EDITING OF RICE TRANSCRIPTION FACTOR *OsMADS26* FOR DROUGHT TOLERANCE THROUGH CRISPR/Cas9 SYSTEM

By

ANJALA K.

(2019-11-004)

DEPARTMENT OF PLANT BIOTECHNOLOGY

CENTRE FOR PLANT BIOTECHNOLOGY AND MOLECULAR BIOLOGY

COLLEGE OF AGRICULTURE

VELLANIKKARA, THRISSUR – 680656

KERALA, INDIA

2021

EDITING OF RICE TRANSCRIPTION FACTOR *OsMADS26* FOR DROUGHT TOLERANCE THROUGH CRISPR/Cas9 SYSTEM

By

ANJALA K.
(2019-11-004)

THESIS

Submitted in partial fulfilment of the requirement for the degree of

Master of Science in Agriculture
(PLANT BIOTECHNOLOGY)

Faculty of Agriculture

Kerala Agricultural University, Thrissur

DEPARTMENT OF PLANT BIOTECHNOLOGY
CENTRE FOR PLANT BIOTECHNOLOGY AND MOLECULAR BIOLOGY
COLLEGE OF HORTICULTURE
VELLANIKKARA, THRISSUR – 680656
KERALA, INDIA

2021

DECLARATION

I, hereby declare that the thesis entitled **"Editing of rice transcription factor *OsMADS26* for drought tolerance through CRISPR/Cas9 system"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me any degree, diploma, fellowship or other similar title of any other University or Society.

Vellanikkara
Date: 20-12-2021

Anjala K.
(2019-11-004)

CERTIFICATE

Certified that the thesis entitled **"Editing of rice transcription factor *OsMADS26* for drought tolerance through CRISPR/Cas9 system"** is a bonafide record of research work done independently by **Ms. Anjala K. (2019-11-004)** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, associateship or fellowship to her.

Vellanikkara

Date: 20.12.2021

Dr. Rehna Augustine
Assistant Professor
Dept. of Plant Biotechnology
College of Agriculture
Vellanikkara

CERTIFICATE

We, the undersigned members of the advisory committee of **Ms. Anjala K. (2019-11-004),** a candidate for the degree of **Master of Science in Agriculture** with major field in **Plant Biotechnology,** agree that the thesis entitled **"Editing of rice transcription factor *OsMADS26* for drought tolerance through CRISPR/Cas9 system"** may be submitted by **Ms. Anjala K.,** in partial fulfilment for the degree.

Dr. Rehna Augustine
Assistant Professor
Dept. of Plant Biotechnology
College of Agriculture
Vellanikkara

Dr. Abida P. S.
Professor and Head
Dept. of Plant Biotechnology
College of Agriculture
Vellanikkara

Dr. Deepu Mathew
Associate Professor
Dept. of Plant Biotechnology
College of Agriculture
Vellanikkara

Dr. Parvathi M. Sreekumar
Assistant Professor
Dept. of Plant Physiology
College of Agriculture
Vellanikkara

DEDICATED TO MY BELOVED FAMILY AND TEACHERS

Acknowledgement

*While looking back to the path traversed during this endeavor, I praise and thanks to the '**Almighty God'** for his boundless blessings which accompanied me throughout my research work to complete it successfully not only for this special case but also for the unconditional love and guidance throughout my life. Secondly, my special thanks to my parents for their tireless efforts in moulding me in a way to face and respond to any kind of situations in life.*

*It is with immense pleasure and happiness, I avail this opportunity for expressing my sincere and heartfelt gratitude and indebtedness to my major advisor **Dr. Rehna Augustine**, Assistant Professor, Department of Plant Biotechnology for continuous untiring help during each stage of my study and research, friendly approach, valuable guidance and support, kind treatment, practical suggestions, inspiring encouragement, immense patience for guiding at all hurdles and timely help throughout my research work and thesis preparation, because of which I have been able to successfully complete the work with fruitful results. I highly appreciated her concern in this experiment, may the almighty God bless her for all success and happiness in life ahead. I would like to express my sincere gratitude to **Dr. Deepu Mathew**, Associate Professor, Department of Plant Biotechnology and member of my advisory committee, for the valuable solutions and support throughout the course. He always showed interest in helping out students at times of dilemma without any reluctance. I would also like to express my sincere thanks and deep sense of honour to **Dr. Abida P. S**, Professor and Head, Department of Plant Biotechnology and member of my advisory committee for her keen interest, precious suggestions, inspiration and needful help in my research work for the unwavering inspire, timely support for my research work. I express my heartfelt gratitude to **Dr. Parvathi M. Sreekumar,** Assistant Professor, Department of Plant Physiology and member of my advisory committee for her valuable suggestions, guidance and pleasant way of finding solutions for the research program and preparation of the thesis. I express my indebtedness to **Smitha Nair**, Asssistant Professor, Department of Plant Biotechnology for her guidance and valuable suggestion for the research work as well as guiding us correctly during our practical*

classes and made us capable to do various molecular techniques in right way.

I am also thankful to ***Vipul bhayya, Shivaji bhayya*** *and* ***Feba chechi*** *for the guidance rendered to me throughout the period of my research work and study. I would like to acknowledge the helping hands extended by each of the non-teaching staff especially* ***Shylaja chechi****. and* ***Simi chechi*** *for their motherly love and support, Research Assistant* ***Nimmi chechi*** *and* ***Arya chechi*** *for their tremendous help. And also, I am very grateful to my classmates* ***Sanjay Sathian*** *and* ***Varsha K*** *for their tremendous support and whole hearted help for my research work as well as thesis preparation. They helped me at times of stress, pulled me out of tension and helped me enjoy each stage of my research with pleasure.*

I'm so happy to express my gratitude and heartfelt thanks to ***Sanjay Sabu, Athira, Midhuna, Anjitha, Swathi, Shafreena, Keerthana, Chinnu, Paru, Shri Hari, Nahla, Gershom, Drishya and Mullai,*** *as their co-operation has in one way or another assisted me to accomplish this work successfully. Many people helped me during the whole period of my study since it is difficult to mention them all I would like to emphasize that it would not have been possible to complete my research work without their cooperation. So, I thank them all, and may almighty God bless them. I would like to extend my gratitude to all those who helped me in the throughout the endeavor.*

Thanking all,

Anjala K

TABLE OF CONTENTS

LIST OF TABLES

LIST OF PLATES

LIST OF FIGURES

LIST OF ANNEXURES

Annexure No.	Title
1	Vector map of pRGEB32 procured from Addgene (CAT#63142)
2	Genomic sequence of OsMADS26 gene sequence from 'Rice Genome Annotation Project'
3	Guide RNA targeting regions in OsMADS26 gene sequence
4	Primers designed for the study

LIST OF ABBREVIATIONS

%	Percentage
=	Equal to
µL	Micro Litre
µM	Micro Molar
2,4-D	2,4-Dichlorophenoxy acetic acid
ABA	Abscisic Acid
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
BT-solution	Bacterial Transformation Solution
CRISPR/Cas	Clustered Regularly Interspaced Short Palindromic Repeats/ CRISPR associated proteins
CTAB	Cetyl Trimethyl Ammonium Bromide
DBT	Department of Biotechnology
DNA	Deoxynucleic Acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylene Diamine Tetra Acetic acid
EMS	Ethyl Methane Sulfonate
FP	Forward primer
g	Grams
GA	Gibberellic Acid
GDP	Gross Domestic Product
h	Hour
HF	High Fidelity
kb	Kilo base pairs
L	Litre
LB	Luria-Bertani
LEA proteins	Late Embryogenesis Abundant Proteins
M	Molar
Mb	Mega base pairs
mg	Milligram
min	Minutes
mL	Milli Litre
mM	Milli Molar
mRNA	messenger RNA

NAA	Naphthalene Acetic acid
ng	Nanogram
°C	Degree Celsius
OD	Optical Density
p.s.i.	Pound force per square inch
pDNA	Plasmid DNA
pH	Hydrogen ion concentration
PVP	Poly Vinyl Pyrrolidine
RNA	Ribonucleic Acid
RNAi	RNA interference
RNase	Ribonuclease
ROS	Reactive Oxygen Species
RP	Reverse primer
rpm	Revolutions per minute
SDS	Sodium dodecyl sulfate
sec	Seconds
snoRNA	Small nucloeolar RNA
SSR	Simple Sequence Repeats
TAE	Tris Acetate EDTA
Taq	*Thermus aquaticus*
T-DNA	Transfer DNA
TE	Tris EDTA Buffer
Tm	Melting Temperature
UV	Ultraviolet
V	Volts

INTRODUCTION

1. INTRODUCTION

Rice (*Oryza sativa* L.) is the most widely consumed staple food of the world's human population belonging to Asia and Africa. Cultivation of rice had evolved along with the tradition of Asian and African plains that could feed the enormous population. Flooding the fields is the most common or rather the earliest way for rice cultivation. India being the second largest producer and the largest exporter of rice in the world, it is very crucial to increase the productivity for ensuring global food security. It contributes a lion's share in the GDP of our country, since we consider agriculture as our backbone.

Kerala is the state that consumes rice to an extent greater than other states. Rice is now mainly cultivated in the districts of Palakkad, Thrissur, Alappuzha and Malappuram. Current status of rice cultivation in Kerala shows a declining curve, which clearly indicates the shift to other crops. But this ends up with the shortage of rice and we have to depend on other states to meet our demands. One of the major reasons for this shift is due to the unpredictability of the climate that is prevailing nowadays. Rice cultivation requires either plenty of moisture or proper irrigation for getting proper yield.

About 45% of the global rice area is under rainfed conditions and faces production loss due to abiotic stress (Lafitte *et al*., 2004). Under this scenario it is necessary to develop varieties tolerant to water deficit to meet the global food demand. Rice being less adaptable to the water-limited conditions due to its semi-aquatic nature, make it more prone to losses from drought (Bouman *et al*., 2005). Even high yielding varieties like IR64 are highly susceptible to drought stress (Lafitte *et al*., 2006). Being a complex trait, drought stress affects different growth stages of rice plant.

The overall level of tolerance of the rice plant is as a result of a coordinated interplay of numerous stress responsive genes with other components that stimulate the signal transduction pathways. Conventional breeding, marker assisted breeding and genetic engineering techniques were proven successful in developing stress tolerant varieties. Due to the complexity of the trait the outcome is not fully

satisfying. Evidence regarding the existence of substantial genetic variability in rice germplasm for drought tolerance and the ways to develop elite breeding programs for developing improved rice varieties *via* Marker Assisted Breeding was given by Serraj *et al*. (2011). There are several abiotic stress related genes and transcription factors which had come to limelight by the endeavors of scientists.

Apart from conventional breeding programs that produce elite varieties, there are several genetic engineering technologies of recent origin for producing crop plants with desired traits. These include RNAi, anti-sense technology, Zinc Finger Nuclease (ZFN), Transcription Activator Like Effector Nucleases (TALENs)and finally the CRISPR/Cas system. There are many protocols developed by scientists around the globe for *Agrobacterium* mediated transformation of plants. Zeng *et al*. (2013) had reported the use of ZFN for enhanced drought tolerance *via* increased ABA biosynthesis. Over expression of few genes also improved tolerance to abiotic stress in rice. ABA receptors and homeobox transcription factors when over expressed induced drought tolerance in rice (Kim *et al*., 2014; Bhattacharjee *et al*., 2017). The transcription factors belonging to the MADS box family were shown to have cross-talk with many downstream pathways that regulate abiotic and biotic stresses (Khong *et al*., 2015).

One of the most advancing genetic manipulation technologies currently in use is the Clustered Regularly Interspaced Short Palindromic Repeats – associated nuclease (CRISPR/Cas) system (Xie *et al*., 2014). It is from 30 years back incident that CRISPRs was first identified from *Escherichia coli* (Ishino *et al*., 1987). Shan *et al.* (2014) had devised a protocol for the CRISPR/Cas platform in rice which made manipulation of complex traits possible. Various traits related to abiotic stress, biotic stress and yield were manipulated according to need using this platform. Zhang *et al*. (2014) showed that about 11 target genes of two subspecies of rice inherited the mutations induced via CRISPR/Cas following the Mendelian laws in further generations. Xie *et al*. (2014) had given a well-structured protocol of the CRISPR/Cas platform in rice. They had used the vector pRGEB31 for cloning and had explained the procedure for the development of CRISPR/Cas construct. Efficient protocols for

transformation of rice *via Agrobacterium* have been developed by Sahoo *et al.* (2011) and Toki *et al*. (2006).

MADS box genes are included in a multi-genic family and the functions of only few genes are deciphered so far. About 75 genes of this family are identified in rice genome (Khong *et al*., 2015). Many of them were discovered to have cross-talk with pathways controlling flower development, flowering time, tillering and stress related processes during development (Arora *et al*., 2007; Guo *et al*., 2013). Among the MADS box genes the stress related responses were found to be regulated only by *OsMADS26* gene (Lee *et al*., 2003, 2008). A severe stress phenotype was identified in rice plants with *OsMADS26* gene overexpressed and when down regulated using RNAi found to develop tolerance to stresses (Khong *et al*., 2015).

Since drought is a major problem related to rice cultivation, developing plants with tolerance to drought stress is a mandatory problem to be addressed. In this context, the down regulation of *OsMADS26* gene *via* CRISPR/Cas9 system, which is the most advanced technology for stable genome editing, was found to have significance. Hence, study was taken up with an objective of developing rice plants with tolerance to abiotic stress by silencing the MADS box gene *OsMADS26* using CRISPR/Cas9 system.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1. Rice

Rice (*Oryza sativa* L.), a monocot plant belonging to Poaceae family, is widely grown as an annual crop. The chromosome number of rice is 2n = 24 and has a genome size of 430 Mb (Kurata *et al.*, 2002; Waghmare *et al.*, 2020). Being the staple food of more than half of the population of the world, it is cultivated widely around Asian and African plains (Muthayya *et al.*, 2014, Gupta *et al.*, 2020). Rice covers about 23 percent of the total area under cereal production around the world (USDA, 2016). Rice belongs to the genus *Oryza* which includes about 22 species, but only two are domesticated and consumed by human population. The only cultivated species are *Oryza sativa* or Asian rice and *Oryza glaberrima* or African rice. Asian rice is cultivated throughout the Asian continent and is divided into three sub-species namely, indica, japonica and javanica.

Japonica and indica are the two widely cultivated subspecies of rice around the world (Chen *et al.*, 2020). It is expected that the world population may increase to a tremendous extend by 2050, which indicates the need to double the rice production by then (Skamnioti and Gurr, 2009). Rice being the crop with its genome sequenced is considered as a model system for cereal genome analysis and is being widely used for genetic manipulation studies widely (Waghmare *et al.*, 2020). Moreover, the ease of in-vitro regeneration of the crop from explants also adds to its advantages for using in such experiments.

The rice cultivar Nipponbare is the temperate variety of japonica rice (Chen *et al.*, 2021). With its genome sequenced, is mostly used in functional genomics studies. It also has genotypes highly amenable to manipulation and for further tissue culture events (Jeon *et al.*, 2000; Sallaud *et al.*, 2004). Many studies had revealed the ability of japonica cultivars for being used for transformation and development of new traits in rice (Jeon *et al.*, 2000; Toki *et al.*, 2006).

2.2. Drought stress and rice cultivation

Rice is one of the crops which is grown under varied agro-ecological conditions and hence have varied characteristics that can influence its productivity (Grover and Pental, 2003). Improvement strategies were developed for various traits like increased yield, resistance to lodging, nutritional enrichment and resistance to various stresses. Rice is highly prone to damages due to environmental stresses. The diseases like blast and bacterial blight are considered devastating due to the vast range of loss in yield (Miah *et al.*, 2017; Wing *et al.*, 2018). Many studies have pointed out that, being a semi- aquatic plant rice is critically susceptible to abiotic stress. Thus, improvement for abiotic stress tolerance in rice become a major problem to be addressed.

Drought, often mentioned as moisture stress condition is a major abiotic stress that adversely affects the normal growth and productivity of crops. Considering all the abiotic factors, drought is the most devastating stress that drastically affect more than 50% of the world's arable land (Singhal *et al.*, 2016). It is one of the crucial factors that affects the production of rice, which is a semi-aquatic plant (Oladosu *et al.*, 2019). Almost 45% of the rice area around the world is under rainfed conditions where its production is reduced due to various abiotic stresses (Lafitte *et al.*,2004).

Abiotic stress severely affects rice yield, mainly at the reproductive stage (Oh *et al.*, 2009). Since rice is one of the most susceptible plants to water deficit, it can be used as a model plant to study the abiotic stress responses in monocots (Jin *et al.*, 2013). It is mainly because of its small root system, thin cuticular wax and swift stomata closure (Sahebi *et al.*, 2018). Therefore, it has become an important issue to be addressed to develop high yielding rice variety with tolerance to both biotic and abiotic stresses for attaining a stable food production (Chukwu *et al.*, 2019). The overall level of tolerance in rice was found to be a result of the coordinated interplay of numerous stress responsive genes with other components that stimulate the signal transduction pathways (Hu *et al.*, 2014).

2.3. Stress responsive genes for drought tolerance in rice

Being a complex trait, drought stress adversely affects the different stages of growth of the rice plant. Evidences regarding the availability of substantial genetic variability in rice germplasm for drought tolerance, and the ways to develop elite breeding programs for developing improved rice varieties by Marker Assisted Breeding were given by Serraj and co-workers (2011). But it took a slower pace in crops like rice. This was mainly because of the fact that the trait for drought tolerance in rice was controlled by polygenes. These polygenes were having differential effects and the unpredictability in the severity of drought in addition made it more difficult (Bernier *et al.*, 2008). Conventional breeding, marker assisted breeding and genetic engineering techniques were successful in developing stress tolerant varieties.

When external stress signals are perceived, a complicated signaling network is effectively initiated and this in turn regulates the expression of an entire set of stress responsive genes to cope up the stress situation (Nakashima *et al.*, 2014; Golldack *et al.*, 2014). Many of the functional and regulatory proteins in the plant system includes the drought responsive genes like water channels, detoxification enzymes, transcription factors (TFs) and phosphatases (Joshi *et al.*, 2016; Gong *et al.*, 2020).

Many studies conducted had revealed the stress responsive genes present in rice. SSR markers were commonly used for the detection and diversity analysis of abiotic stress tolerance in rice (Salam *et al.*, 2017). The genotypic variations against high temperature stress tolerance at flowering period had been evaluated by Jagadish and co-workers (2008). Markers linked to the genes responsible for heat tolerance were identified and this simplified the marker assisted selection in order to transfer the reproductive phase heat tolerance to rice (Yang *et al.*, 2011). The endogenous concentration of Abscisic acid (ABA) changes according to the environmental stimuli, like drought (Qi *et al.*, 2018). Therefore, comprehensive studies were carried out on the ABA biosynthetic and signaling pathways in *Arabidopsis* and rice (Cutler *et al.*, 2010; Sato *et al.*, 2018). The overexpression of ABA receptor *OsPYL/RCAR5* in rice under the maize ubiquitin promoter had induced the expression of many abiotic stress responsive genes, which led to the salt and drought tolerance in rice. But fine regulation of its expression had to be studied since it slightly affects few of the

agricultural traits (Kim *et al.*, 2014). The overexpression of the rice *GLYCINE-RICH PROTEIN 3* (*OsGRP3*) conferred drought tolerance in rice through regulating the ROS scavenging related genes (Shim *et al.*, 2021).

