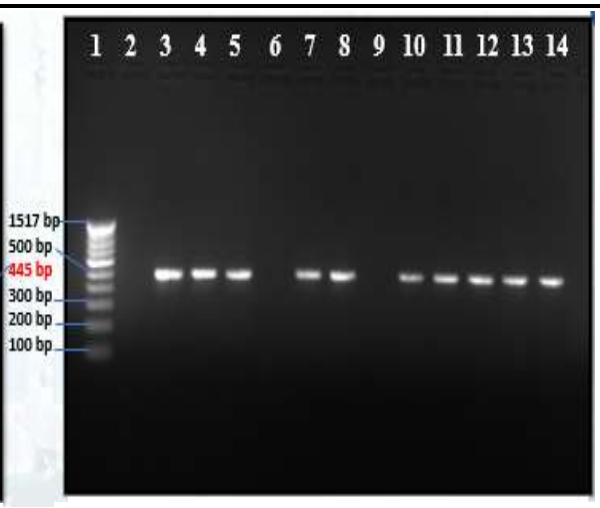
PCR for sgRNA: Lane 1: 100 bp ladder, Lane 2: Negative control of chilli, Lane 3: Colony PCR product of *Agrobacterium*, Lane 4-15: PCR product of transformed chilli plant samples (78, 80, 76, 75, 49, 48, 55, 53, 1, 46, 85, 77 respectively)

PCR for sgRNA: Lane 1: 100 bp ladder, Lane 2: Negative control of chilli, Lane 3: Colony PCR product of *Agrobacterium*, Lane 4-14: PCR product of transformed chilli plant samples (2, 8, 12, 41, 42, 45, 46, 47, 52, 69, 74 respectively)

Plate 5: PCR for sgRNA from upper leaves of T_0 generation chilli plants



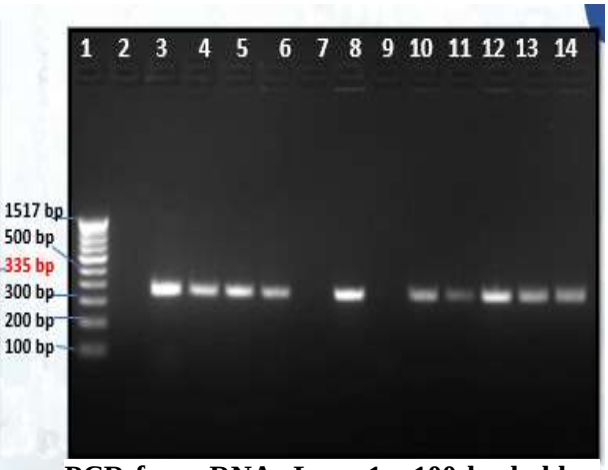
PCR for Cas9: Lane 1: 100 bp ladder, Lane 2: Negative control of chilli, Lane 3: Colony PCR product of *Agrobacterium*, Lane 4-15: PCR product of transformed chilli plant samples (78, 80, 76, 75, 49, 48, 55, 53, 1, 46, 85, 77 respectively)



PCR for Cas9: Lane 1: 100 bp ladder, Lane 2: Negative control of chilli, Lane 3: Colony PCR product of *Agrobacterium*, Lane 4-14: PCR product of transformed chilli plant samples (2, 8, 12, 41, 42, 45, 46, 47, 52, 69, 74 respectively)

Plate 6: PCR for Cas9 from lower leaves of T_0 generation chilli plants

PCR for sgRNA: Lane 1: 100 bp ladder, Lane 2: Negative control of chilli, Lane 3: Colony PCR product of *Agrobacterium*, Lane 4-15: PCR product of transformed chilli plant samples (78, 80, 76, 75, 49, 48, 55, 53, 1, 46, 85, 77 respectively)



PCR for sgRNA: Lane 1 : 100 bp ladder, Lane 2 : Negative control of chilli, Lane 3: Colony PCR product of *Agrobacterium*, Lane 4-14: PCR product of transformed chilli plant samples (2, 8, 41, 12, 42, 45, 46, 47, 52, 69, 74 respectively)