Transcription factors acts as a class of major regulatory proteins as they have vital role in the regulation and transcription of functional genes by binding to the upstream elements of genes (Joshi *et al.*, 2016). By now, there are numerous evidences that indicates the direct role of transcription factors (TFs) and TF families in drought tolerance and resistance (Todaka *et al.*, 2015; Mohanty *et al.*, 2016). Many of these TFs regulate the drought stress signaling pathways in rice. Few of these TFs belongs to bZIP, AP2/ERF, MYB, NAC, WRKY, NF-Y, Homeodomain, CAMTA and bHLH families. These TF families plays major role in the responses of plants towards abiotic and biotic stresses (Chen *et al.*, 2012; Shao *et al.*, 2015).

In ABA independent pathway for drought tolerance, the DREB (dehydration-responsive element-binding protein) transcription factors have vital role to play. DREB TFs like DREB/CBF and DREB2 are involved in drought stress tolerance (Srivasta *et al.*, 2010). Similarly, Hong and co-workers have shown that overexpression of a stress-responsive NAC transcription factor gene *ONAC022* enhances drought and salt tolerance in rice (2016). A large number of NAC TFs have been identified for their role in abiotic stress responses (Puranik *et al.*, 2012). The NAC TFs belong to a large family comprising of 151 members in rice (Fang *et al.*, 2008).The ten rice NAC genes such as *ONAC002* (Hu *et al.*, 2006), *ONAC068* (Kaneda *et al.*, 2009), *ONAC048* (Nakashima *et al.*, 2007), *ONAC009* (Song *et al.*, 2011; Jeong *et al.*, 2013), *ONAC058* (Liang *et al.*, 2014), *ONAC122* (Sun *et al.*, 2013), *ONAC131*(Sun*et al.*, 2013), *ONAC054* (Yoshii *et al.*, 2009), *ONAC045*(Zheng *et al.*, 2009), and *ONAC017* (Yokotani *et al.*,2014) were found to play important roles in abiotic and biotic stress responses. But the biological function of most *ONAC* genesis not known (Hong *et al.*, 2016).

Jiang *et al.*, (2019) have reported the overexpression of another NAC transcription factor gene *OsNAC2* improves drought and salt tolerance in rice. The SNPs induced by EMS in membrane transporter genes like *OsAKT1, OsHKT6, OsNSCC2, OsHAK11* and *OsSOS1* led to varied expression levels and tolerance to

salt stress treatments (Hwang *et al.*, 2016). Overexpression of LEA protein genes such as *OsEM1* and *OsLEA3-1*, also lead to drought stress tolerance in rice (Xiao *et al.*, 2007, Yu *et al.*, 2016). Other TFs like *OsDRAP1*, *OsMYB6* and *OsbZIP62* were also reported to increase drought tolerance in transgenic rice (Huang *et al.*, 2018; Baillo *et al.*, 2019). Overexpression of the homeobox transcription factors like *OsHOX24* confers abiotic stress tolerance in transgenic rice *via* modulating stress responsive gene expression (Bhattacharjee *et al.*, 2017). Under drought stress conditions, genes like *DEEPER ROOTING 1* increases rice yield and control the root system architecture (Uga *et al.*, 2013). *OsHSP50*, one of the heat shock proteins in rice when overexpressed improved heat tolerance (Xiang *et al.*, 2018). Tang *et al.*, (2019) had reported the natural variation in *OsLG3* that enhances the drought tolerance by inducing ROS scavenging in rice. *OsMADS26* gene has role in regulating drought tolerance and has been studied *via* RNAi mediated silencing (Khong *et al.*, 2015).

2.4. MADS-box Transcription factors in rice

MADS-box transcription factors being a multi-genic family, are extensively found in yeasts, plants, insects, nematodes, lower vertebrates and mammals. They control different aspects of development and cell differentiation (Shore and Sharrocks, 1995). The *MADS-BOX PROTEIN REQUIRED FOR INFECTIOUS GROWTH1/ RESISTANCE TO LEPTOSPHAERIA MACULANS1* transcription factor confers for the pathogenicity of the causal agent of blast disease in rice (Mehrabi *et al.*, 2008). Some of the transcription factors belonging to the MADS-box family were found to have major role in controlling the stress-related developmental processes like abscission, fruit ripening and senescence (Fernandez *et al.*, 2000). There are evidences showing that these MADS-box transcription factors act as negative regulators of such developmental processes (Fang and Fernandez, 2002). SHATTERPROOF1 (SPH1) and SHATTERPROOF2 (SPH2) have role in cell specification of the dehiscence zone in *Arabidopsis* fruit (Liljegren *et al.*, 2000).

There are about 75 genes for encoding the MADS-box transcription factors in rice genome. The notation of MADS have its origin from the first letters of its founding members like, *Mini Chromosome Maintenance 1 (MCM1)* of yeast (*Saccharomyces cerevisiae*), *Agamous* of *Arabidopsis*, *Deficiens* of Snapdragon

(*Antirrhinium majus*) and *Serum Response Factor* (*SRF*) of human (Arora *et al.*, 2007). MADS-box TFs have 60 amino acids containing DNA binding domain called as the MADS-box domain present at the N-terminal region of the protein (Cho *et al.*, 1999; Yang *et al.*, 2003). The entire MADS-box family is divided into two major groups. The type I includes the *ARG80*/*SRF*-like genes in animals and fungi which is also notated as M-type genes in plants. These type I proteins are further divided into four namely, Mα, Mβ, Mγ and Mδ based on the phylogenetic relationships between MADS-box regions (Parenicova *et al.*, 2003). Type II includes *MEF2*-likegenes in animals and yeast as well as MIKC-type genes of plants (Alvarez-Buylla *et al.*, 2000). Type II genes are also further grouped into MIKC^c and MIKC* classes based on structural features (Kofuji *et al.*, 2003). Among the 75 genes identified in rice, 38 genes belong to MIKC^c, six genes to MIKC*, nine genes to Mβ, 13 genes to Mα and 10are grouped into Mγ type (Arora *et al.*, 2007).

Apart from these classifications, the MADS-box genes in rice are further divided into five classes. They are *APETALA1* (*AP1*) like genes, B-class, C-class, D-class and SEP-like genes. Rice genome contains at least three *AP1*-like MADS-box genes, *OsMADS14*, *OsMADS15*, and *OsMADS18* (Yamaguchi and Hirano, 2006). *AP1* like genes are involved in the establishment of floral meristem and for sepal-petal identity (Mandel *et al.*, 1992). B-class genes consists of *OsMADS2*, *OsMADS4* and *superwoman1* (*spw1*) or *OsMADS16* which controls floral development (Yamaguchi and Hirano, 2006). The rice genome contains two C-class genes, *OsMADS3* and *OsMADS58*expressed in the stamen and carpel whorl (Kang *et al.*, 1998). It regulates floral meristem determinacy and in normal carpel development (Yamaguchi and Hirano, 2006). There are two MADS-box genes in rice, *OsMADS13* and *OsMADS21* that show high similarity to D-class genes and they regulate ovule identity (Lee *et al.*, 2003; Colombo *et al.*, 1995). Five *SEP*-like MADS-box genes, *OsMADS7* (also known as *OsMADS45*) and *OsMADS8* (*OsMADS24*), *LHS1* (*OsMADS1*), *OsMADS5*, and *OsMADS34*, are found in rice and they regulate the floral meristem development (Lee *et al.*, 2003).

The most wonderful aspect regarding the MADS-box gene family is the diverse functions of its members in plant growth and development (Saedler *et al.*,

2001). Maximum number of these genes were identified to be located on chromosome 1 whereas, chromosome 10and 11 has only one gene each (Arora *et al.*, 2007). But the function of only few are identified till date (Khong *et al.*, 2015). Most of the genes which came into limelight have functions related to control of tillering, flower development and flowering time (Guo *et al.*, 2013). Among them, few are involved in the controlling the stress related processes, like *OsMADS3* involved in homeostasis of reactive oxygen species during anther maturation and *OsMADS29* that controls cell degeneration during seed development (Hu *et al.*, 2011). Many MADS-box genes in rice were studied by ectopic expression and some like, *OsMADS6*, *OsMADS14*, *OsMADS1*, *OsMADS5*, *OsMADS45* and *OsMADS24* were found to cause early flowering and dwarfism when overexpressed (Pelucchi *et al.*, 2002). The extreme phenotypes were observed for *OsMADS6* and *OsMADS14*, since they regulate early stages of flower development (Moon *et al.*, 1999). It was found that the expression of *OsMADS15, OsMADS18, OsMADS26, and OsMADS58* were slightly up-regulated under drought and *OsMADS23*was down-regulated over two-fold (Jin *et al.*, 2013).

The role of rice MADS-box genes in stress response has been reported mainly for the *OsMADS26* gene. It is an ortholog of *AGL12* and is expressed in roots, shoot, leaf and panicle throughout all developmental stages (Pelucchi *et al.*, 2002; Lee *et al.*, 2008). When overexpressed *OsMADS26* causes a severe stress phenotype in rice that leads to plant death and pigment accumulation. If expressed under the control of a dexamethasone-inducible promoter, induces differential expression of the gene involved in jasmonic acid biosysnthesis as well as regulates the reactive oxygen species production (Lee *et al.*, 2008). Another study reported that expression levels of four MADS-box genes, *OsMADS18, 22, 26* and *27* were up regulated by over two folds in response to cold and dehydration stress treatments (Arora *et al.*, 2007). In addition to this, it was identified that the transcript levels of *OsMADS26* was higher in older leaves and roots implying its role in senescence or maturation process (Lee *et al.*, 2008).

OsMADS26 gene was widely studied by the scientists regarding its role in abiotic and biotic stress regulation. To characterize the gene and its functions in rice, both overexpressed and RNAi-mediated down regulated plants were analyzed (Lee *et*

al., 2008; Khong *et al.*, 2015). The overexpressed plants showed defective growth, chlorosis, cell death, pigment accumulation, spotted leaves and senescence (Lee *et al.*, 2008). When *OsMADS26* was down regulated *via* RNAi, showed no dramatic changes in their development and were found to have tolerance to drought stress as well as gained resistance towards *Magnaporthe oryzae* and *Xanthomonas oryzae* pv *oryzae* (Khong *et al.*, 2015).

It was reported that the stress responses of *OsMADS26* gene may be related to hormonal activity. *OsMADS26* overexpressed plants when analyzed using microarray showed that it induces few jasmonic acid biosynthesis genes like, *LOX, OsLOX3, OsAOS1, OsAOS4, OsAOS5, OsOPR2, OsOPR12, OsOPR13 and OsJMT4.* It was also identified that this gene has cross-talk with jasmonic acid, salicylic acid and ethylene biosynthesis pathways (Lee *et al.*, 2008). Two genes in ethylene biosynthesis in rice, *1-AMINOCYCLOPROPANE-1-CARBOXYLATE OXIDASE 3* and *ACIREDUCTONE DIOXYGENASE 1* are down regulated in the OsMADS26 overexpressed plants were also identified by Khong and co-workers (2015). Since MADS-box genes regulates different stages of plant growth and development, there were misconceptions regarding the phenotype of plants with these genes manipulated. Khong and co-workers (2015) proved that the misregulation of *OsMADS26* gene, even if exhibited retarded growth at early stages of development, after maturation regained their normal growth. Similarly, they also identified that, the *OsMADS26* gene overexpressed plants showed more symptoms of *Magnaporthe oryzae* (blast) when compared to the down regulated lines. The *OsMADS26* down regulated lines *via* RNAi showed small and dark spots, which indicates resistance to blast disease. Thus, these studies suggests that *OsMADS26* negatively regulates blast resistance in rice. Similar characters were observed regarding bacterial blight resistance in *OsMADS26* down regulated plants *via* RNAi. Therefore, *OsMADS26* negatively regulates both blast and bacterial blight diseases in rice.

Plant tolerance against drought stress were analyzed in *OsMADS26* overexpressed as well as down regulated lines. *OsMADS26* overexpressed lines wilted and almost died when stress was induced. On the other hand, those down regulated lines fully recovered from water stress. In these plants, the expression of two drought

responsive genes namely, *RAB21* (a rice dehydrin) and *SALT STRESS-INDUCED PROTEIN* (Claes *et al.*, 1990) were found to be higher (Lee *et al.*, 2008; Khong *et al.*, 2015). Other stress related transcription factors were also expressed differentially in accordance with the down regulation of *OsMADS26*. The TFs like *OsNAC103* was known to be up regulated by water stress, salt stress and jasmonate treatment (Fang *et al.*, 2008; Nuruzzaman *et al.*, 2012) was up regulated on down regulating *OsMADS26* gene. Similarly, *OsWRKY24* that represses ABA and GA signaling in aleurone cells (Xie *et al.*, 2005) was also up regulated (Khong *et al.*, 2015). All these studies summarize that the *OsMADS26* gene is a negative regulator for abiotic and biotic stress tolerance in rice.

2.5. CRISPR/Cas9 technology

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR associated protein 9 (CRISPR/Cas9) system is currently in limelight for molecular biology research in plants. It was from 30 years back, the incident that identified the first CRISPRs from *Escherichia coli* took place. Yoshizumi Ishino, a scientist was conducting research on the genes related to the isozyme conversion of the enzyme Alkaline Phosphatase (Ishino *et al.*, 1987). But he couldn't identify the exact functions of these repeats due to the lack of sequence evidences and technologies that time. These interspaced and palindromic DNA repeat sequences were found in the intergenic region upstream of the *iap* gene in *E. coli* (Kim and Kim, 2014). Afterwards, another scientist, Mojica identified similar repeats from the archaea, *Haloferax mediterranei* (Mojica *et al.*, 1993). In 2002, Jansen named the repeats as CRISPR (Jansen *et al.*, 2002).

The first finding regarding the biological activity of CRISPR was found four years later by Eugene that the CRISPR/Cas system was a defense mechanism based on RNA-interference (RNAi). In early 2000s works were going on to come out of the CRISPR puzzle. Attempts for strain differentiation *via* molecular methods were in progress for the bacterium *Streptococcus thermophilus*. They came across CRISPR, then called it as SPIDR (Spacers interspaced Direct Repeats) in the genome sequencing of the strain CNRZ1066. They also came across the CRISPR loci multiple times while assembling the draft genome of the lactic acid bacteria like *Lactobacillus*

acidophilus. By 2012, CRISPR technology was in use to cut the target DNA outside the cell. From the very next year the system taken from *Streptococcus pyogenes* was used for targeted genome editing in various organisms (Liang *et al.*, 2015; Tahir *et al.*,2020).

The availability of genomic sequences helped scientists to compare the CRISPR gene sequences of various organisms. This paved way for the discovery of four genes that are conserved and found regularly with the adjacent regions of CRISPR regions in genome. These genes were denoted as CRISPR associated genes 1 to 4 (Cas1 to Cas4). Cas1 and Cas4 do not show similarity with functional domains of any other known proteins. Cas3 was related to the superfamily 2 helicases and Cas4 related to RecB exonucleases which functions with RecBCD complex for the homologous recombination of double stranded breaks. Therefore, Cas3 and Cas4 were predicted to have function in DNA metabolism, repair, recombination and chromosome segregation. That means these two Cas genes helps in the CRISPR loci genesis (Ishino *et al.*, 2018).

Cas9 is an endonuclease possessing two RNA molecules, i.e., crispr-RNA (cr-RNA) and transactivating cr-RNA (tracr-RNA). It can identify and degrade any nucleic acids which makes it useful in the field of genome editing (Barman *et al.*, 2019). It has two nuclease domains which creates a double stranded break in the genome (Cong *et al.*, 2013; Mali *et al.*, 2013). The two different domains of it include a large globular recognition (REC) specific functional domain, connected to a smaller nuclease (NUC) domain. The NUC domain further contains two nuclease sites, RuvC and HNH, and also a PAM-interacting site (Doudna and Charpentier, 2014; Jinek *et al.*, 2014). One domain bind to the DNA strand which is complementary to the sequences in the SgRNA and the other domain binds to the non-target strand.

There are two classes of CRISPR/Cas systems based on the nature of the effector nuclease that helps in targeting. It is either a multi-protein complex for class 1, or a single protein for class 2. The classification also includes six main types (I–VI) and over 19 sub types based on the CRISPR/Cas machinery and the mode of action of the system (Makarova *et al.*, 2011). For class 1, a canonical type I CRISPR–Cas system is seen, with the Cas3 exonuclease and Cascade. For class 2, a canonical

type II CRISPR–Cas system is shown, with the Cas9 endonuclease. In class 1, type I systems, the Cas3 exonuclease nicks and then degrades the target DNA strand while in type III systems, the Cas10 nuclease cleaves mRNAs in a ruler–anchor mechanism manner, which is coupled in some systems to target DNA degradation. The mechanism of targeting is still uncharacterized in type IV systems. In class 2 systems, the type II systems use the Cas9 endonuclease to generate two nicks that gives a double stranded DNA break while type V systems use Cas12 to generate two offset nicks. The type VI systems use Cas13 to get a cut in the target mRNA and then processes it non-specifically to produce a collateral damage. For type I, II, V and VI, interference depends on PAM sequences to initiate target recognition (Barrangou and Horvath, 2017).

The CRISPR/Cas system was first analyzed completely from *S. pyogenes* and is classified as type II system. As name suggests, CRISPR has short repeats of 21-40bp in length are unique among organisms but has similar length and separated from each other by spacers. Repeats are palindromic and the spacers has role in bacterial immunity, as they store the memory of the sequences from previous attacks. The number of spacers may vary in different species.

The single guide RNA (sgRNA) presents along with the CRISPR/Cas construct is user designed according to the target gene sequences to be edited. SgRNA has a 20-nucleotide spacer sequence that defines the genomic target to be modified (Sander and Joung, 2014). The tracrRNA is the universal sequence from *S. pyogenes* near to which the sgRNA is cloned in the construct. Cas9 and sgRNA can be introduced into the target cells by techniques like *Agrobacterium*-mediated transformation and biolistic transformation (Miao *et al.*, 2013). The efficiency of CRISPR/Cas9-medaited genome editing is depended on the use of promoters for sgRNA and the stability of Cas9 enzyme expression in the host system. In plants, RNA Polymerase II-dependent promoters such as CaMV35S are used for efficient expression of Cas9. RNA pol III dependent promoters such as U3 or U6 promoters were good for sgRNA expression (Kumar and Jain, 2015). Once the CRISPR/Cas construct is ready in a binary vector, it will be used to transform or cause editing in

the plant genome by following the *Agrobacterium* mediated transformation which was found to be the best way for plant transformation (Hiei *et al.*, 1994; Char *et al.*, 2016)

The Protospacer Adjacent Motif (PAM) is present just downstream to the target DNA. It is "NGG" for *S. pyogenes* (Barrangou, 2013; Tahir *et al.*, 2020). SgRNA recognizes the PAM sequence and binds to the respective region in target. Cas9 protein is activated on loading the guide RNA, which further undergoes a conformational rearrangement and form a central channel for RNA-DNA heteroduplex binding and recognition of canonical PAM motifs (Anders *et al.*, 2014). The Cas9 protein cleaves the target at a region 3 bp upstream of the PAM sequence (Jaganathan *et al.*, 2018). The double stranded break in the DNA is repaired by the natural repair system like Non-Homologous End Joining (NHEJ) which is highly error prone. NHEJ lead to the efficient introduction of insertion/deletion mutations (indels), which can interrupt the translational reading frame of a coding sequence or the transcription factors binding sites in promoters or enhancers in the genome (Cong *et al.*, 2013). This creates mutations at the target that lead to either activation or knock out of the gene (Barman *et al.*, 2019).

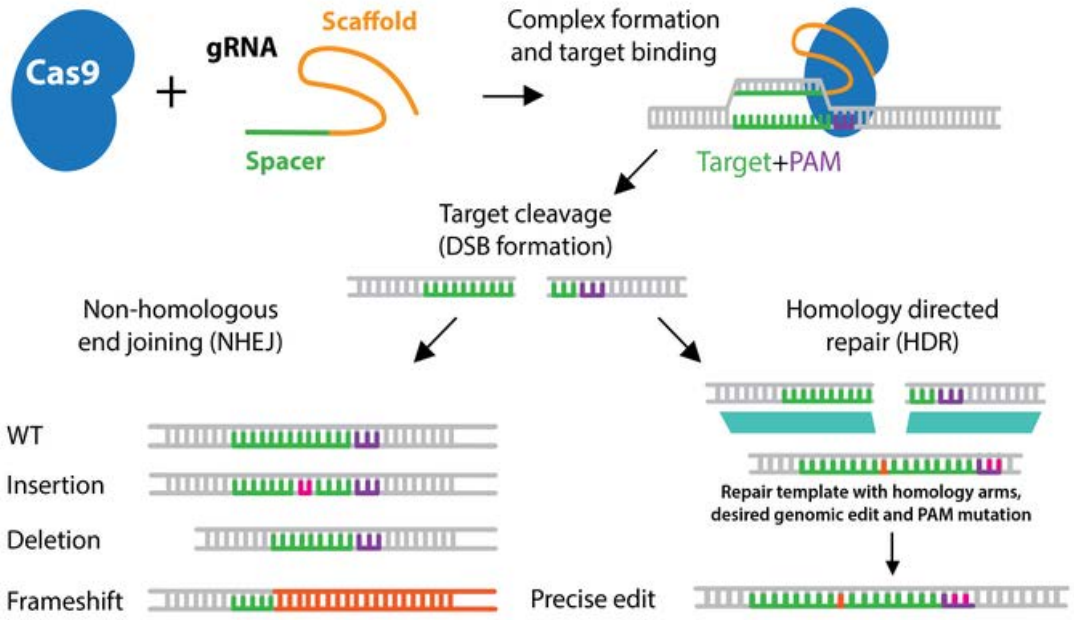


Figure 1.1 Overview of CRISPR/Cas9 system. The gRNA-Cas9 complex create double stranded break in genomic target. The break is repaired either by NHEJ or by HDR (adapted from https://www.addgene .org/crispr//)

2.6. CRISPR/Cas9 technology for abiotic stress tolerance in rice

CRISPR/Cas9 system as a tool for gene editing has been adopted in about 20 crop species till now (Ricroch *et al.*, 2017) for manipulating various traits including yield improvement, biotic and abiotic stress management (Jaganathan *et al.*, 2018). CRISPR/Cas9-based genome editing has been used to enhance crop disease resistance and also to improve tolerance to abiotic stresses like drought and salinity. Due to small genome size, availability of genetic resources, high transformation efficiency and greater genomic synteny with other cereals, it is well studied and serves as a model crop for monocots in functional genomics studies (Mishra *et al.*, 2018). The rice genome has abundance of potential PAM (1 in10 bp) sites (Xie and Yang, 2013). CRISPR technology can thus be used to target any trait of interest in the rice genome for developing new traits.

Shan *et al.* (2013) showed that sequence-specific CRISPR/Cas9 mediated genomic modification of three rice genes, *phytoene desaturase* (*OsPDS*), *betaine aldehyde dehydrogenase* (*OsBADH2*) and *mitogen-activated protein kinase* (*OsMPK2*) genes that are involved in regulating responses to various abiotic stress signals for the first time in crop plants *via* both protoplast as well as particle bombarded rice calli systems. The editing rates observed for *OsPDS* and *OsBADH2* genes were about nine and seven percent, respectively. Xie and Yang (2013) have explained a RNA-guided genome editing methodology by developing two vectors, pRGE3 and pRGE6 suitable for genome editing in rice. A negative regulator of biotic and abiotic stresses in rice *OsMPK5*, was selected for targeted mutagenesis using three guide RNAs and was tested in rice protoplasts. Off-targets were reduced by using more precise method of guide RNA designing (Jaganathan *et al.*, 2018). The mutation efficiency and off-target effects of few genes in rice like *OsDERF1, OsPMS3, OsEPSPS, OsMSH1* and *OsMYB5* was analyzed by Zhang and co-workers (2014). About 11 % homozygous lines were observed from T_2 generation.

The basic protocol for CRISPR/Cas9 has been developed in various crops through cumbersome trials. Shan *et al.* (2014) had demonstrated a method for the CRISPR/Cas platform in rice which throwed light for further genome editing in future. The credibility of this system of editing genome was found more efficient in

rice with less off- target effects. Thus, these aspects studied by Hui Zhang *et al.* (2014) found that about 11 target genes of two subspecies of rice inherited the mutations induced *via* CRISPR/Cas following the Mendelian laws in further generations.

Initially CRISPR/Cas system was used to search for the genes or transcription factors related to abiotic stress tolerance. Duan *et al.* (2016) have explained the role of rice *OsRAV2* transcription factor family in the induction of salt tolerance by identifying the *in-situ* and *ex-situ* promoters using CRISPR/Cas9. Similarly, another gene namely, the Annexin gene *OsAnn3*, a multigene family in rice when knocked down showed their role in cold stress tolerance (Shen *et al.*, 2017). The targeted editing ability of the CRISPR/Cas system further helped a lot to identify the genes involved in various stress related traits. Huang *et al.* (2018) explained the role played by the *OsNCED3* gene in increasing the multi-abiotic stress tolerance by knocking out this gene *via* CRISPR platform.

The *9-cis epoxycarotenoid dioxygenase* (*NCED*), which is an intermediate in ABA biosynthesis pathway is induced by NaCl, hydrogen peroxide stress and PEG, which provides the reason for its role in multi-stress tolerance. A similar kind of result was also found by Lou *et al.* (2018) by producing loss of function mutants for the *sucrose non-fermenting-1 related protein kinases SAPK1* and *SAPK2* using CRISPR/Cas9. The overexpression of these two genes in rice led to tolerance to drought, PEG and NaCl treatments. Sadanandom *et al.* (2019) also showed another target gene in rice that confers salt tolerance. *OsOTS1* is a SUMO protease encoding gene that was identified by him through CRISPR/Cas9 directed mutation. CRISPR/Cas9 system was found to be useful to easily knock out genes or create desirable mutations at the target sites in order to get required traits in rice. The *OsRR22* gene in rice when knocked out using Cas9-OsRR22-gRNA achieved 64.3% salinity tolerant mutant plants in T_0 transgenic plants and also without any exogenous T-DNA (Zhang *et al.*, 2019). The transporters present in the bio-membranes were also found to assist in providing salt tolerance to crop plants. The *HKT* and *NHX* type transporters were found to be activated by the salt ions (mainly Na^+). CRISPR technology was used to create variations in these transporters to enhance salt tolerance

in rice (Farhat *et al.*, 2019). A combined salt and drought tolerance was observed by the overexpression of the NAC transcription factor, *OsNAC2* which is targeted by microRNA, *miR164b*. This was achieved by preventing the microRNA from targeting the *OsNAC2* gene, which positively regulates the salt and drought tolerance in rice plants (Jiang *et al.*, 2019).

CRISPR/Cas9 targeted mutation in the rice ethylene responsive factor, *OsERF922* has been successfully established to enhance resistance to blast disease caused by *M. oryzae* (Liu *et al.*, 2012). The expression of the disease susceptibility gene, *OsSWEET13* in rice is essential for infection by *Xanthomonas oryzae* pv. *oryzae* to cause bacterial blight. Knock-out mutants of *OsSWEET13* produced using CRISPR/Cas9 that target its promoter led to improved resistance to bacterial blight disease in indica rice, IR24 (Zhou *et al.*, 2015).

A variant form of CRISPR/Cas which includes a chimeric guide RNA (cgRNA) was developed to go for HDR RNA repair prototype in rice. cgRNA directed repair helped in developing herbicide resistance in rice (Butt *et al.*, 2017). Different variations of CRISPR/Cas system have come in use for efficient and error less genome editing for enhancing the crop production. Many recent researches focus towards the scope of using CRISPR/Cas system in solving present and future problems in rice production.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The research work entitled 'Editing of rice transcription factor *OsMADS26* for drought tolerance through CRISPR/Cas9 system' was carried out at the Centre for Plant Biotechnology and Molecular Biology, College of Agriculture, Kerala Agricultural University, Vellanikkara, Thrissur, Kerala, India during the period from 2019 to 2021. This chapter includes the materials used and the methodologies adopted for the research work.

3.1. Materials

3.1.1. Plant material

Oryza sativa sub species *japonica* cultivar Nipponbare was used in the study The seeds were procured from DBT-National Institute of Plant Genome Research, New Delhi, India. The seeds were stored at room temperature for use. Callus initiated from mature seeds were used as explant for rice genetic transformation.

3.1.2. CRISPR/Cas9 vector

The CRISPR/Cas9 binary vector pRGEB32 was used in the study for designing guide RNA (gRNA) constructs. The vector was purchased from the Addgene, a non-profit plasmid repository. The culture was obtained as stab cultures. Cultures were revived on LB agar medium supplemented with Kanamycin. For long term storage, glycerol stocks were prepared and stored in deep freezers. Plasmids were isolated and used for cloning purpose.

3.1.3. Bacterial strains

The bacterial strains used in the study were obtained from DBT-National Institute of Plant Genome Research, New Delhi, India. The cultures were maintained as streak plates as well as glycerol stocks. *E. coli* strain DH5α was used for cloning and *Agrobacterium tumefaciens* strain EHA105 was used for rice genetic transformation.

3.1.4. Laboratory chemicals, glassware and plasticware

The chemicals used for this work were purchased mainly from, Sigma-Aldrich, Merck, Duchefa Biochemie, Netherlands, HiMedia laboratories, Thermo Scientific, Invitrogen, New England BioLabs (NEB), Macherey-Nagel (MN) and Puregene. All the chemicals used for plant tissue culture and genetic transformation of rice were plant culture tested. Other chemicals used were of molecular biology grade. DNA isolation, gel extraction, plasmid DNA isolation and PCR clean-up kit were purchased from MN. The primers and gRNAs were got synthesized from Sigma-Aldrich. Plasticwares purchased from Tarsons India Ltd. and glasswares from Borosil, India Ltd. were used in the study. The Sanger sequencing was done at Agrigenome Labs, Pvt., Ltd., Kochi.

3.1.5. Equipment and machinery

The research work was carried out using the facilities available at CPBMB, CoA, Vellanikkara, Thrissur, Kerala. The details of equipments and their model and make are provided in the table below

Table 1. List of equipments with their make and model

Sl. No.	Equipment name	Make and model
1	Thermal cycler	Agilent, Sure cycler 8800
2	Gel documentation system	Bio-Rad, Gel Doc XR+
3	Nanodrop spectrophotometer	Thermo Scientific, ND 1000
4	UV-transilluminator	Wealtec
5	Electrophoresis unit	Bio-Rad
6	Incubator shaker	Lab companion, SI-600
7	Centrifuge	HITACHI, Eppendorf 5418R
8	Heat block	Labnet
9	Pipettes	Eppendorf
10	Water bath	Rotek
11	Weighing balance	Shimadzu
12	pH meter	Eutech
13	Vortex mixer	GeNei^{TM}
14	Iceflaking machine	Icematic, F100 compact
15	Autoclave	Equitron
16	Refrigerator	LG, India

3.2. Methods

3.2.1. Retrieval of gene sequence data of *OsMADS26* gene

The sequence information of the rice transcription factor gene *OsMADS26* was obtained from the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu). The Locus ID of the genomic sequence of the gene was identified along with the location of the gene in the genome. The sequence of gene was downloaded as FASTA file and saved for use.

3.2.2. Designing of guide RNAs (gRNAs)

The guide RNAs (gRNAs) of 20 bp for editing of *OsMADS26* gene were designed using the free online tool CRISPR-P v2.0 (Xie and Yang, 2013). Best three guide RNAs were selected based on the on-score values, GC content, off-target sites and location in the genome. The PAM (Protospacer Adjacent Motif) was selected as NGG and gRNA scaffold from *Streptococcus pyogenes* (Meng *et al.*, 2017; Jiang *et al.*, 2013). For the expression of the gRNA cassette, SnoRNA promoter of rice OsU3 was selected (Char *et al.*, 2016). The guide RNAs were synthesized as top and bottom strand oligos separately from Sigma-Aldrich after adding BsaI restriction enzyme sites for facilitating cloning in to CRISPR/Cas9 vector.

3.2.3. Designing of primers

Primers were designed manually. Primers for amplification of partial gene sequences of *OsMADS26* from rice, hygromycin resistance (*hpt*) and *Cas9* gene of vector pRGEB32 were designed.

3.2.4. Evaluation of the primers

The primers designed were evaluated for their characteristics using the free online software 'Oligoevaluator', by Sigma-Aldrich. The primers were got synthesized from Sigma-Aldrich.

3.2.5. Primer dilution

The primers synthesized from Sigma-Aldrich (including the gRNA oligos) were resuspended in sterile nuclease free water to a final concentration of 100 µM as

prescribed in the primer details provided by the manufacturer. From this stock, working stock (10 µM or 10 picomoles) was prepared with 1:10 dilution. For this, 2.0 µL of primer was mixed with 18.0 µL of sterile nuclease free water.

3.2.6. Genomic DNA isolation of rice and estimation of DNA

3.2.6.1. Isolation of genomic DNA

Seeds of cultivar Nipponbare were germinated on moist cotton. Tissues were harvested when plants reached 4-5 leaf stage. DNA isolation was carried out from the leaves following CTAB method (Rogers and Bendich, 1994).

Reagents used

1. Extraction buffer preheated at 65ºC
2. 10 % CTAB solution
3. TE buffer (pH 8.0)
4. RNase A (10 mg/L)
5. Chloroform: Isoamyl alcohol (24:1)
6. Absolute alcohol, 70% ethanol
7. Isopropanol, PVP, β-mercaptoethanol
8. Liquid nitrogen

Procedure

- Leaf sample was ground in to fine powder using pre-chilled mortar and pestle with a pinch of PVP and 50.0 µL of β-mercaptoethanol using liquid nitrogen.
- The homogenized sample was transferred into a sterile 2.0 mL centrifuge tube and 1.0 mL of extraction buffer was added (Table 2).

Table 2: **Reagents used for CTAB method of DNA extraction**

Sl.No.	Reagent	Composition
1.	Extraction buffer/ CTAB buffer (2X)	2% CTAB (w/v) 100 mM Tris (pH 8.0) 20 mM EDTA (pH 8.0) 1.4 M NaCl 1 % PVP (PVP and β-mercaptoethanol added after autoclaving)
2.	10 % CTAB solution	10 % CTAB (w/v) 0.7 M NaCl
3.	TE buffer (pH 8.0)	10 mM Tris HCl (pH 8.0) 1.0 mM EDTA (pH 8.0)

- The contents were mixed thoroughly and incubated at 65°C for 30 min. with occasional mixing by gentle inversion.
- An equal volume of Chloroform: Isoamyl alcohol (24:1) was added and mixed by inversion. The mixture was centrifuged at 10,000 rpm for 15 min. at 4°C.
- The top aqueous phase was transferred to a clean microcentrifuge tube and 1/10th volume of 10% CTAB solution was added followed by an equal volume of Chloroform: Isoamyl alcohol (24:1). The contents were mixed gently by inversion.
- The contents were centrifuged at 10,000 rpm for 15 min. at 4°C.
- The aqueous phase was transferred to a clean tube and 2-3 µl of RNase A (10 mg/mL) added. The tubes were incubated at 37°C for 1.5 hrs. in a water bath.
- An equal volume of Chloroform: Isoamyl alcohol (24:1) was added to the tubes and the mixture was centrifuged at 10,000 rpm for 10 min. at 4°C.
- The upper aqueous phase was transferred to a fresh tube and 0.6 volume of chilled isopropanol was added and mixed by gentle inversion. The samples were incubated at -20°C for half an hour for complete precipitation.
- The samples were centrifuged at 10,000 rpm for 15 min. at 4°C and the supernatant was gently poured-off.

- The DNA pellet was washed twice with 70% ethanol.
- The pellet was air dried until the alcohol smell has disappeared and it was dissolved in 50.0 µL of TE buffer/sterile water and stored at -20°C for long term storage.

3.2.6.2. Estimation of quality and quantity of isolated DNA

Quality and quantity of the isolated rice genomic DNA was assessed using Nanodrop spectrophotometer and agarose gel electrophoresis (Sambrook *et al.*, 1989).

3.2.6.2.1. Agarose gel electrophoresis

Reagents used for electrophoresis

1. Tris acetic acid EDTA (TAE) buffer 50X (pH 8.0)

    - Tris buffer (1 M): 242.2 g/L
    - Glacial acetic acid: 57.1 mL
    - 0.5 M EDTA (pH 8.0): 100 mL
    - Total volume made: 1.0 L

2. Agarose (1%)

3. Tracking or loading dye (6X), Invitrogen Life Sciences

4. 1kb Plus DNA ladder, Invitrogen

5. Ethidium bromide (10 mg/mL stock)

Procedure

- The gel casting tray was cleaned using 70% ethanol and was placed properly in the gel caster with comb.
- Agarose gel was prepared by adding 0.6 g agarose to 60.0 mL of TAE (1X) buffer. The agarose solution was heated until the entire agarose melted completely.
- Gel was casted by adding of ethidium bromide to it and left to solidify at room temperature.