Plate 7: PCR for sgRNA from lower leaves of T_0 generation chilli plants

4.3.3 Detection of *Agrobacterium* in T_0 generation

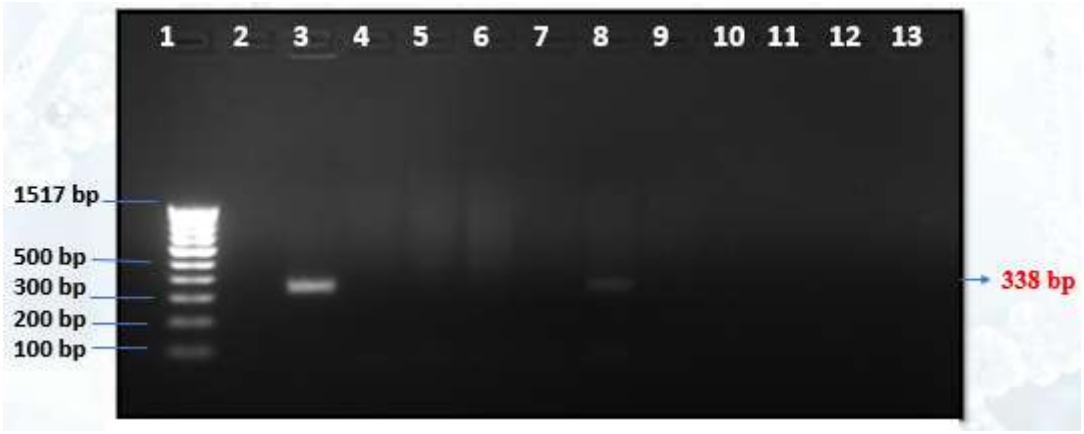
To avoid false PCR-positive plants and to check for the integration of the inserted genes into the plant genome, PCR for a region specific to the non T-DNA region was carried out. The Vir D_2 region is present outside the T-DNA region of the plasmid of *Agrobacterium*. In T_0 generation plants, PCR for Vir D_2 was done for 10 plants. One plant sample was PCR positive for the Vir D_2 region (Plate 8).

4.3.4 Kanamycin sensitivity for germination of wild type (non-transformed) seeds of chilli

To screen true transformed plants from the large number of seeds obtained from T_0 generation plants, there need to be some selection pressure to avoid non-transformed plants early during their growth. Seeds of wild type (non-transformed) plants were screened on varying concentration of kanamycin. Fifty per cent reduction in germination of seeds was observed at 375 mg/L of kanamycin. Whereas, 75 percent reduction in germination of wild type seeds was found at 750 mg/L kanamycin (Plate 9). So, seeds obtained from T_0 generation plants were made to germinate on 750 mg/L of kanamycin (Plate 10). Sixteen of the 18 seeds from the 78th T_0 plant were made to germinate, and 16 of them germinated. Seventeen seeds from the 74th T_0 plant were made to germinate, and seven of them germinated, whereas only one seed germinated from 18 seeds of 53rd T_0 plant. The germinated seeds were transferred to greenhouse and planted in soil for their further growth.

4.3.5 Confirmation of CRISPR/Cas9 construct in T_1 generation chilli plants

PCR was done for the 22 T_1 generation plant using Cas9 and sgRNA specific primers. Seven plants were PCR positive in T_1 generation. In the figure showing the presence of sgRNA gene (Plate 11), the same seven PCR positive plants were reconfirmed with Cas9 primers for the presence of the gene, where all of them were positive (Plate 12).



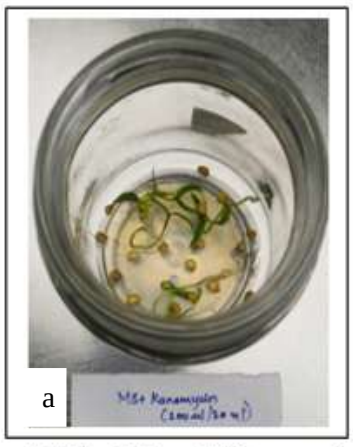
PCR for Vir D_2: Lane 1: 100 bp ladder, Lane 2: Negative control of chilli, Lane 3: Colony PCR product of *Agrobacterium*, Lane 4-13: PCR product of transformed chilli plant samples (80, 49, 78, 76, 75, 48, 53, 55, 46, 85 respectively)