- After proper solidification of the gel, the comb was removed gently without breaking the wells and the gel was kept into the electrophoresis tank with 1X TAE buffer.
- Samples were loaded in 6X gel loading dye. One kb plus ladder was used for determining the approximate size of DNA.
- Electrophoresis was carried out at a voltage of 90 V and the gel was run until the dye reached bottom of the gel.

3.2.6.2.2. Nanodrop spectrophotometer

The concentration and purity of the isolated DNA was checked using the Nanodrop®ND-1000 spectrophotometer. The purity of the samples was estimated by measuring the absorbance at 260 and 280 nm and A_{260}/A_{280} ratio was noted.

To measure the absorbance of the samples, the pedestal of the nanodrop was wiped properly using clean tissue paper after switching on the system. Initially the pedestal was cleaned by loading 1.0 µL sterile water onto the lower pedestal and the sampling arm was closed. Then the blank was loaded to avoid the background noise. For samples, 1.0 µL was loaded and the absorbance was measured. The samples were considered pure only if the OD_{260}/OD_{280} ratio was between 1.8 and 2.0.

3.2.6.2.3. Gel documentation

The electrophoresed gel was initially visualized for the presence of bands in UV-transilluminator and then documented in Biorad gel documentation system using PDQuest™ software.

3.2.7. Confirmation of the *OsMADS26* gene in rice genome

The sequences of transcription factor gene *OsMADS26* flanking the gRNA target was confirmed by Polymerase Chain Reaction (PCR) using gene specific primers. The reaction was setup as described in Table 3. All the reagents except *Taq* DNA polymerase were thawed on ice before setting the reactions.

Table 3: **PCR reaction mix to confirm *OsMADS26* gene fragment**

Reagents	**Quantity**
Rice DNA	2.0 µL
10X buffer with $MgCl_2$	5.0 µL
dNTP mix (2.0 mM each)	4.0 µL
OsMADS26 FP (10 picomoles)	2.0 µL
OsMADS26 RP (10 picomoles)	2.0 µL
Taq DNA Polymerase	1.0 µL
Sterile nuclease free water	34.0 µL
Total reaction volume	50.0 µL

The PCR program was setup as shown in Table 4.

Table 4. **The PCR program for the amplification of *OsMADS26* gene fragment**

Initial denaturation	95°C	5 min.	
Denaturation	95°C	30 sec.	35 cycles
Annealing	60°C	30 sec.	
Extension	72°C	30 sec.	
Final extension	72°C	10 min.	
Hold	4°C	∞	

3.2.7.1. Analysis PCR product using agarose gel electrophoresis

The PCR amplified *OsMADS26* gene fragment was checked on 1.2 % agarose gel along with 1.0 kb Plus ladder.

3.2.7.2. Confirmation of the PCR product by Sanger sequencing

The PCR products were eluted from gel using Nucleospin Gel extraction kit (MN). The expected DNA fragment was excised from the agarose gel using clean, sharp scalpel and weighed. Gel elution was proceeded according to manufacturer's

instructions. The eluted PCR product was confirmed by Sanger sequencing. The samples were sequenced using gene specific forward and reverse primer.

3.2.8. Maintenance of bacterial cultures

The *E. coli* and *Agrobacterium tumefaciens* cultures were maintained as both streaked plates and glycerol stocks. Table 5 shows the antibiotic supplement required and culture conditions for each strain. Glycerol stocks (15%) were prepared from the liquid cultures of the bacteria. 850 µL of overnight grown culture was taken in sterile cryovials and mixed with 150 µL of sterile absolute glycerol and stored at -20°C for medium term up to one year.

Table 5. **Culture requirements for bacterial strains/clones used for the study**

Bacterial strain	Antibiotics used	Temperature of incubation
DH5α	LB agar	37°C
DH5α + pRGEB32	LB agar +50 mg/L kanamycin	37°C
EHA105	LB agar + 20 mg/L rifampicin	28°C
OsMADS26 gRNA clones in EHA105	LB agar +50 mg/L kanamycin + 20 mg/L rifampicin	28°C

Kanamycin stock of 50 mg/mL and Rifampicin stock of 20 mg/mL were prepared. Kanamycin stock was prepared in water and rifampicin stock was prepared in Dimethyl Sulfoxide (DMSO) and stored in -20°C for further use.

3.2.9. Cloning of OsMADS26 gRNAs to pRGEB32 vector

3.2.9.1. Isolation of pRGEB32 vector plasmid

The vector pRGEB32 plasmid DNA was isolated using the Nucleospin plasmid isolation kit (MN). The procedure for the isolation of high copy number plasmid from *E. coli* was followed with additional steps to increase the yield.

Procedure

- A single colony of the bacterial culture was inoculated from a freshly streaked plate to 5.0 mL LB broth containing Kanamycin (50 mg/L). The culture was incubated at 37°C at 180 rpm overnight (16-18 hrs.).
- Before starting isolation, the elution buffer (AE) was diluted to 1:2 using autoclaved nuclease free water and preheated in a dry block set at 70°C.
- The overnight grown bacterial culture was transferred to a sterile microcentrifuge tube (2.0 mL) and was pelleted down at 11,000 rpm for 30 sec.
- Buffer A1 (250 µL) was added to the culture and the contents were resuspended by vortexing.
- Buffer A2 (250 µL) was added and the contents were mixed rapidly by inverting 6-8 times. The samples were incubated at room temperature for up to 5 min.
- Buffer A3 (300 µL) was added and contents were mixed thoroughly by inverting tubes 6-8 times until the blue colour disappeared to colourless.
- The samples were centrifuged for 5-10 min. at 11,000 rpm at room temperature and the step was repeated until the supernatant is clear
- A NucleoSpin® column was placed in a 2.0 mL collection tube and the clear supernatant (maximum 700 µL) was pipetted out carefully into the center of the column.
- The samples were centrifuged at 11,000 rpm for 1 min. The flowthrough was discarded and the column was placed back to the collection tube.
- Now 600 µL of buffer A4 was added and the contents were centrifuged at 11,000 rpm for 1 min. The flowthrough was discarded and the column was placed back to the empty collection tube.
- The empty column was centrifuged for 2 min. at 11,000 rpm to dry the membrane and the collection tube was discarded.
- The column was placed into a clean autoclaved 1.5 mL microcentrifuge tube and 50.0 µL of AE buffer was added and incubated at 70ºC in dry block for 2 min.

- Then incubated at room temperature for 1 min. and centrifuged at 11,000 rpm for 1 min.

The plasmid DNA was checked using Nanodrop spectrophotometer and agarose gel electrophoresis (1%). The gel was documented using the gel documentation system. The samples were stored for further use at -20ºC.

3.2.9.2. Restriction digestion of pRGEB32 vector

The vector used in the current work, pRGEB32 contains BsaI restriction sites for gRNA cloning. The gRNAs were synthesized with BsaI cut sites at both ends.

Reagents used

1. BsaI restriction enzyme (NEB)
2. CutSmart buffer (NEB)
3. Nuclease free water

Procedure

The restriction digestion reaction was set up as follows (Table 6).

Table 6. Reaction set up for vector digestion

Reagents	Quantity	Reaction condition
pRGEB32 (pDNA)	25.0µL	Incubated at 37ºC for 2 hrs. and then heat inactivated the reaction at 65ºC for 10 min. in dry block
Nuclease free water	19.5 µL	
CutSmart buffer	5.0 µL	
Bsa1	0.5 µL	
Total reaction	50.0 µL	

The digested vector was checked on agarose gel and was visualized using gel documentation system. The digested vector was stored at -20ºC for cloning.

3.2.9.3. Purification of digested vector

The BsaI digested vector was purified using the Nucleospin® PCR clean-up kit. Before starting the procedure, ethanol was added to wash buffer. Volume of the digested vector was made up to 100 µL by adding 55.0 µL of sterile water.

- Two volumes of NTI buffer was mixed with every one volume of sample. 200 µL of buffer NTI was added to 100 µL of digested vector.
- A NucleoSpin® Gel and PCR Clean-up column was placed into the collection tube and the reaction mix was loaded to the column.
- The columns were centrifuged at 11,000 rpm for 30 sec.
- The NT3 buffer of 700 µL was added to the column and centrifuged at 11,000 rpm for 30 sec. and the flow-through was discarded.
- Empty collection tube was centrifuged for 1 min. at 11,000 rpm to remove the buffer NT3 completely.
- Prior to elution, the columns were incubated for 2 min. at 70ºC in dry block to remove ethanol. The column was placed into a clean autoclaved 1.5 mL microcentrifuge tube.
- Sterile water of 30.0 µL was added to the centre of the column and incubated at room temperature for two min followed by centrifugation at 11,000 rpm for 1 min.
- The eluted sample was stored at -20ºC for further use.

3.2.9.3. Guide RNA annealing

The guide RNAs synthesized as separate top and bottom strand oligos were annealed prior to the ligation with the digested vector. The gRNAs were annealed using either of the two following protocols.

3.2.9.3.1. The gRNA annealing protocol without PNK phosphorylation

Reaction components were used from NEB for this protocol.

Procedure

- The gRNA stock solutions (100 µM) were diluted to 1:10 and used as working solution for the annealing reaction.
- Each of the top and bottom strand of the diluted respective gRNAs of 1.0 µL were added to 48.0 µL of sterile nuclease free water in a PCR tube (0.2 mL) and the contents were mixed and a short spin was given.

- The reaction was incubated in thermal cycler and a program of 98°C for 5 min. was setup.
- After the PCR reaction, the samples were placed outside the thermal cycler to cool down to room temperature gradually.
- These samples were stored at 4°C until ligation reaction was set up.

3.2.9.3.2. The gRNA annealing protocol with PNK phosphorylation

This protocol was done with the components from Thermo-scientific**.**

Reagents used

1. 10x T4 DNA ligase buffer

2. T4 Poly Nucleotide Kinase (PNK)

3. gRNAs of Os*MADS26*

4. Autoclaved Sigma water

Procedure

- The required gRNAs were used for annealing without dilution.
- The reaction mix was prepared in PCR tubes (0.2 mL) and mixed well by giving a short spin. The annealing reaction was carried out in a thermal cycler.
- The annealing reaction was prepared as given in Table 7.

Table 7. **The gRNA annealing reaction with PNK**

Reagents	**Quantity**	**PCR program**
gRNA top strand	1.0 µL	• 37°C for 60 min. • 95°C for 10 min.
gRNA bottom strand	1.0 µL	
10X T4 DNA ligase buffer	1.0 µL	
T4 PNK	0.5 µL	
Autoclaved Sigma water	6.5 µL	
Total reaction	10.0 µL	

- After the incubation, the tubes were kept at room temperature to cool down gradually.

3.2.9.4. Ligation of gRNAs to the vector

The ligation of the gRNAs to the vector pRGEB32 was done using two different protocols as the annealing was done in two different ways.

3.2.9.4.1. Ligation of non-phosphorylated gRNAs

This protocol was done using the reaction components from NEB.

Reagents used

1. T4 DNA ligase buffer
2. T4 DNA ligase enzyme (HF)
3. Sterile nuclease free water
4. Annealed gRNAs

Procedure

- The annealed gRNAs were ligated to the vector pRGEB32 following the ligation reaction as described in Table 8.
- The reaction mix was prepared in 0.2 mL PCR tubes and incubated at 4ºC overnight.

Table 8. Ligation reaction (NEB) for non-phosphorylated gRNAs

Reagents	Quantity
pRGEB32 (digested and purified)	5.0 µL (~50 ng)
Annealed gRNAs	1.0 µL
10X T4 DNA ligase buffer	1.0 µL
T4 DNA ligase enzyme	1.0 µL
Nuclease free water	2.0 µL
Total reaction	10.0 µL

3.2.9.4.2. Ligation of phosphorylated gRNAs

Ligation reaction was setup using components from Thermo-scientific.

Reagents used

1. 10XT4 DNA ligase buffer

2. T4 DNA ligase enzyme (HF)

3. Sterile nuclease free water

4. Annealed gRNAs

Procedure

- The annealed gRNAs were diluted by adding 190.0 µL of sterile nuclease free water.
- The ligation reaction for phosphorylated gRNAs was set up as given in Table 9.
- The reaction mix was prepared in 0.2 mL PCR tubes and incubated at 22°C for 1 h in thermal cycler.
- After the incubation, the reaction mixture was stored at -20°C.

Table 9. Ligation reaction (Thermo-scientific) for phosphorylated gRNAs

Reagents	Quantity
pRGEB32 digested and purified	4.0 µL (~50 ng)
Annealed gRNAs	2.0 µL
10X T4 DNA ligase buffer	1.0 µL
T4 DNA ligase enzyme	1.0 µL
Nuclease free water	2.0 µL
Total reaction volume	10.0 µL

3.2.9.5. Competent cell preparation and transformation to *E. coli*

The *E. coli* strain DH5α was transformed with the ligation mixture containing ligated gRNAs in pRGEB32 binary vector. For this, the competent cells of DH5α were prepared and transformed using the 'Bacterial Transformation kit GeneSure™' from Puregene life sciences. The procedure given in the product manual was followed with minor modifications. All the procedures should be performed on ice and with gentle handling. All centrifugations were performed at room temperature.

Procedure (for two transformations)

Day 1:

- The C-medium provided with the kit was thawed to room temperature and 1.5 mL of C-medium aliquoted in to 2.0 mL tube and stored at 4°C.
- A single fresh colony of DH5α was inoculated into 2.0 mL of thawed C-medium taken in a falcon tube using a sterile loop.
- The culture was incubated overnight (16-18 hrs.) at 37°C with continuous shaking at 180 rpm in incubator shaker.

Day 2

- C-medium was pre-warmed at 37°C for at least 20 min.
- BT-solution was prepared by adding 250 µL of each of both BT-A and BT-B solutions was kept on ice.
- 150 µL of overnight grown culture of DH5α was added to 1.5 mL of pre-warmed C-medium and incubated at 37°C for 30 min. to 1h in an incubator shaker.
- The cells were centrifuged at 10,000 rpm for 1 min. at room temperature to pellet down the cells and the pellet was resuspended in 300 µL of BT-solution and incubated on ice for 5 min. Followed by centrifugation at 10,000 rpm for 1 min.

- The pelleted cells were resuspended in 120 µL of BT-solution and incubated on ice for 5 min. Divided it into two tubes of 60.0 µL each and kept on ice. The competent cells are ready now.
- The ligation mixture was then added to competent cells, mixed by gentle tapping and incubated on ice for 5 min.
- Total mixture (70.0 µL) was plated immediately on to the LB agar plate supplemented with the antibiotic Kanamycin (50 mg/L) and incubated at 37ºC overnight (16-18 hrs.) in incubator. The colonies were observed on next day and number of colonies were recorded.

3.2.10. Confirmation of the vector-gRNA construct in DH5α

The transformed DH5αcolonies were streaked on to LB agar plates supplemented with Kanamycin (50 mg/L), followed by colony PCR, plasmid isolation and Sanger sequencing to confirm recombinants/ positive clones.

3.2.10.1. Colony PCR of transformed colonies

Streaked colonies were used for colony PCR using forward primer specific to gRNA cloned and reverse primer specific to vector (Ratnayake and Hettiarachchi, 2010). Individual colonies were picked and dissolved in 10.0 µL of sterile water and used as template for PCR. Care was taken to avoid too much culture during resuspension.

Reagents used

1. 10X PCR buffer with $MgCl_2$

2. *Taq* DNA polymerase

3. dNTP mix (2.0mM each)

4. Sterile water (Sigma)

The PCR reaction was setup and program are given below (Table 10, Table 11)

Table 10. Colony PCR reaction set up

Reagents	**Quantity**
Template	10.0 µL
10x buffer with $MgCl_2$	2.5 µL
dNTP mix	2.0 µL
Forward primer (RA027)	1.0 µL
Reverse primer (RA055)	1.0 µL
Taq DNA polymerase	0.5 µL
Sterile water	8.0 µL
Total reaction	25.0 µL

Table 11. PCR program to amplify the gRNA cloned in pRGEB32 vector

Initial denaturation	95°C	10 min.	
Denaturation	95°C	30 sec.	35 cycles
Annealing	58°C	30 sec.	
Extension	72°C	20 sec.	
Final extension	72°C	10 min.	
Final hold	4 °C	∞	

The PCR products were analyzed on 1.4 % agarose gel to visualize the amplified products.

3.2.10.2. Plasmid isolation and Sanger sequencing

The PCR positive colonies were inoculated into the 3.0 mL LB broth containing Kanamycin (50 mg/L) in screw cap tubes. These liquid cultures were incubated at 37°C overnight with shaking at 180 rpm in the incubator shaker. These overnight grown liquid cultures were used to isolate plasmid DNA. The isolation was carried out using the Nucleospin plasmid isolation kit (MN) as described in section 3.2.9.1. The isolated sample was used for Sanger sequencing. Before sending the samples for sequencing, the plasmids were checked on 1% agarose gel and Nanodrop

spectrophotometer. About 15.0 µL of each sample were aliquoted to 1.5 mL tube and was properly labelled and packed to send for sequencing to 'Agrigenome Labs Pvt. Ltd, Kochi'. Universal M13 Reverse primer, present on the vector was used for sequencing the plasmids.

3.2.10.3. Analysis of sequencing results

The sequencing result obtained was analyzed for the presence of respective gRNAs using Clustal Omega multiple sequence alignment program and manual analysis. The sequence confirmed positive clones were further used for *Agrobacterium* genetic transformation. The glycerol stocks of the positive clones were prepared and maintained.

3.2.11. Transformation of the positive clones into *Agrobacterium* strain EHA105

The *OsMADS26* CRISPR/Cas9 constructs were further mobilized into EHA105 (Hood *et al.*,1986). The competent cells of EHA105 were prepared manually and transformed using Freeze-thaw method (Holsters *et al.*, 1978; de Framond *et al.*, 1983; Weigel and Glazebrook, 2006).

3.2.11.1. Preparation of Competent cells of EHA105

Competent cells were prepared following the Calcium chloride method. All the solutions were made with utmost care so that transformation efficiency of the cells was not affected. Entire procedure was carried out gently on ice so that the cells were not disturbed.

Reagents used

1. 0.5 M NaCl

2. 20 mM $CaCl_2$

3. Sterile absolute glycerol

4. Liquid nitrogen

Procedure

Day 1:

- Primary culture was given in 5.0 mL LB Broth supplemented with Rifampicin (20 mg/L) in a screw cap tube by inoculating a single colony of *A. tumefaciens* strain EHA 105 from freshly streaked plates (not older than 10 days).
- The culture was grown in an incubator shaker at 28°C with shaking at 220 rpm, overnight.