Plate 8: PCR for Vir D_2 from T_0 generation chilli plants

a) MS + 0 mg/L Kanamycin

b) MS + 250 mg/L kanamycin

c) MS + 375 mg/L kanamycin

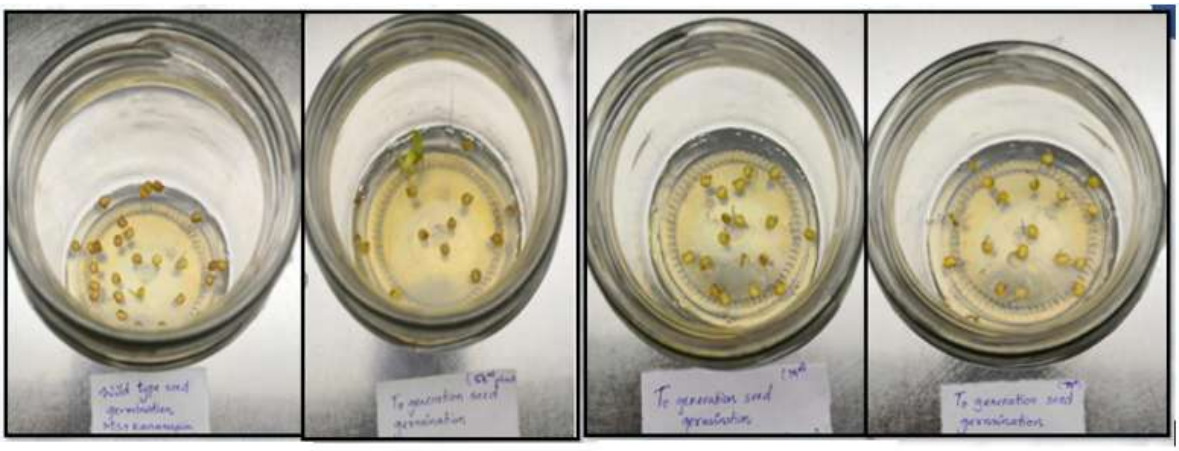


d) MS + 500 mg/L Kanamycin

e) MS + 625 mg/L kanamycin

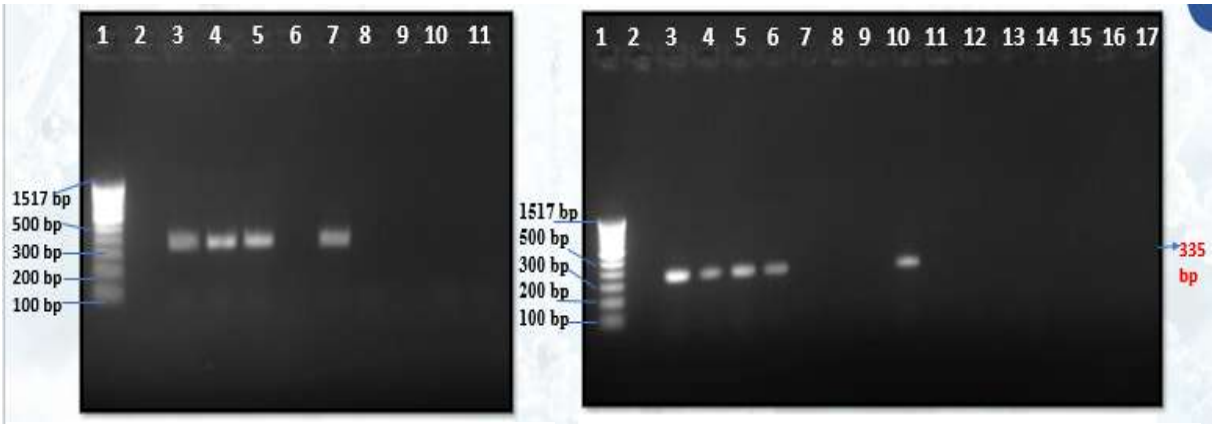
f) MS + 750 mg/L kanamycin

Plate 9: Kanamycin sensitivity for germination of wild type seeds of chilli



a.Wild type seed germination (Control) | b. Seed germination from 53rd T_0 plant | c. Seed germination from 74th T_0 plant | d. Seed germination from 78th T_0 plant

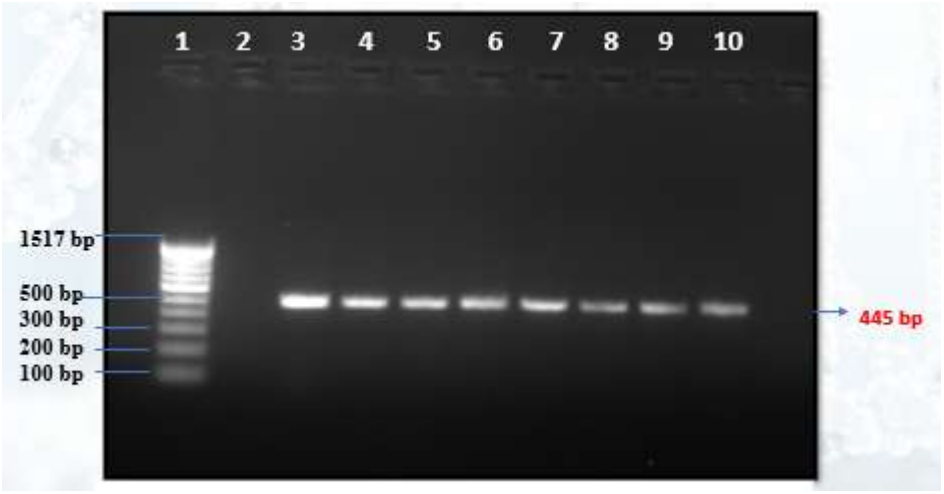
Plate 10: Seed germination of transformed T_0 generation plants on kanamycin (750 mg/L)



PCR for sgRNA: Lane 1: 100 bp ladder, Lane 2: Negative control of chilli, Lane 3: Colony PCR product of *Agrobacterium*, Lane 4-11: PCR product of transformed chilli plant samples (78_1, 78_2, 78_3, 78_4, 78_5, 78_6,76_2,76_3 respectively)

PCR for sgRNA: Lane 1: 100 bp ladder, Lane 2 : Negative control of chilli, Lane 3: Colony PCR product of *Agrobacterium*, Lane 4-17: PCR product of transformed chilli plant samples (76_4,76_7, 76_8, 76_9, 76_{10}, 76_{11}, 74_1, 74_2, 74_3, 74_5, 74_6, 74_7,74_9, 53_1 respectively)

Plate 11: PCR for sgRNA in T_1 generation plants



PCR for Cas9: Lane 1: 100 bp ladder, Lane 2: Negative control of chilli, Lane 3: Colony PCR product of *Agrobacterium*, Lane 4-10: PCR product of transformed chilli plant samples (78_1, 78_2, 78_4,76_7, 74_1, 76_4, 76_8 respectively)

Plate 12: PCR for Cas9 in T_1 generation plants

5. DISCUSSION

Chilli pepper (*Capsicum annuum* L.), popularly known as hot pepper, is a spice crop in the *Solanaceae* family. India has a wide range of chilli varieties, each with its own set of excellent characteristics. Chillies are sensitive susceptible to a variety of diseases, including viruses, which result in significant yield losses. So far, 65 viruses have been reported around the world, including *begomoviruses* that infect chillies and cause Chilli leaf curl virus disease (ChLCVD) (Nigam *et al.,* 2015). Plant breeding techniques had limited success to provide resistant chilli types, especially those which are viral virus resistant. Although a variety of insecticides have been successful in the past in controlling whitefly, it has the ability to acquire resistance very quickly. The CRISPR/Cas9 technology for genome editing was employed to control Chilli leaf curl viral infection in chilli plants by inhibiting viral DNA replication for virus resistance in the current study.

An attempt was undertaken to transform chilli with the CRISPR/Cas9 construct targeting the promoter region of the *Rep* gene of the *Chilli leaf curl virus*. Byadgi kaddi is a famous chilli type popular in northern Karnataka for its rich red colour and mild pungency.

5.1 Optimisation of *in vitro* direct regeneration protocol

The variety of a plant has a significant impact on explant regeneration, necessitating the development of *in vitro* propagation techniques specific to certain cultivars (Alejo and Moreno, 1990).