Day 2:

- Around 1.0 mL of primary culture was inoculated into 25.0 mL LB Broth in a 250 mL conical flask and incubated for 3-4 hrs. at 28°C, 220 rpm in incubator shaker till the OD_{600} reached > 0.5.
- The cell culture was transferred into pre-chilled sterile oakridge tube and kept on ice for 30 min.
- The culture was centrifuged at 5000 rpm for 5 min. at 4°C and the supernatant was discarded.
- The cell pellet was resuspended in 10.0 mL of pre-chilled 0.5 M NaCl by gentle swirling. The tubes were placed back on ice intermittently to avoid any heat shock injury to the cells.
- The cells were harvested at 5000 rpm for 5 min. at 4°C and the supernatant was discarded.
- The cell pellet was resuspended in 850 µL of pre-chilled 20 mM $CaCl_2$ by gentle swirling as in the previous step.
- Around 150 µL (13 drops) of chilled glycerol was added to cells mixed gently and aliquoted (100 µL) into 1.5 mL microcentrifuge tubes and stored at -80°C deep freezer after snap freezing in liquid nitrogen.

3.2.11.2. Genetic transformation of *A. tumefaciens* EHA105

The transformation of *Agrobacterium* strain EHA105 was done following the Freeze-thaw method (Holsters *et al.*, 1978).

Procedure

- The *Agrobacterium* competent cells were taken from -80℃ and thawed on ice for 20 min.
- 10.0 -15.0 µL (~500ng) of the isolated plasmid DNA of the positive clones were added into the competent cells and incubated on ice for 30 min.
- The cells were initially frozen in liquid nitrogen for 5 min. followed by thawing at 37℃ for 5 min. in a heat block.
- 900 µL of LB broth was added to the tubes and the tubes were incubated with gentle shaking at 28℃ (220 rpm) for 2-4 hrs.
- The cells were pelleted at 6000 rpm for 5 min. at room temperature and supernatant was discarded.
- The pellet was resuspended in 100 µL of LB broth and plated onto LB Agar plates supplemented with Kanamycin (50 mg/L) and Rifampicin (20 mg/L).
- The plates were incubated in dark (covered with aluminium foil) at 28℃ for 48 hrs. and number of colonies appeared were recorded.

3.2.12. Confirmation of the CRISPR/Cas9 construct in EHA105

The colonies obtained after transformation were further streaked onto fresh LB agar plates containing Kanamycin (50 mg/L) and Rifampicin (20 mg/L). The CRISPR/Cas9 constructs were further confirmed in EHA105 using colony PCR using hygromycin resistance gene specific primers and also using plasmid isolation followed by plasmid PCR. Colony PCR was done as mentioned in section 3.2.10.1, only the elongation time for the hygromycin specific primers (RA048 and RA049) of 1.5 min. is varied.

3.2.12.1. Plasmid isolation from EHA105

The plasmid isolation from EHA105 was carried manually following the Alkali lysis method (Green and Sambrook, 2016).

Reagents used

1. **Solution I:**

- Glucose – 50 mM
- EDTA - 10 mM
- Tris - 25 mM (pH 8.0)

  Stored at 4ºC

2. **Solution II:**

- 10 N NaOH
- 10 % SDS

For 1.0 mL solution 2: 20.0 µL 10 N NaOH+ 100.0 µL 10% SDS+ 880.0 µL water

The components were made separately as stocks and need not autoclave. Solution II was prepared freshly during each isolation.

3. **Solution III:**

- Potassium acetate – 3 M, pH 5.8
- 5 M Potassium acetate: 60.0 mL
- Glacial acetic acid: 11.5 mL
- Sterile water: 28.5 mL

Resulting solution is 3 M in concentration and stored at 4ºC.

Procedure

Day 1:

- The culture was given in 5.0 mL LB Broth supplemented with Kanamycin (50 mg/L) and Rifampicin (20 mg/L) in a screw cap tube by inoculating a single colony of *A. tumefaciens* strain EHA105 with respective gRNA construct from freshly streaked plates (not older than 10 days).
- The culture was grown in an incubator shaker at 28ºC with shaking at 220 rpm, overnight.

Day 2:

- The overnight grown culture was transferred into 2.0 mL tubes.
- The cells were harvested by centrifuging at 9000 rpm for 2 min. at 4°C and discarded the supernatant.
- The cell pellet was resuspended by vortexing in 200 µL of ice-cold solution I along with 5.0 µL of RNase A (2.0 mg/ mL).
- The vortexed cells were incubated at room temperature for 5-10 min.
- After incubation, 300 µL of ice cold freshly made solution II was added and inverted rapidly for about 5-6 times to mix the contents thoroughly.
- The cells were then incubated at room temperature for 5 min. (time strict).
- To these cells added 300 µL of ice-cold solution III and mixed the contents by gentle inversion.
- Mixed cells were then incubated on ice for 5 min. (not more than 5 min).
- The cells were centrifuged at 13,000 rpm for 10 min. at room temperature.
- Supernatant was transferred to clean 1.5 mL tube and equal volume of chilled isopropanol was added to precipitate the plasmid DNA.
- The contents were mixed gently by inversion.
- The tubes were further centrifuged at 13,000 rpm for 10 min. at room temperature.
- Supernatant discarded and the DNA pellet was washed with 70 % ethanol.
- Centrifuged the tubes at 13,000 rpm for 5 min. at room temperature to remove the ethanol content.
- Discarded the ethanol completely from the tube.
- Air dried the pellet for 10-15 min. until the ethanol was dried up completely.
- The DNA pellet was dissolved in 50.0 µL of sterile water and stored at -20°C for long term usage.

3.2.12.2. Confirmation of the plasmid isolated from EHA105

3.2.12.2.1. Amplification of hygromycin resistance gene using plasmid PCR

Hygromycin resistance (*hpt*) gene in vector pRGEB32 facilitates for the plant selection after plant genetic transformation. Recombinant plasmids in EHA105 were confirmed by plasmid PCR using primers specific to hygromycin resistance gene (Ratnayake and Hettiarachchi, 2010). Empty pRGEB32 plasmid was also used as a control.

Reagents used

1. 10X PCR buffer with $MgCl_2$
2. *Taq* DNA polymerase
3. dNTP mix (2.0 mM each)
4. Sterile nuclease free water (Sigma)

The PCR reaction was setup as given in Table 12.

Table 12. **Plasmid PCR reaction set up**

Reagents	**Quantity**
Template plasmid DNA	1.0 µL
10X buffer with $MgCl_2$	2.5 µL
dNTP mix	2.0 µL
Forward primer	1. 0 µL
Reverse primer	1.0 µL
Taq DNA polymerase	0.5 µL
Sterile nuclease free water	17.0 µL
Total reaction volume	25.0 µL

The PCR program was setup as given in Table 13.

Table 13. **PCR program setup for plasmid PCR**

Initial denaturation	95°C	10 min.	
Denaturation	95°C	30 sec.	35 cycles
Annealing	58°C	30 sec.	
Extension	72°C	1.5min.	
Final extension	72°C	10 min.	
Final hold	4°C	∞	

The PCR products were visualized on 1.0 % agarose gel.

3.2.13. Rice genetic transformation

The plant material used for the current work was *O. sativa* ssp. *japonica* cv. Nipponbare. Transformation of the CRISPR/Cas9 construct into rice was carried out by *Agrobacterium* mediated transformation following the protocols developed by Toki *et al.*, 2006 and Sahoo *et al.*, 2011, with minor modifications.

3.2.13.1. Media preparation

Compositions of the various culture media like callus induction, co-cultivation, selection and regeneration are provided in Table 14. The media was sterilized for 20 min. at 121^0C and 15 p.s.i. in standard autoclave or pressure cooker.

Hygromycin (50 mg /L) and Augmentin (300 mg/L) were added to selection medium after autoclaving. The composition of resuspension medium for *Agrobacterium* is given in Table 15.

Table 14. Composition of various media in rice genetic transformation

Components	**Callus induction (CIM) mg/L**	**Co-cultivation (N6-AS) mg/L**	**Selection (CIM+ antibiotics) mg/L**	**Regeneration (RE) mg/L**
N6 salts (Duchaefa)	4000	4000	4000	MS salts (4.4g/L)
Organic components				
Casein hydrolysate	300	300	300	2000
L- Proline	2878	-	2878	-
myo- Inositol	100	100	100	100
Phytohormones				
2,4-D	3.0	3.0	3.0	-
NAA				0.02
Kinetin				2.0
Acetosyringone (AS)		150 µM	150 µM	
Carbon source				
Maltose	30,000	30,000	30,000	30,000
Sorbitol	-	-	-	30,000
Phytagel	0.4%	0.4%	0.4%	0.4%
pH	5.8	5.2	5.8	5.8

The 150 mM. stock solution of acetosyringone was prepared by dissolving 0.195g acetosyringone in 5.0 mL of 100 % Dimethyl Sulfoxide (DMSO) and filter sterilized. Aliquots were made for single use. The tubes were covered with Al foil and stored at -20°C.

Table 15. Composition of *Agrobacterium* resuspension medium (MSR)

Components	g/L
MS salts	4.0
Maltose	68.0
Glucose	36.0
KCl	3.0
$MgCl_2$	4.0
Acetosyringone	150 µM
pH	5.2

3.2.13.2. Callus induction from rice seeds

3.2.13.2.1. Dehusking of seeds

The seeds were dehusked manually with utmost care, not to damage the embryo. Only visually healthy, bold seeds were used for callusing. Immature and darkened seeds with damaged embryo were discarded.

3.2.13.2.2. Seed sterilization

Rice seeds were subjected to surface sterilization prior to inoculation into callus induction medium (CIM). Surface sterilization was carried out using standard protocols (Sahoo *et al.*, 2011). About 200- 400 seeds were inoculated for each set of transformation.

Seeds were taken in 250 mL conical flask and were treated with 70% ethanol for 1 min with intermittent shaking followed by washing with sterile distilled water twice. The seeds were surface sterilized with 2.5% of Sodium hypochlorite (4% w/v) with a drop of Tween20 for 30 min. with intermittent shaking. The seeds were rinsed with autoclaved distilled water for about 5-8 times or until the foam disappeared. The seeds were blot dried on sterile filter paper for 15-20 min. before inoculation.

3.2.13.2.3. Rice seed inoculation

The seeds were inoculated into the petri plates containing callus induction medium after proper blot drying. On an average 25-30 seeds were inoculated in each plate. The plates were sealed properly with parafilm and were kept under continuous white light for 5 days at 32°C. Separate sets of seeds were inoculated for *OsMADS26* gRNA constructs, vector control and wild type (untransformed).

3.2.13.3. Co-cultivation of calli

3.2.13.3.1. *Agrobacterium* culture preparation

Single colony from confirmed EHA105 clones were inoculated into 5.0 mL LB broth supplemented with antibiotics Kanamycin (50 mg/L) and Rifampicin (20 mg/L). The primary culture was incubated at 28°C with 220 rpm shaking for overnight. From the primary culture, 1.0 mL culture was inoculated into 25.0 mL LB broth supplemented with proper antibiotics to initiate secondary culture.

3.2.13.3.2. Co-cultivation of calli with *Agrobacterium*

When the OD of the secondary culture reached 0.1 (after 2-4 hrs.), the culture was placed on ice to arrest further growth of the bacterium. The cultures were further transferred to pre-chilled oakridge tubes and centrifuged at 8000 rpm for 15 min. at 4°C to collect the cells. *Agrobacterium* cells were resuspended in resuspension medium supplemented with 150 µM acetosyringone. The cells were mixed by gentle mixing.

The calli for infection were transferred to sterile conical flask and to this the resuspended *Agrobacterium* culture harboring desired gRNA construct was added. Infection was given for 1.5-2 min. at room temperature with gentle intermittent shaking at dark. The bacterial suspension was decanted and the calli were blot dried on sterile filter papers. Calli were then transferred to co-cultivation media over laid with Whatman No. 1 filter paper disc moistened with liquid MSR medium. Petri plates were sealed with parafilm. The plates were incubated at dark for two days at 28°C.

3.2.13.4. Selection of co-cultivated calli on selection medium

3.2.13.4.1. Washing of co-cultivated calli to remove *Agrobacterium*

The calli were transferred to sterile conical flasks for washing. The calli were initially washed two times with autoclaved distilled water with continuous shaking. Thereafter, the calli were washed with sterile distilled water containing Augmentin (300 mg/L) 3-4 times for 15 min. each with continuous shaking. After washing, the calli were blot dried on plates containing sterile filter paper.

3.2.13.4.2. Inoculation of calli on to selection medium

After blot drying for around 30 min., the calli were carefully inoculated on to the selection medium containing Hygromycin (50 mg/L) and Augmentin (300 mg/L) using sterile forceps. 22-25 calli were placed in each plate on an average. These plates were incubated under continuous white light for 14 days at 28°C. The calli were observed daily for any contamination, *Agrobacterium* over growth and other morphological changes. Plates showing *Agrobacterium* resurgence were sub-cultured as and when required.

3.2.13.5. Regeneration of selected calli

The transformed calli which showed proliferation of micro-calli were selected and transferred to regeneration medium (RE) supplemented with NAA (0.02 mg/L) and Kinetin (2.0 mg/L) along with Hygromycin and Augmentin. The calli were trimmed carefully to remove dead and browning tissues before inoculation in to regeneration media. The plates were kept for regeneration in culture racks set at photoperiod of 16 hrs light/8 hrs dark at 28°C.

RESULTS

4. RESULTS

The results of the study on 'Editing of rice transcription factor *OsMADS26* for drought tolerance through CRISPR/Cas9 system' was undertaken during the period from 2019 to 2021 at the Centre for Plant Biotechnology and Molecular Biology, College of Agriculture, Kerala Agricultural University, Vellanikkara, Thrissur, are presented in this chapter.

4.1. Procurement of CRISPR/Cas9 vector pRGEB32

The CRISPR/Cas9 binary vector pRGEB32 (Xie *et al.*, 2014) was procured from 'Addgene' (catalog number 63142). The vector contains rice codon optimized *Cas9* gene under rice ubiquitin promoter, BsaI restriction site for guide RNA (gRNA) cloning and selectable markers for both bacteria (Kanamycin) and plant (Hygromycin) selection after genetic transformation. The single guide RNA (sgRNA) cassette is cloned under the control of rice U3 promoter (Figure 4.1).

4.2. Retrieval of *OsMADS26* gene sequence

The sequence information of *OsMADS26* gene was downloaded from the 'Rice Genome Annotation Project'(http://rice.plantbiology.msu.edu) and the locus ID and genomic position were identified (Figure 4.2). The locus ID of *OsMADS26* gene was LOC_Os08g02070 and the gene was found to be located on chromosome number 8 of rice genome. The FASTA file of the sequence was downloaded for further studies.

4.3. Designing of guide RNAs (gRNAs)

Guide RNAs for the *OsMADS26* gene were designed using the online tool CRISPR-P v2.0 (Xie and Yang, 2013) and the best three gRNAs were selected based on their on-score value, off-target sites, GC content and location in genome (Figure 4.3). The gRNAs located on the coding sequences (CDS) were majorly considered. The first two gRNAs were selected from the CDS region and the third gRNA was selected from the 5'UTR (untranslated region). In other words, the first two gRNAs were selected from the plus strand and the third gRNA was from the minus strand (Table 16, Table 17). The selected gRNAs were analyzed using the online tool 'Oligoevaluator' before synthesis to check general properties like primer secondary structure and primer

dimer. The restriction sites for the restriction enzyme BsaI were added to top and bottom strands before ordering.

Table 16. The gRNAs selected for *OsMADS26* gene

Sl. No.	Guide RNA name	Strand	Sequence (5'- 3')
1	OsMADS26 gRNA 1 (G1)	Top strand	GGAGCTCTCCATCCTCTGCG
		Bottom strand	GGCCTTCTTCAGCAGGCCGG
2	OsMADS26 gRNA 2 (G2)	Top strand	CGCAGAGGATGGAGAGCTCC
		Bottom strand	ACTAGTTTGGACTAGCTTCG
3	OsMADS26 gRNA 3 (G3)	Top strand	CCGGCCTGCTGAAGAAGGCC
		Bottom strand	CGAAGCTAGTCCAAACTAGT

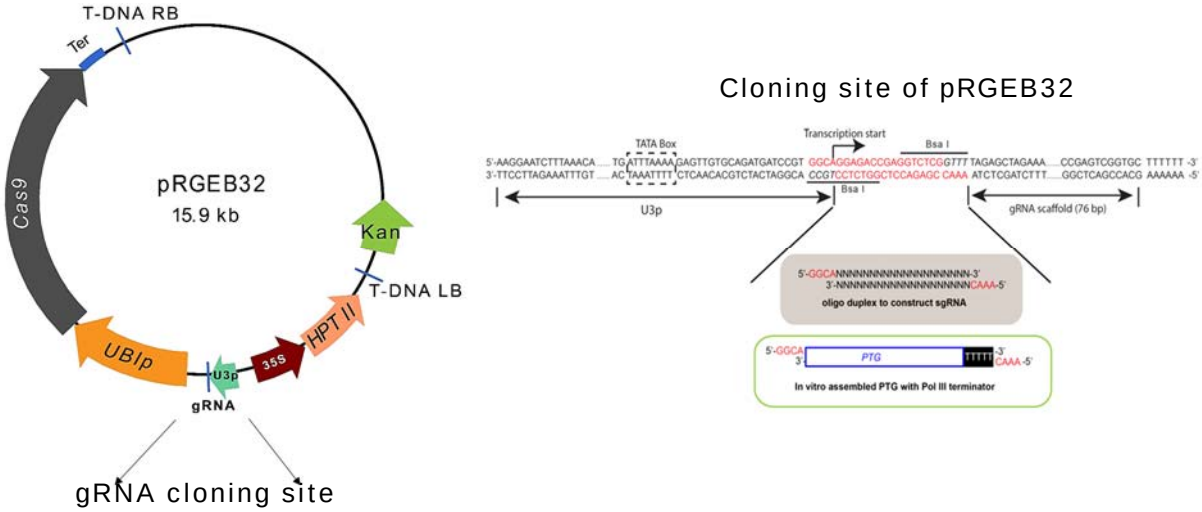


Figure 4.1. Vector map of pRGEB32 vector

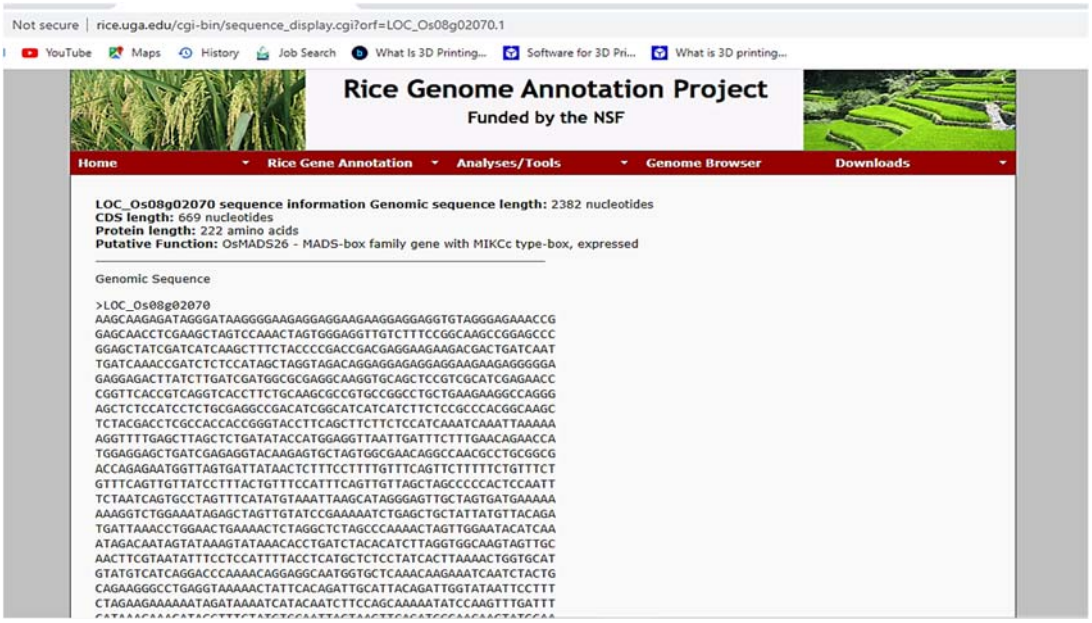


Figure 4.2. FASTA sequence of OsMADS26 obtained from Rice Genome Annotation Project

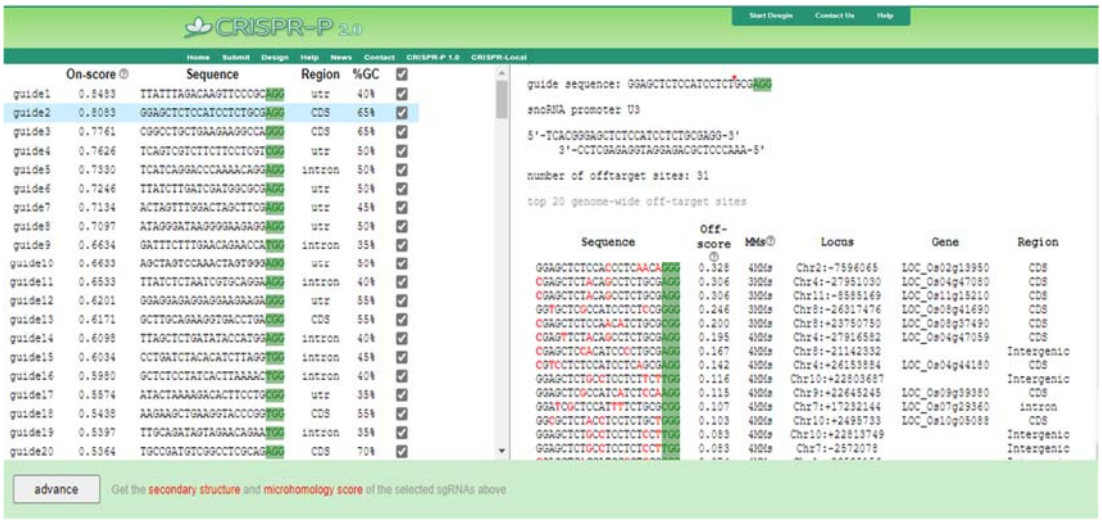


Figure 4.3. CRISPR-P v2.0 result window of first gRNA selected

Table 17. Details of the features of the gRNAs selected for the study

gRNAs	On-score value	Location in genome	Length (bp)	GC content (%)	PAM	off-target sites (No.)
gRNA 1	0.808	CDS (+357bp)	20	65	AGG	31
gRNA 2	0.022	CDS (+335bp)	20	70	AGG	39
gRNA 3	0.713	UTR (-68bp)	20	45	AGG	11

4.4. Designing and evaluation of primers

Different primer sets were designed for the study. Primers were designed manually and the primers were evaluated using the online tool 'Oligoevaluator' to check the GC content, Tm, number of GC clamp, primer dimer and secondary structure if any. The primers designed and used in the study are listed and used are presented in Table 18 and Table 19.

Table 18. List of primers designed for the study

Sl. No.	Purpose	Name of primer	Sequence (5'- 3')
1	Primers for amplifying partial genomic region of *OsMADS26* gene for flanking gRNA target	*OsMADS26* FP	GGATAAGGGGAAGAGGAGGA
		OsMADS26 RP	TGATGGAGAAGAAGCTGAAGG
2	Primers for amplifying hygromycin resistance gene	Hygro FP	ATGAAAAAGCCTGAACTCACCGC
		Hygro RP	CTTTGCCCTCGGACGAGTGCTG
3	Primers for amplifying partial Cas9 gene	Cas9 FP	GACGAGTACAAGGTGCCCAG
		Cas9 RP	TTTTCCAGGATGGGCTTGAT
4.	M13 Rverse primer for sequencing plasmid clones	M13 RP	GAATTTGTGGACCTGCAGGC

Table 19. Features of primers designed for the study

Sl. No	Primer	Length (bp)	Tm (^{0}C)	GC content (%)	GC clamp (No.)	Primer dimer (Yes/No)	Secondary structure (Yes/No)
1	*OsMADS26* FP	20	63.2	55	2	No	No
2	*OsMADS26* RP	21	63.4	47.6	2	No	No
3	Hygro FP	23	69.2	47.8	4	No	Yes (Very weak)
4	Hygro RP	22	74.1	63.6	2	No	Yes (Weak)
5	Cas9 FP	20	64.7	60	3	No	No
6	Cas9 RP	20	65.5	45	1	No	Yes (Weak)
7	M13 RP	20	66.8	55	3	No	No

4.5. Dilution of primers and gRNAs

All the primers and gRNAs were initially diluted to 100 µM concentration using sterile nuclease free water. The oligonucleotides were stored at -20˚C till further use.

4.6. Genomic DNA isolation of rice and estimation of quality and quantity of isolated DNA

Rice genomic DNA was isolated using CTAB method and quality and quantity of isolated DNA was checked using Nanodrop spectrophotometer. The isolated DNA had a concentration of about 351.2 ng/µL with an absorbance ratio of A_{260}/A_{280} of 1.97. Further, the DNA was electrophoresed in 0.8% gel at 90 volts and the presence of genomic DNA was visualized and documented using gel documentation unit (Figure 4.4). A clear band of genomic DNA was observed. Presence of RNA was also detected in the gel.

4.7. Confirmation of the *OsMADS26* gene sequences in rice genome

OsMADS26 gene was confirmed by Polymerase Chain Reaction (PCR) using the isolated rice genomic DNA with the gene specific primers. A clear sharp band was obtained at around 450 bp in the 1.2% agarose gel (Figure 4.5). The PCR product was

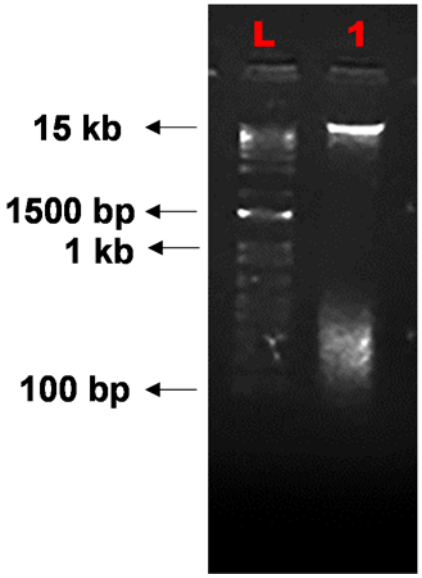


Figure 4.4. Gel picture of isolated rice DNA

L- 1kb Plus Ladder, **1**- Rice DNA isolated using CTAB method

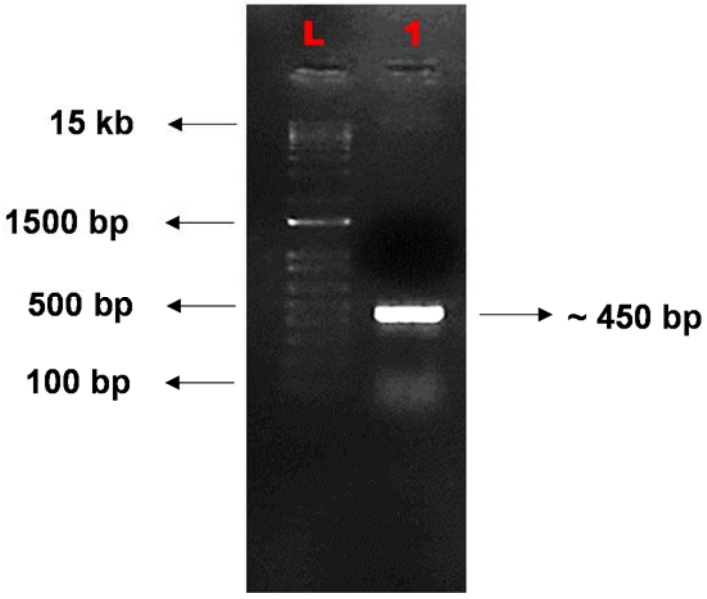


Figure 4.5. Gel picture of PCR amplified *OsMADS26* gene (~450 bp)

L- 1 kb Plus Ladder, **1**- PCR amplified *OsMADS26* gene from rice genome

eluted and sequenced. The sequencing result was analyzed using BLAST and Clustal omega programs to confirm the correctness of sequences. The sequencing result showed 100% sequence similarity with *OsMADS26* gene of reported japonica cultivar Nipponbare (Figure 4.6 and Figure 4.7).

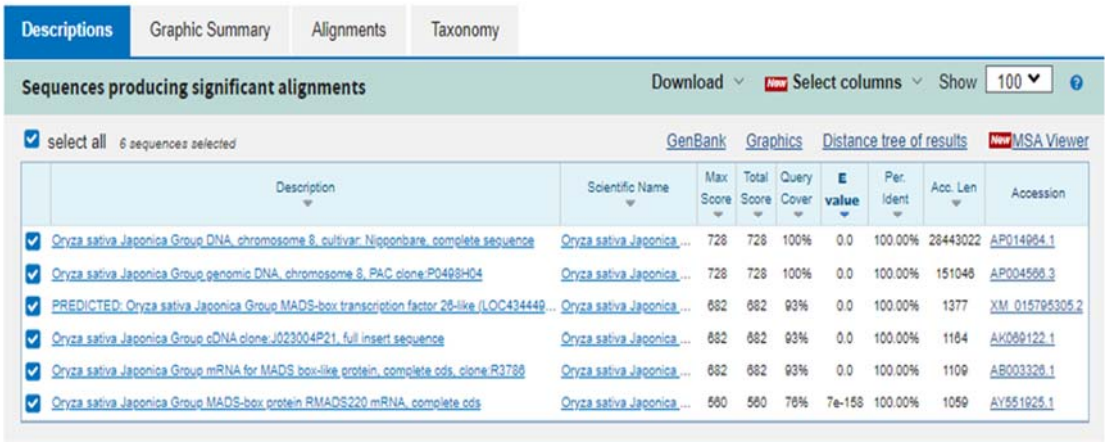


Figure 4.6. Blast result of *OsMADS26* PCR product

```
OsMADS26 PCR product ----------------------GCTAGTCCAAACTAGTGGGAGGTTGTC
LOC_08g02070 GGAGAAACCGGAGCAACCTCGAAGCTAGTCCAAACTAGTGGGAGGTTGTC
                                           ****************************

OsMADS26 PCR product TTTCCGGCAAGCCGGAGCCCGGAGCTATCGATCATCAAGCTTTCTACCCC
LOC_Os08g02070 TTTCCGGCAAGCCGGAGCCCGGAGCTATCGATCATCAAGCTTTCTACCCC
               ************************************************

OsMADS26 PCR product GACCGACGAGGAAGAAGACGACTGATCAATTGATCAAACCGATCTCTCCA
LOC_Os08g02070 GACCGACGAGGAAGAAGACGACTGATCAATTGATCAAACCGATCTCTCCA
               ************************************************

OsMADS26 PCR product TAGCTAGGTAGACAGGAGGAGAGGAGGAAGAAGAGGGGGAGAGGAGACTT
LOC_Os08g02070 TAGCTAGGTAGACAGGAGGAGAGGAGGAAGAAGAGGGGGAGAGGAGACTT
               ************************************************

OsMADS26 PCR product ATCTTGATCGATGGCGCGAGGCAAGGTGCAGCTCCGTCGCATCGAGAACC
LOC_Os08g02070 ATCTTGATCGATGGCGCGAGGCAAGGTGCAGCTCCGTCGCATCGAGAACC
               ************************************************

OsMADS26 PCR product CGGTTCACCGTCAGGTCACCTTCTGCAAGCGCCGTGCCGGCCTGCTGAAG
LOC_Os08g02070 CGGTTCACCGTCAGGTCACCTTCTGCAAGCGCCGTGCCGGCCTGCTGAAG
               ************************************************

OsMADS26 PCR product AAGGCCAGGGAGCTCTCCATCCTCTGCGAGGCCGACATCGGCATCATCAT
LOC_Os08g02070 AAGGCCAGGGAGCTCTCCATCCTCTGCGAGGCCGACATCGGCATCATCAT
               ************************************************

OsMADS26 PCR product CTTCTCCGCCCACGGCAAGCTCTACGACCTCGCCACCACCGGGTACCTTC
LOC_Os08g02070 CTTCTCCGCCCACGGCAAGCTCTACGACCTCGCCACCACCGGGTACCTTC
               ************************************************
```

Figure 4.7. Multiple sequence alignment of *OsMADS26* PCR product with *OsMADS26* gene

4.8. Maintenance of bacterial strains and plasmids

The *E. coli* and *Agrobacterium tumefaciens* strains were maintained in both LB agar plates and glycerol stocks supplemented with suitable antibiotics. *Agrobacterium* strains were streaked on LB agar medium with Rifampicin and incubated at 28°C for 48 hrs. (Plate 4.1 A). DH5α was streaked and maintained in plain LB agar (Plate 4.1 B). The binary vector pRGEB32 was maintained LB agar medium supplemented with Kanamycin (Plate 4.1 C). The cultures were re-streaked at regular intervals of every 30 days to maintain the cultures.

4.9. Cloning of *OsMADS26* gRNAs to the CRISPR/Cas9 vector pRGEB32

4.9.1. Isolation of pRGEB32 plasmid and quality analysis

The pRGEB32 plasmid DNA was isolated using the Macherey Nagel (MN) plasmid isolation kit and the quality was checked using Nanodrop and agarose gel electrophoresis. The yield of plasmid DNA was approximately 93.14 ng/ µL. Agarose gel electrophoresis showed a clear band of around 15.9 kb which was the expected size (Figure 4.8). The plasmid DNA was stored at -20ºC for further cloning experiments.

4.9.2. Vector digestion, gRNA annealing and ligation

The BsaI digested pRGEB32 and annealed gRNAs were ligated into the vector. The digested plasmid was checked on agarose gel which showed the linearization of vector indicating complete digestion (Figure 4.9).

The top and bottom strands of gRNAs were annealed and used for ligation in to digested pRGEB32 vector. The gRNAs, G1 and G2 were annealed following phosphorylation of ends with PNK (Poly Nucleotide Kinase), and G3 without phosphorylation.

4.9.3. Competent cell preparation and genetic transformation of *E. coli,* DH5α

The competent cells of DH5α were prepared and transformed using the 'Bacterial Transformation kit of GeneSure™'. Bacterial colonies for each guide construct were obtained on the plates after overnight incubation (Table 20; Plate 4.2).

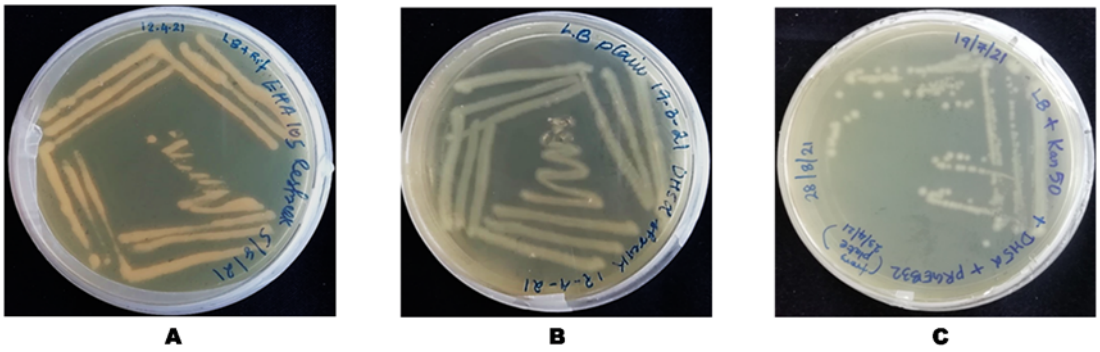


Plate 4.1. Bacterial cultures used in the study

A - *Agrobacterium* strain EHA105 culture plate

B - Culture plates of DH5α

C - DH5α transformed with pRGEB32 vector

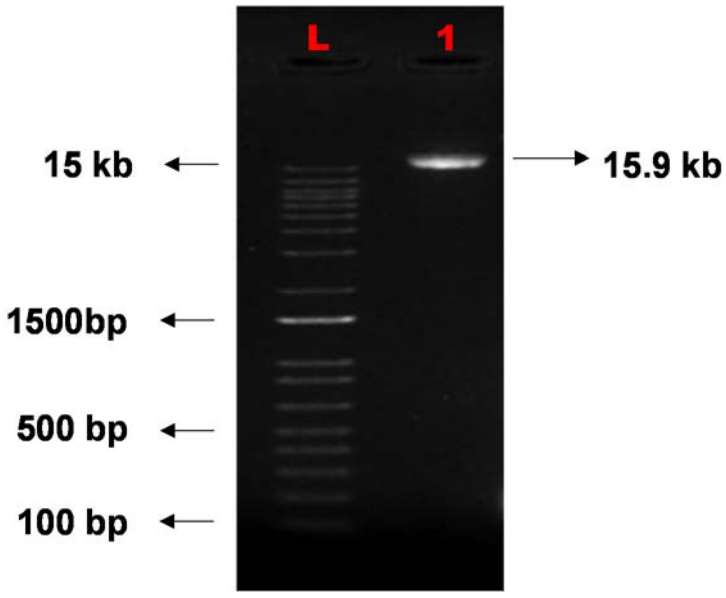


Figure 4.8. Gel picture of pRGEB32 isolated using MN kit

L- 1 kb Plus Ladder, **1**- pRGEB32 isolated using MN plasmid isolation kit

Table 20. The number of colonies obtained for each gRNAs in DH5α

gRNA	No. of colonies observed
gRNA 1 (G1)	9
gRNA 2 (G2)	3
gRNA 3 (G3)	4

4.9.4. Confirmation of positive clones in DH5α

The colonies were initially screened by streaking the colonies onto fresh LB agar plates supplemented with antibiotic Kanamycin (Xie and Yang, 2013) which showed bacterial growth after overnight incubation (Plate 4.3 A and B). Colony PCR was performed with vector and gRNA specific primers (Figure 4.10 A and B). Clear bands of expected size of 250 bp were observed on 1.5% agarose gel. The constructs were labelled as OsMADS26#G1, #G2 and G3 respectively based on the gRNA transformed. Three colonies each were selected and used for further plasmid isolation and sequencing. Plasmids isolated from the colonies were checked for the concentration before sequencing, the results of which are presented in Table 21.