In the present study, MS + 8 mg/L zeatin worked best with six multiple shoots out of five distinct media compositions used. Manoharan *et al.* (1998) employed the chilli cultivar "Pusa Jwala" and found that it responded better to media containing zeatin (9.12 µM). According to Arrillaga *et al.* (2001), zeatin was used for increased shoot organogenesis in wild tomatoes (*Lycopersicon cheesmanii*). Explants grown for 2 weeks on media supplemented with 0.1-0.3 mg/L IAA and 2-5 mg/L zeatin aggressively developed normal shoots in over 90 per cent of the cases (Lee*,* 1996).

The MS media with 6 mg/L thidiazuran (TDZ) was the second best working medium found, with four shoots developing. The cytokinin employed had a big impact on *in vitro* regeneration and maximum shoot bud regeneration with thidiazuran was reported by Szasz *et al.* (1995); and Manoharan *et al.* (1998). In TDZ-containing medium, maximal shoot bud

regeneration was achieved (Fig 1). A wide range of plants responded well to TDZ for cytokinin-dependent shoot regeneration (Malik and Saxena, 1992; Huetteman and Preece, 1993).

5.2 Genetic transformation

5.2.1 *Agrobacterium*-mediated tissue culture dependent transformation methodology

Due to many difficulties that jeopardise the *in vitro* response, pepper (*Capsicum* spp.) is considered to be a recalcitrant species (Kothari *et al.,* 2010).

5.2.1.1 Chilli seed germination on MS media:

Capsicum germination *in vitro* is extremely slow, and inconsistent germination has been reported (Hegde *et al.,* 2017). The non-starchy endospermic tissues that surround radical tips may function as a mechanical barrier to the expanding embryo, resulting in delayed and inconsistent germination. To improve seed germination, seeds were steeped in distilled water with 2 mg/L GA_3 for two days before being sown *in-vitro* to produce sterile seedlings (Watkins and Cantlife, 1983). In the present experiment, seeds were steeped in distilled water for a day and were surface sterilized and used further to grow into seedlings.

5.2.1.3 Pre-induction media:

5.2.1.3.1 Use of cotyledonary nodal explant as explant of choice

Cotyledons are the preferred explants because they respond better to *in vitro* plant regeneration treatments (Romero *et al.,* 2001; Venkataiah *et al.,* 2001; Mathew 2002; Golegaonkar and Kantharajah, 2006). The meristem-based regeneration approach is one of the transformation strategies that can be employed in both direct and indirect transformation (shoot apex method). The shoot apex is advantageous for *Agrobacterium*-mediated gene transformation because it has a low incidence of tissue culture-induced genetic alterations and allows for simple and direct transformation of plants (Gould *et al.,* 1991). In the present study, the Byadgi kaddi chilli cultivar with explants of the cotyledonary nodal region was used as an explant.

5.2.1.3.2 Explant pre-culture is a crucial step.

The explants were pre-cultured on pre-culture media for 24-48 hours. *Agrobacterium*-mediated transformation of several plant species has been reported to benefit from explant

preculture (Lawrence and Koundal, 2000; Barik *et al.,* 2005; Pasapula *et al.,* 2009). The hypersensitive response was minimised when the explants were pre-cultured on the regeneration medium for two days compared to co-cultivation with *Agrobacterium* without pre-culture (Prakash and Gurumurthi, 2009)

5.2.1.4 Co-cultivation media:

The hypervirulent strain EHA-105 has been demonstrated to have a greater frequency of *Agrobacterium*-mediated transformation than the C58C1 or LBA 4404 strains of *Agrobacterium* (Orczyk and Orczyk, 2000). The same strain, EHA-105, was used in the present study.

Explants were co-cultivated for 48 hours in present investigation. Due to their natural physiological condition, overgrowth of bacteria likely activates defence/senescence reactions in cotyledonary leaf explants, resulting in a reduction in transformation efficiency when co-cultured for longer than 48 hours (Mahto *et al.,* 2018).