Table 21. Estimation of yield of plasmids isolated from DH5α colonies

Sl. No	Construct	Colony number	Nanodrop concentration (ng/µL)
1	OsMADS26# G1	4	18.4
2	OsMADS26# G2	1	34.1
3	OsMADS26# G3	1	51.3

The isolated plasmids were sequenced using universal M13 reverse primer and the sequence result was confirmed using multiple sequence alignment (Figure 4.11). The insertion of gRNA in the vector was also identified by searching the gRNA

sequences in the chromatogram obtained from sequencing result (Figure 4.12 A and B) of OsMADS26#G1 and #G3. For OsMADS26#G1 colony-1 (pRGEB32:OsMADS26#G1-1) was found to be positive clone and for OsMADS26#G3, colony-3 was found to be positive (pRGEB32:OsMADS26#G3 -3).

After sequencing and sequence analysis, positive clones were obtained only for OsMADS26# G1 and OsMADS26#G3. No positive clones were obtained for OsMADS#G2. Hence further experiments were carried out using OsMADS26# G1 and OsMADS26#G3 only.

4.10. Mobilization of CRISPR/Cas9 gRNA construct into *A. tumefaciens*

The CRISPR/Cas9 gRNA construct for OsMADS26#G1 and G3 were further mobilized into EHA105 strain of *A. tumefaciens* (Hood *et al.*,1986). The competent cells of EHA105 were prepared using Calcium chloride method (Cohen *et al.*, 1972). The recombinant plasmids, pRGEB32:OsMADS26#G1-1 and pRGEB32:OsMADS26#G3 -3 were mobilized to EHA105 using Freeze-Thaw method (Holsters et al, 1978). After 48 hrs. of incubation at 28˚C, colonies were observed in both G1 and G3 transformed plates (Plate 4.4 A and B). The number of colonies obtained in each plate were counted and recorded (Table 22).

Table 22. Number of colonies obtained for each gRNA construct in *A. tumefaciens* EHA105

Construct name	No. of colonies
OsMADS26#G1	13
OsMADS26#G3	12

4.11. Confirmation of the CRISPR/Cas9 gRNA construct of *OsMADS26* in EHA105

Few colonies were streaked freshly onto LB agar plates containing Kanamycin (50 mg/L) and Rifampicin (20 mg/L) (Plate 4.5 A and B). The clones were confirmed by colony PCR using primers specific to hygromycin resistance gene. Expected amplicon size of around 1.0 kb was observed on 1% agarose gel (Figure 4.13).

The plasmids isolated manually from EHA105 colonies were confirmed by PCR amplification of the *hpt* gene. The PCR products were run on 1% gel and clear bands

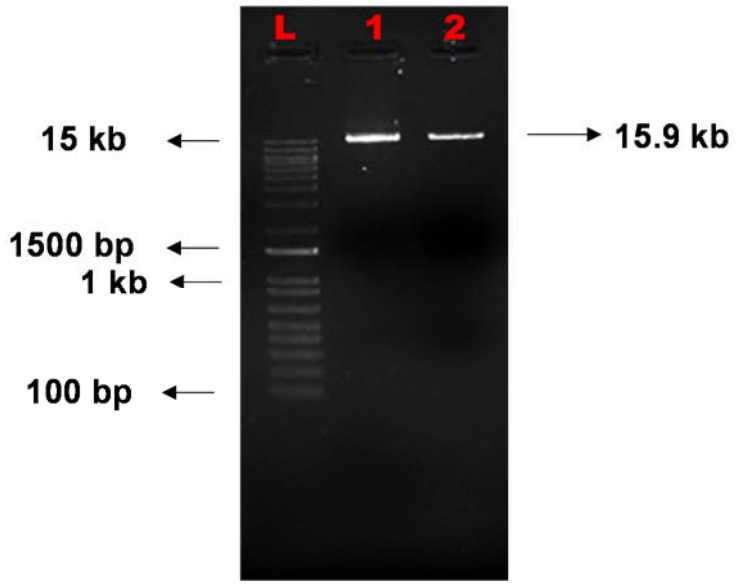


Figure 4.9. Gel picture of undigested and digested pRGEB32

L- 1 kb Plus Ladder, **1**- Undigested vector pRGEB32, **2**- Digested vector pRGEB32

Plate 4.2. Transformed colonies in DH5α

A- DH5α colonies of gRNA 1 (G1) construct

B - DH5α colonies of gRNA 2 (G2) construct

C - DH5α colonies of gRNA 3 (G3) construct

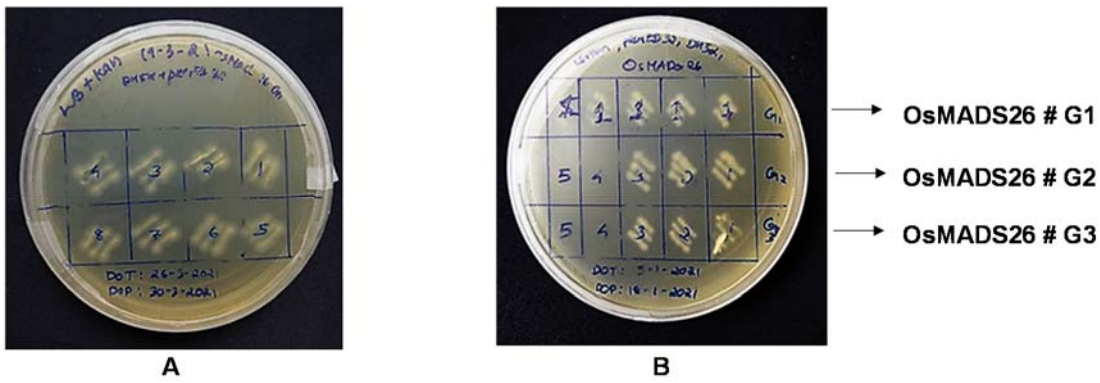


Plate 4.3. Patched culture plates of DH5α transformed colonies

A - Patched colonies of DH5α with OsMADS26 # G1 construct

B - Patched colonies of DH5α with OsMADS26 # G1, G2 and G3 constructs

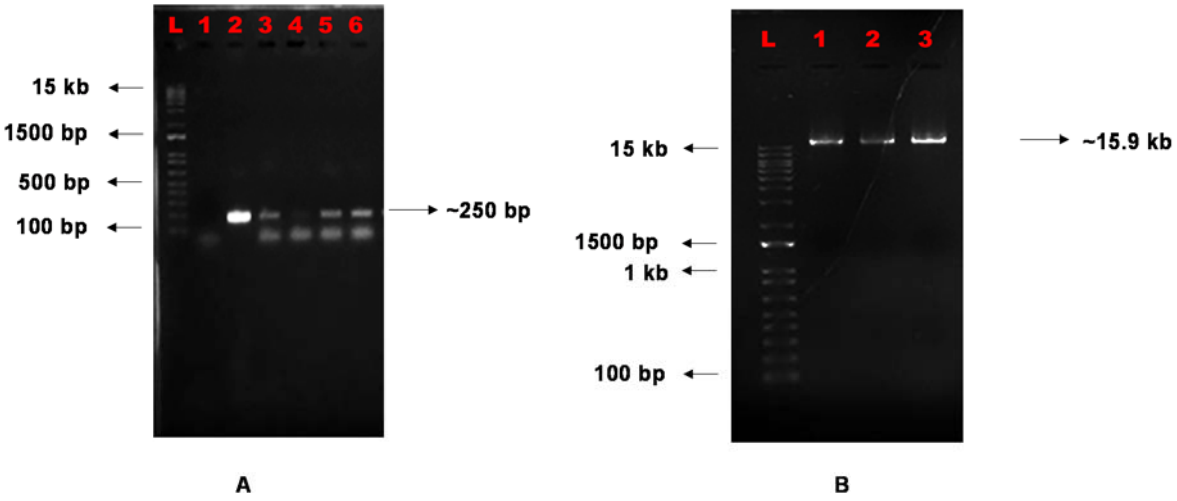


Figure 4.10. Gel picture of colony PCR of DH5α colonies and isolated plasmids

A - Gel picture of colony PCR of DH5α colonies to amplify guide cloned region

L- 1 kb Plus Ladder, **1**- Non-template control, **2**- Positive control (G3), **3-** G1 colony 2, **4**- G1 colony 4, **5**- G1 colony 7, **6**- G1 colony 9.

B - Gel picture of plasmids isolated from DH5α containing G1, G2 and G3 constructs respectively.

L- 1 kb Plus Ladder, **1**- G1 plasmid, **2**- G2 plasmid, **3**- G3 plasmid

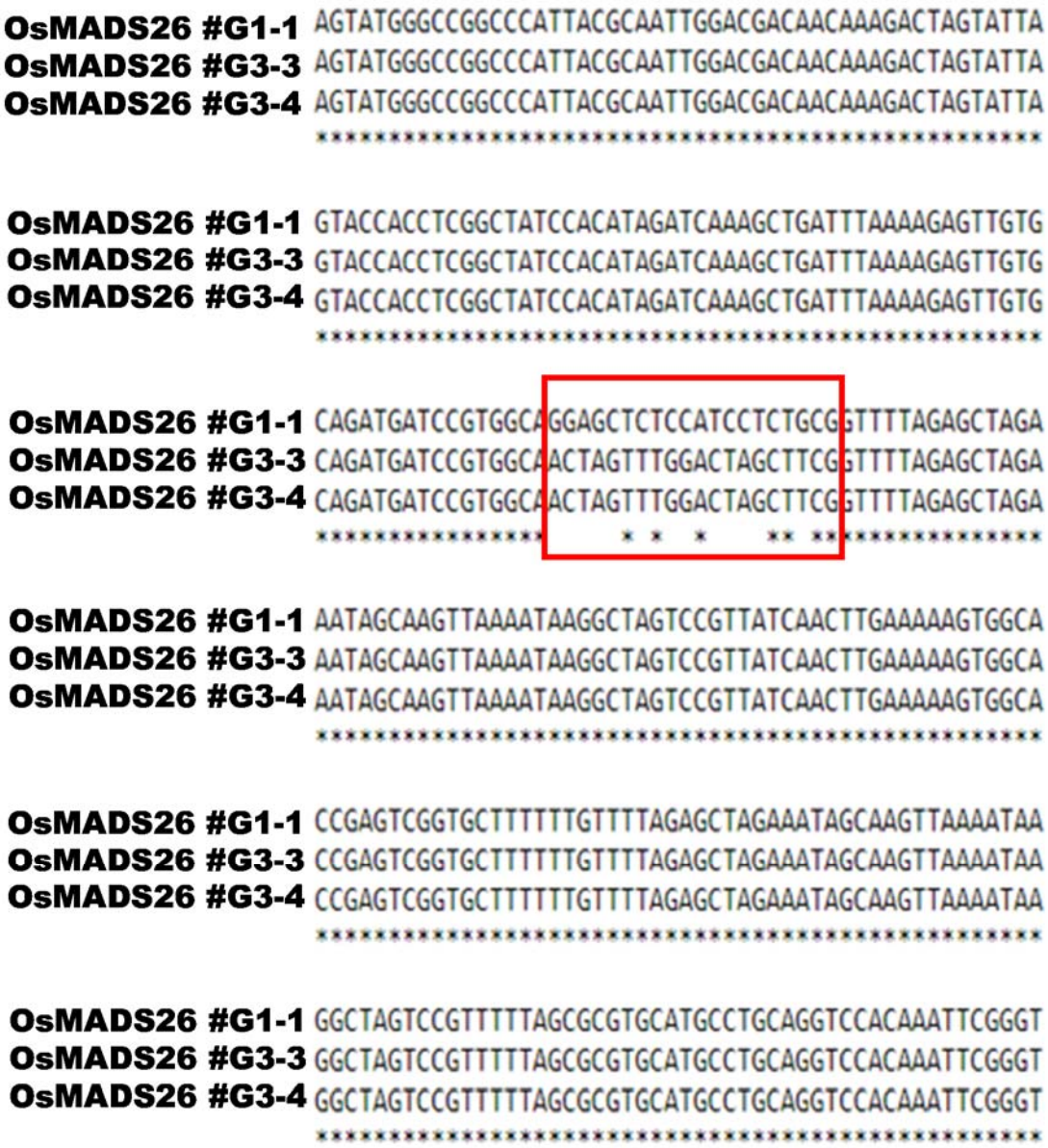


Figure 4.11. Multiple sequence alignment of OsMADS26 #G1 and G3 clones using ClustalW

The guide sequence cloned region in the backbone of vector pRGEB32 is marked in the figure.

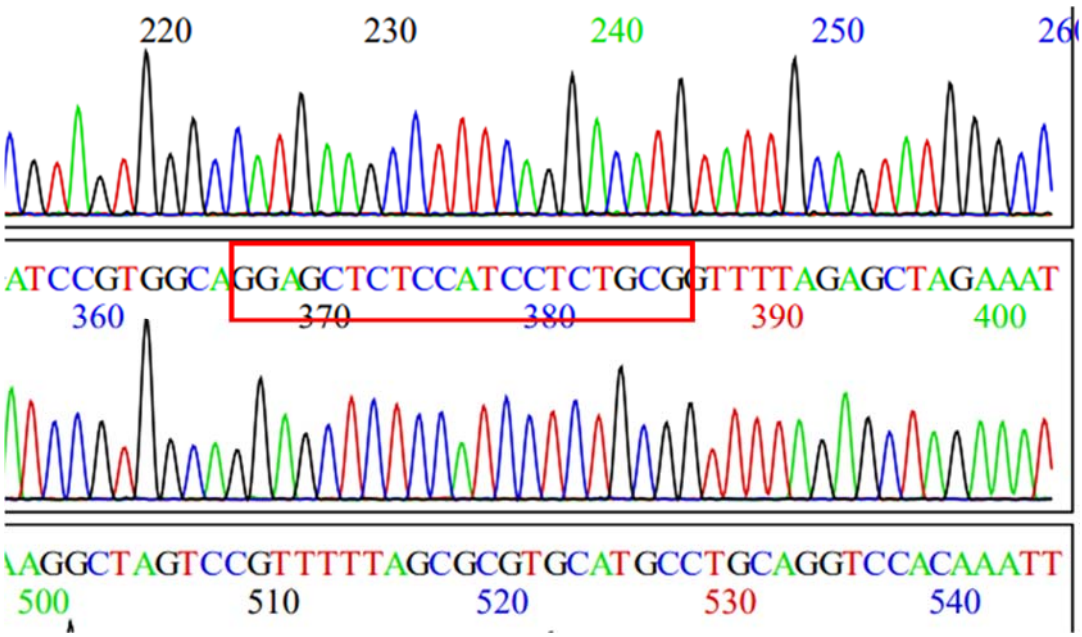


Figure 4.12. A. Chromatogram obtained with sequencing result showing the G1 cloned to pRGEB32 backbone

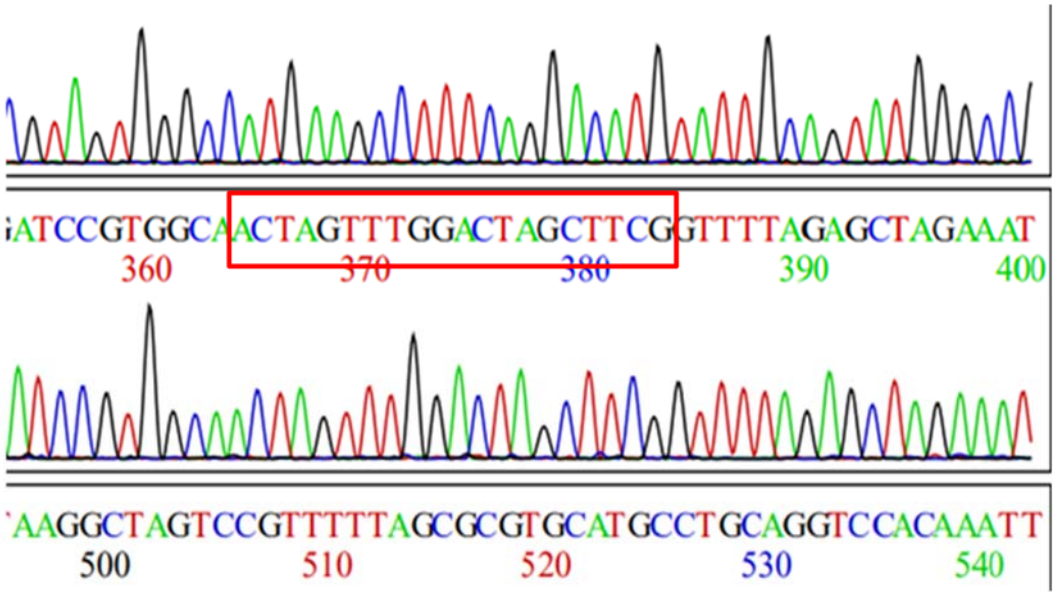
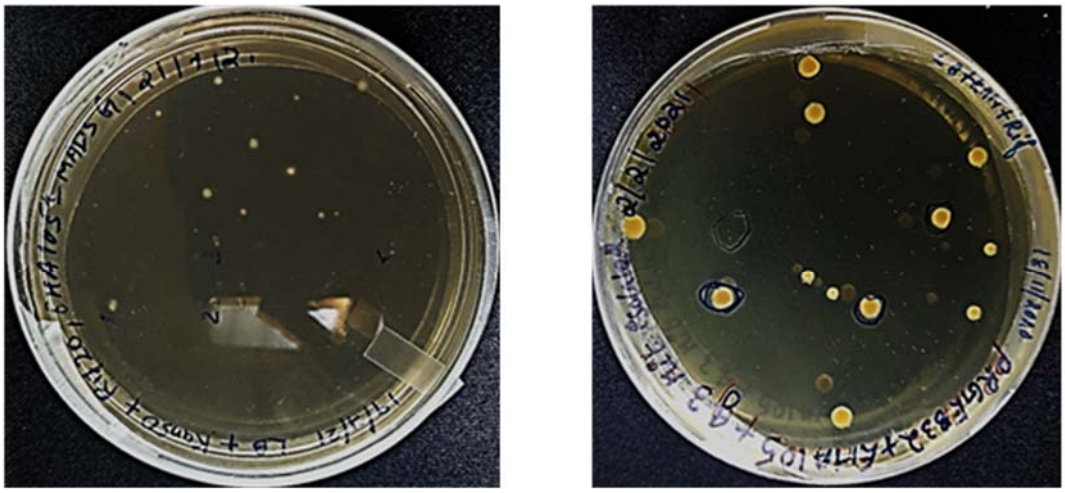