5.2.1.5 Adventitious shoot induction/multiple shoots induction

In recalcitrant species, Lee *et al.* (1993) found that zeatin was more effective than BAP at triggering maximum shoot bud induction. In the chilli cultivars G4 and LCA334, Maligeppagol *et al.* (2016) used zeatin in combination with the auxin phenyl acetic acid (PAA) to stimulate shoot development.

5.2.1.6 Elongation of shoots:

GA_3 is commonly used in the shoot elongation media in combination with varying concentrations of other growth hormones. Incorporation of GA_3 in shoot elongation media at 0.5 mg/L concentration enhanced the elongation in two cultivars, LCA-235 and Supper. However, further increases in GA_3 concentration (1-2 mg/L) had no benefit (Kumar *et al.,* 2012).

In the present study, MS medium supplemented with 7-8 mg/L zeatin and 2 mg/L GA_3 gave significantly higher number of elongated shoots per explant. Regenerated shoots elongated and rooted well on MS medium containing 2 mg/L GA_3 + 1 mg/L IAA (Bhutia *et al.,* 2016).

The kanamycin concentration utilised as a selective agent in this investigation was 50 mg/L. Above this concentration it was poisonous and deadly to plants, as reported by Manoharan *et al.,* (1998); Chanappagoudar, (2007).

5.2.1.7 Rooting and hardening of regenerants:

In the present study, root induction media consisted of half MS with 2 mg/L NAA. Rooting was accomplished by transferring normal elongated shoots to MS medium supplemented with IAA (0.5-1 mg/L) or NAA (0.2-1.0 mg/L). NAA favoured thick roots while IAA favoured thin, slender roots. Complete plantlets developed in 2-3 weeks in 100 per cent of the shoots kept for rooting, and NAA had a better rooting response than IAA (Husain *et al.,* 1999).

5.2.2 *In-planta* transformation methodology

The fundamental issue with the *Agrobacterium*-mediated tissue culture dependent transformation approach is that certain plant species and mutant lines are recalcitrant to regeneration, and plant regeneration requires sterile conditions. The development of a genotype-independent gene transformation method is of great interest in many plants. For individual plants and crops, some tissue culture-independent *Agrobacterium*-mediated gene transformation methods have been reported. These techniques are commonly referred to as "*in planta* gene transformation", or "*In-planta* transformation". Other crops, such as buckwheat (Kojima *et al.,* 2000), mulberry (Ping *et al.,* 2003), kenaf (Kojima *et al.,* 2004), soybean (Chee *et al.,* 1989), and rice (Supartana *et al.,* 2005), have standardised *in planta* transformation protocols. *In planta* transformation methods are free of somaclonal variation, making them easier, faster and simpler than tissue culture-based transformation methods.

To develop chilli transformants with CRISPR/Cas9 construct targeting promoter region of ChLCV *Rep* gene in this study, a tissue culture-independent *in planta* transformation protocol was used.

5.2.2.1 Preparation of seedlings

Chilli seeds of Byadgi kaddi cultivar were sown in germination protrays. Chilli seeds germinated and set their two leaf stage of growth after 2 weeks

5.2.2.2 Inoculation of seedlings at two-leaf stage with *Agrobacterium*

Seedlings at the two-leaf stage were chosen for the current study. It is consistent with the findings of Kalbande and Anita (2016).

Other methods, such as *in-vitro* fruit injection and floral dip, use plant fruit or floral parts as an explant for *Agrobacterium* infection (Yasmeen *et al.,* 2009)

5.2.2.3 Hardening of putative transformants

These putative transformants in cups were initially covered with polythene bags for 1 month to maintain high humidity. After one month of new leaf emergence, they were kept in the shade for a month for hardening before being moved to the greenhouse. The hardening process employed a sand-to-soil (1:1) ratio.

5.2.2.4 Flowering and Fruiting in *In-planta* plants

The chilli plants is an alternate bearing, with produced 5-15 fruits per plant in each picking. After 4 months of growth, *in-planta* plants produced flowers and fruits. Wild Wild-type plants, on the other hand, produced fruits in 2-3 months. The infected, damaged meristematic region inoculated with *Agrobacterium* might have taken time in establishing new growth and leaves.

5.2.2.5 T_1 generation plants

Some selection pressure is required to avoid non-transformed plants early in their growth in order to select the transformed ones from the large number of seeds obtained from T_0 generation plants. Seeds of wild wild-type plants were screened with varying concentrations of kanamycin to find complete germination arrest, which is discussed further in the subheadings.

5.3 Analysis and confirmation of *in-planta* putative transformants

Confirmation analysis of transformants could be done in many ways: Reporter gene expression, Southern blotting, PCR analysis, RT-PCR and Western blotting.

5.3.1 Confirmation of CRISPR/Cas9 construct in T_0 generation chilli plants from upper and lower leaves

In the current study, PCR was used to confirm the presence of T-DNA between left and right borders. According to the findings, the current transformation methodology had 22

per cent transformation efficiency. Scientists observed similar results; Kumar *et al.* (2009) reported 17.8 and 11.4 per cent of T_0 transgenic chilli plants (chimeric) in Arka Gaurav and Arka Mohini, respectively, and 35 and 29.7 percent were identified as stable transformants in the T_1 generation, respectively, based on PCR analysis.

It is well known that some plant genotypes are amenable to *Agrobacterium*-mediated tissue-culture dependent genetic transformation while others are recalcitrant; indicating that plant genotype has an impact on transformation suitability (Kumar *et al.*, 2012). *Agrobacterium*-mediated tissue culture dependent transformation efficiency is also influenced by the type of explant used and the length of time the explants were co-cultured with *Agrobacterial* cells to promote infection without provoking explant defence responses.

Genetic transformation in *Capsicum* with low transformation efficacy could be achieved in several varieties such as Pusa Jwala with about 5-12.2 per cent efficiency (Manoharan *et al.*, 1998; Kumar *et al.,* 2012), Nockkwang with 0.6 per cent (Ko *et al.,* 2007), California wonder with 1.3-2.9 per cent (Verma *et al.,* 2013a, b) and *C. annuum* inbred lines P101, P410, P409 and P915 with an efficiency of 0.03-0.19 per cent (Lee *et al.*, 2004). Unlike other members of the *Solanaceae* family, the efficiency of *A. tumefaciens*-mediated tissue-culture dependent genetic transformation of *Capsicum* is by and large very low. The current study's high transformation efficiency (22%) demonstrates that the *in-planta* method of transformation is more feasible, efficient, and compatible with a wider range of plants than *Agrobacterium*-mediated tissue culture-dependent transformation.

5.3.2 Detection of *Agrobacterium* in T_0 generation

To rule out the possibility of the presence of *Agrobacterium* in the chilli transformants, which might result in false-positives. PCR for $virD_2$ region which is specific for the *Agrobacterium* and not for the T-DNA was included in the experiment. If the result of the PCR shows no amplification for $virD_2$ region in the plant DNA, then it confirms that the PCR amplification in the transgenic plant DNA is because of the integration of the transgene into plant genome and not due to the persistence of the *Agrobacterium*.

No colonies appeared for the homogenate obtained from the young leaves at the shoot apices of mature wheat plants (T_0 and T_1) inoculated onto LB medium containing selection pressure for the LBA4404 strain harbouring a binary vector, indicating the absence of *A. tumefaciens* used for the transformation in the shoot apices of the transformants (Supartana *et al.,* 2005).

5.3.3 Kanamycin sensitivity for germination of wild type (non-transformed) seeds of chilli

Kanamycin resistance is conferred to transformed plants by the presence of the *npt* II gene within the CRISPR/Cas9 construct, whereas non-transformed plants succumb to kanamycin over time. Seeds from wild type were tested for kanamycin concentrations ranging from low (0 mg/L) to high (750 mg/L) in this study to determine the concentration at which non-transformants would die. The findings from the present study were in line with those of Bibi *et al.* (2013), who found that the highest kanamycin concentration level of 600 mg/L resulted in a maximum reduction of 78 percent in seedling survival, but they differed from those of Karthik *et al.* (2021), who used lower kanamycin concentrations of 150 mg/L to screen the T_1 plants.

5.3.4 Confirmation of CRISPR/Cas9 construct in T_1 generation chilli plants

Agrobacterium is targeted at the wounded apical meristem of the differentiated seed embryo in this *in-planta* approach. As a result, *Agrobacterium tumefaciens* inserts the gene into the genomes of various cells that are already programmed to develop into specific organs, while the meristematic cells must still be differentiated. As a result, the primary transformants (T_0) are chimeric by nature. That is why the transgenic plants need to be analysed in the T_1 generation.

In the present study, seven plants were PCR positive in T_1 generation out of 22 plants analysed. Similar findings were made in the study by Keshamma *et al.* (2008), where Plant No. I produced 3 transformants out of 24 plants in the T_1 generation based on PCR analysis for the *uidA* gene, and Plant No. IV produced 11 transformants out of 59 plants.

5.4 Future line of work

1) Sequencing to confirm T_0 plants that are PCR positive for the CRISPR construct but PCR negative for *Agrobacterium* contamination.
2) Zygosity status of CRISPR/Cas9 locus in chilli transformants
3) Determination of copy number of transgenic chilli.
4) Cas9 protein expression and sgRNA expression using cas9 specific antibody and by RT-PCR respectively.
5) Virus protection screening, i.e. phenotypic expression (resistance to ChLCV)

6. SUMMARY AND CONCLUSION

- The CRISPR/Cas9 construct with 20 nucleotide single gRNA targeting the promoter region of *rep* gene of the ChLCV was used for chilli transformation.
- In the *Agrobacterium*-mediated tissue-culture dependent transformation method, the shoot induction media (MS media) with growth regulator of 8 mg/L of zeatin was found to be effective for shooting. The shoot elongation media consisting of MS media with 8 mg/L of zeatin supplemented with 2 mg/L of GA_3 and the root induction media consisting of MS media supplemented with 2 mg/L of NAA were used.
- In the *in-planta* method of plant transformation proved to be efficient, effective and less time consuming and non-labourious approach compared to tissue-culture dependent approach of plant transformation.
- *In-planta* method of chilli transformation, out of 100 inoculated plants, 40 survived after *Agrobacterium* inoculation, 23 survived after hardening and 22 were PCR positive plants for the CRISPR/Cas9 construct.
- Screening of large number of seeds obtained from T_0 transgenic plants was done by sowing seeds in MS media with antibiotic selection pressure of kanamycin (750 mg/L).
- Presence of *Agrobacterium* in putative chilli transformants was checked to avoid false positives, by PCR using Vir D_2 specific primers in T_0 generation. Out of 10 T_0 generation plants, 9 found to be PCR negative for Vir D_2.

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AGROBACTERIUM-MEDIATED TRANSFORMATION OF CHILLI (*Capsicum annuum* L.) WITH CRISPR/CAS9 CONSTRUCT TARGETING *CHILLI LEAF CURL VIRUS*

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ABSTRACT

Chilli (*Capsicum annuum* L.) is a self-pollinated dicot plant in the *Solanaceae* family. Chillies are succulent crops, infected by more than 40 viruses, the most destructive of which is the *Chilli leaf curl virus* (ChLCV). In the previous study, the CRISPR/Cas9 construct targeting the promoter region of ChLCV *Rep* gene was confirmed with restriction digestion, PCR, and sequencing. The construct was then electroporated into an *Agrobacterium* strain (EHA-105) and was evaluated for transient expression of scaffold RNA and Cas9 RNA in tobacco and chilli by agroinfiltration and reverse-transcriptase PCR. In the present study this CRISPR/Cas9 construct was used for chilli transformation by two methods. In tissue-culture dependent transformation method shoot induction media (MS media) with growth regulators of 8 mg/L of zeatin was found to be effective. For shoot elongation, MS media with 8 mg/L of zeatin and 2 mg/L of GA_3 and for root induction, MS media supplemented with 2 mg/L of NAA was used.

In the *In-planta* method of chilli transformation, out of 100 inoculated plants, 40 survived after *Agrobacterium* inoculation, 23 of them survived after hardening, and 20 were found to be PCR positive for the CRISPR construct. Screening of seeds obtained from T_0 transgenic plants was done by sowing seeds in MS media with kanamycin (750 mg/L). The presence of *Agrobacterium* was checked to avoid false positives by PCR for the Vir D_2 region of *Agrobacterium* in T_0 plants. Transgenic chilli lines identified will be further used for ChLCV resistance study.