Figure 4.12. B. Chromatogram obtained with sequencing result showing the G3 cloned to pRGEB32 backbone

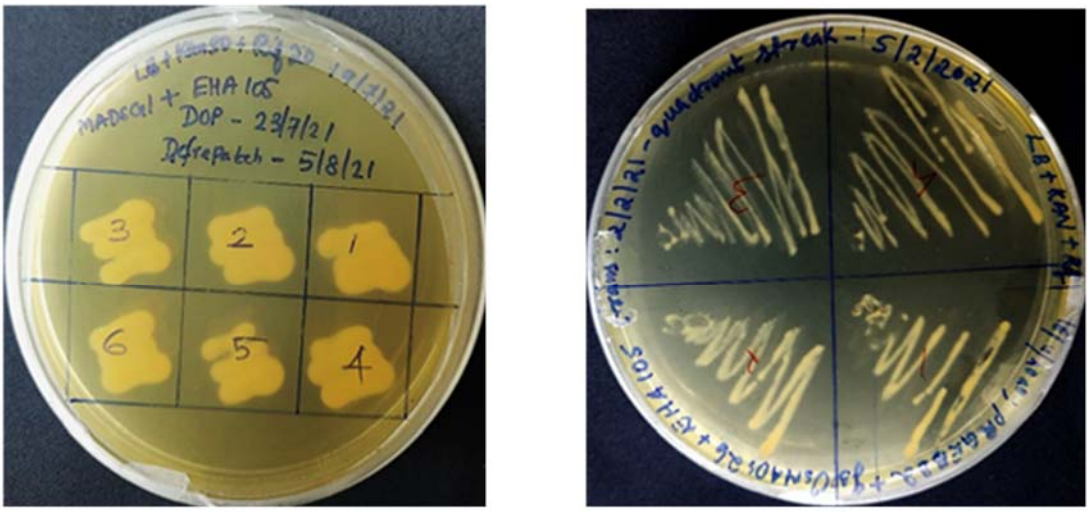


A B

Plate 4.4. Colonies observed in EHA105 after transformation

A - Colonies observed in the EHA105 for pRGEB32:OsMADS26#G1 -1 construct

B - Colonies observed in the EHA105 for pRGEB32:OsMADS26#G3 -3 construct



A B

Plate 4.5. Patched culture plates of EHA105 transformed colonies

A – Patched colonies of EHA105 transformed with pRGEB32:OsMADS26#G1 -1 construct

B - Patched colonies of EHA105 transformed with pRGEB32:OsMADS26#G3 -3 construct

were obtained when visualized on gel documentation unit (Figure 4.14). Thus confirmed the clones obtained in EHA105.

4.12. Genetic transformation of rice cultivar Nipponbare

4.12.1. Callus induction from rice seeds

The seeds of *Oryza sativa* ssp. *japonica* cv. Nipponbare was used for genetic transformation experiments. The seeds were dehusked manually with utmost care without damaging the embryo (Plate 4.6 A and B). The dehusked seeds were surface sterilized, blot dried and inoculated into callus induction medium (Plate 4.6 C) and incubated at 28˚C for 5 days under continuous white light in a culture room.

For each set of transformations about 400 seeds were inoculated for callus induction. In each plate 25-30 seeds were placed. The rice transformation was carried out following the protocol suggested by Toki *et al.* (2006) with minor modifications. Callus induction potential was not found to be uniform (Plate 4.7 A, B and C).

4.12.2. Co-cultivation of calli with *A. tumefaciens* harboring OsMADS26 gRNA constructs

On the 6th day, the proliferating calli were transferred to conical flasks and were infected with *Agrobacterium* harboring respective OsMADS26 gRNA construct (Plate 4.8 A). The Guide 1 (G1) and Guide 3 (G3) constructs in EHA105 were separately resuspended for co-cultivation. These resuspended constructs were then used for infecting the same set of calli. In another set of transformation kept back-to-back, these constructs were used to infect separate set of calli so that editing events for each construct can be identified easily. Along with the OsMADS26 gRNA constructs, an empty pRGEB32 was also transformed to rice calli as control (vector control). (Plate 4.8 B; Plate 4.9 A and B).

4.12.4. Washing of calli and inoculation into selection medium

The 48 hours co-cultivated calli were transferred to separate conical flasks for each guide RNA construct and vector control. The calli were washed thoroughly with sterile distilled water containing Augmentin to remove excess *Agrobacterium* load.

After washing, the calli were blot dried and inoculated into the selection medium (Plate 4.10 A and B). These plates were incubated at 30˚C for 14 days. If resurgence of *Agrobacterium* was observed, the healthy calli were sub-cultured in to fresh selection medium.

Initially the calli turned brownish when kept on selection medium but later few calli started proliferating (Plate 4.11 A and B). The proliferated micro calli were creamish-yellow in colour. These calli were expected to be transformed and were sub-cultured further after each 14 days interval (Plate 4.12). Multiple sets of transformations were performed, the details of which are given in Table 23. Currently a total of 60 calli were selected on selection medium. For OsMADS26#G1 and OsMADS26#G3 co-transformed, 37 calli were screened from selection medium. In vector control around 20 calli were selected.

4.12.5. Regeneration of calli selected on selection medium

The calli screened on selection medium, with proliferation of micro calli were transferred to regeneration medium. Vector control showed proliferation of micro calli in one plate (Plate 4.13 A; Table 23) and so they were further inoculated into regeneration medium (Plate 4.13 B). The untransformed Nipponbare callus was also kept along with the transformations as wild type control. These wild type calli were initially inoculated and regenerated following the same tissue culture procedures in order to test the adaptability of the protocol to our conditions. The untransformed calli showed proliferation after three weeks of incubation in regeneration medium (Plate 4.14 A, B and C). About 37 calli of OsMADS26#G1 and OsMADS26#G3 co-transformed screened and sub-cultured in selection medium that showed proliferating micro calli were transferred into regeneration medium (Plate 4.15 A and B)

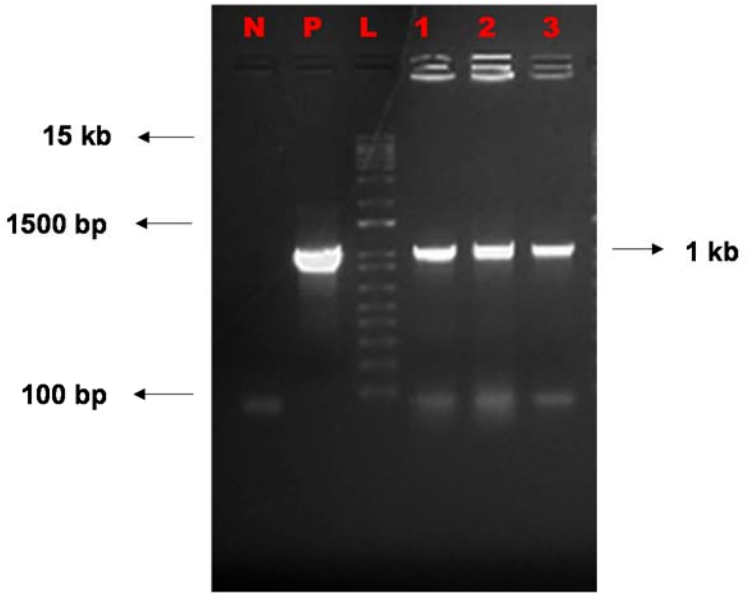


Figure 4.13. Colony PCR of colonies obtained in *A. tumefaciens,* EHA105

N- non template control, **P**- positive control (empty pRGEB32), **L**- 1 kb Plus Ladder, **1**- G1 colony 1, **2**- G1 colony 2, **3**- G3 colony 1

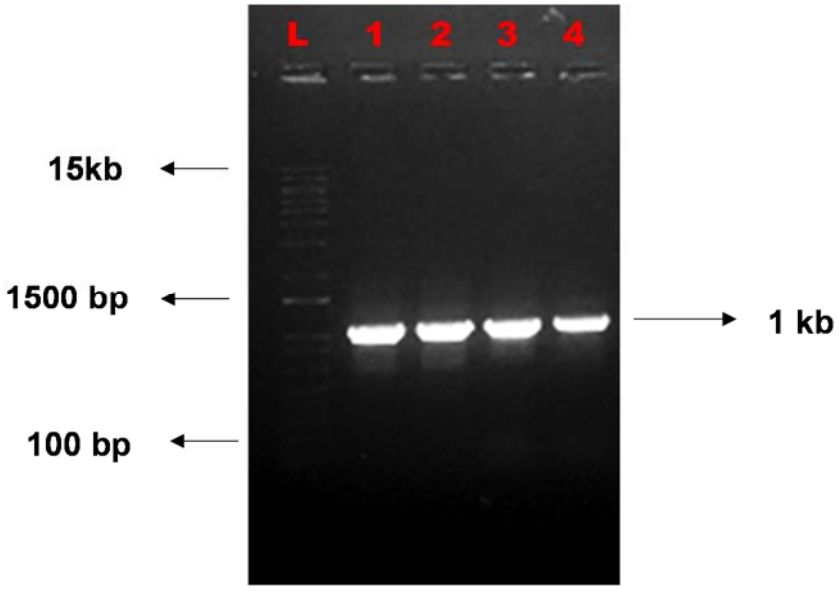


Figure 4.14. Gel picture of *hpt* gene PCR product of plasmids isolated from EHA105 clones

L- 1 kb Plus Ladder, **1**- pCAMBIA (have hygromycin gene), **2**- pRGEB32 empty vector, **3**- G1 cloned plasmid, **4**- G3 cloned plasmid. Both pCAMBIA and pRGEB32 positive controls.

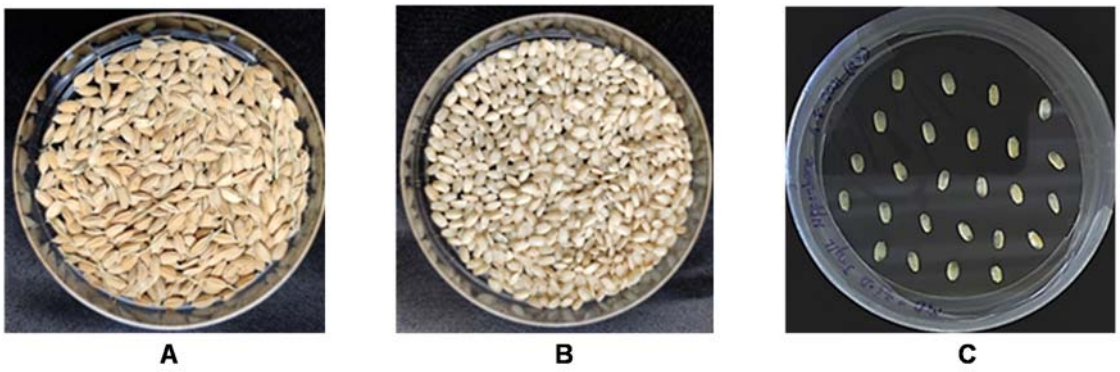


Plate 4.6. A. Nipponbare seeds B. Dehusked Nipponbare seeds after surface sterilization C. Seeds inoculated in CIM

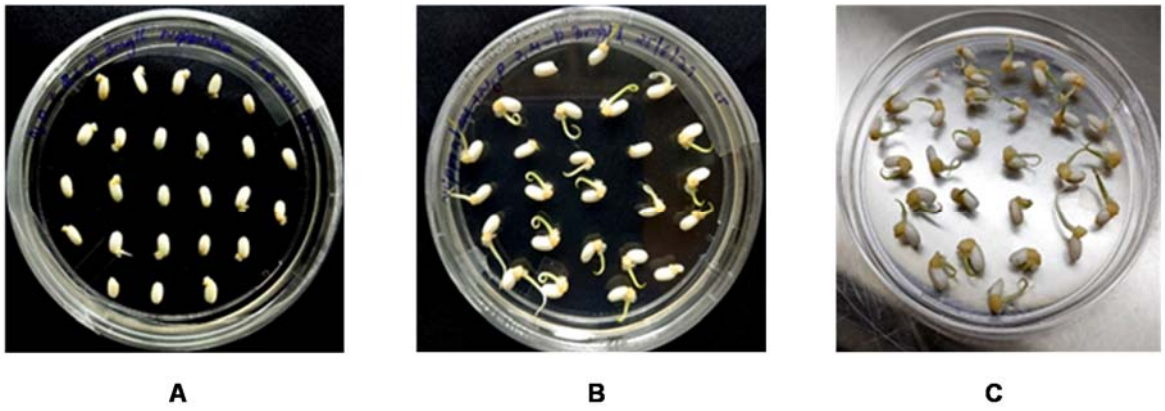
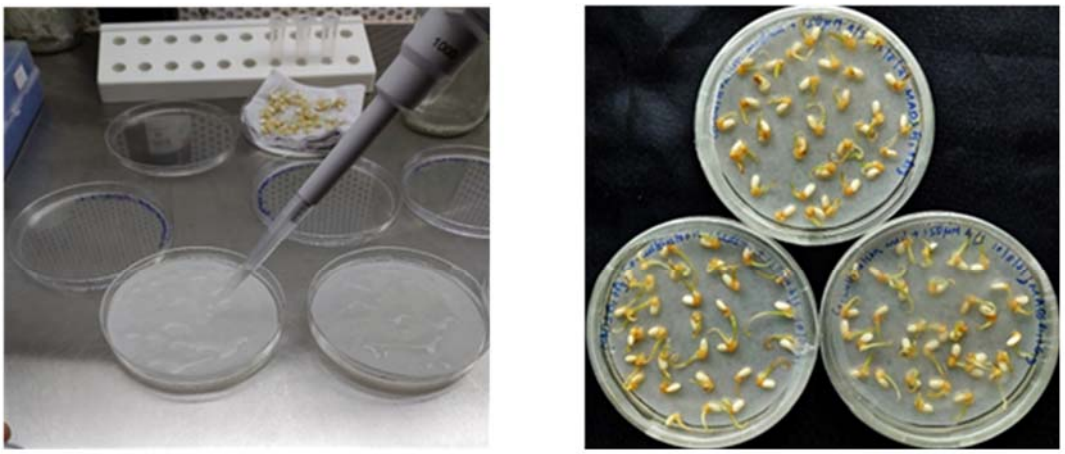


Plate 4.7. A. 2nd day of callus induction B. 4th day of callus induction C. 5th day of callus induction

A B

Plate 4.8. A. Rice calli transferred to conical flask for co-cultivation B. Infection of rice calli with *A. tumefaciens* containing OsMADS26 gRNA constructs

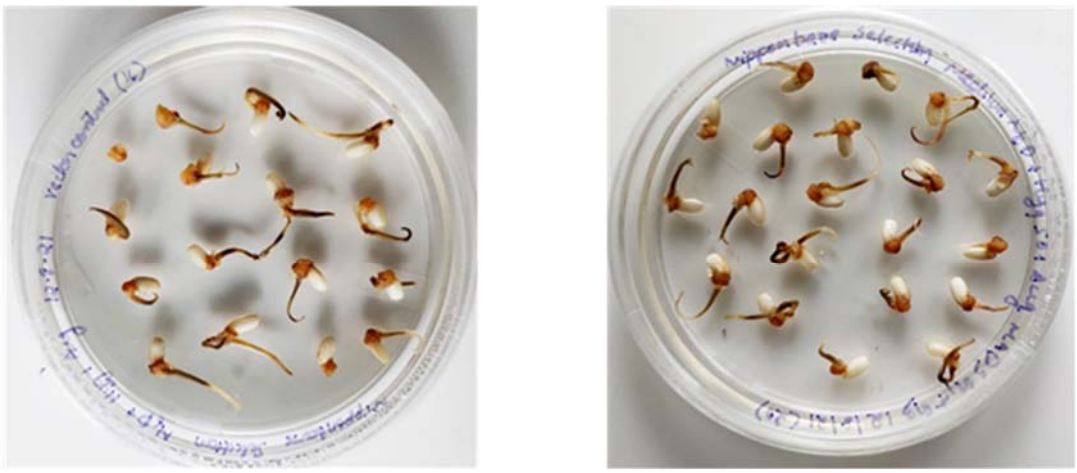


A B

Plate 4.9. A. Moistening of the overlayed filter paper on co-cultivation medium with resuspension medium B. calli co-cultivated with EHA105 clones

A B

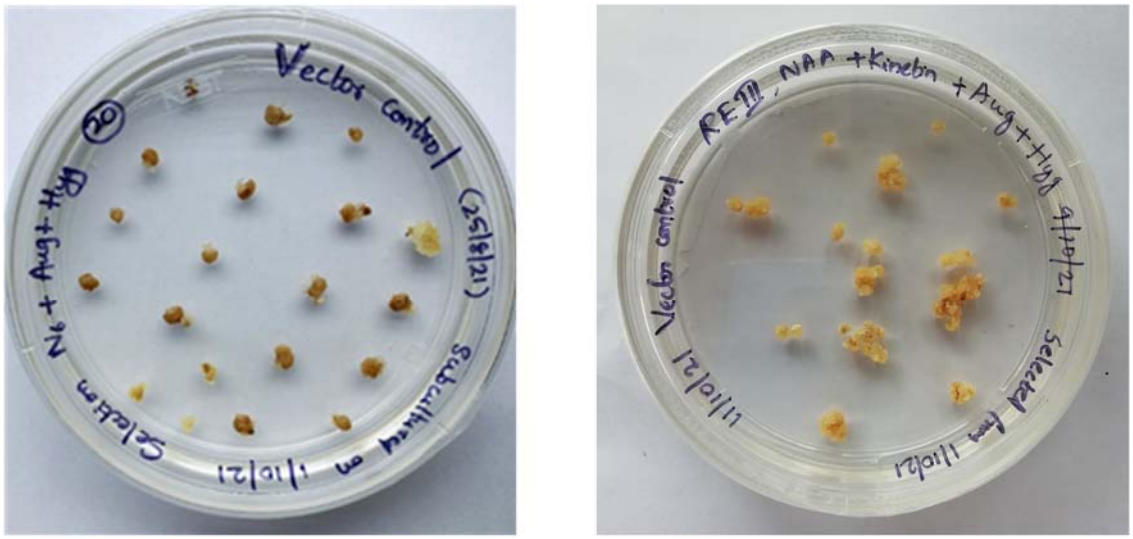
Plate 4.10. A. Calli blot dried on sterile filter paper B. Calli on selection medium-OsMADS26#G1 and OsMADS26# G3 co-transformed



A B

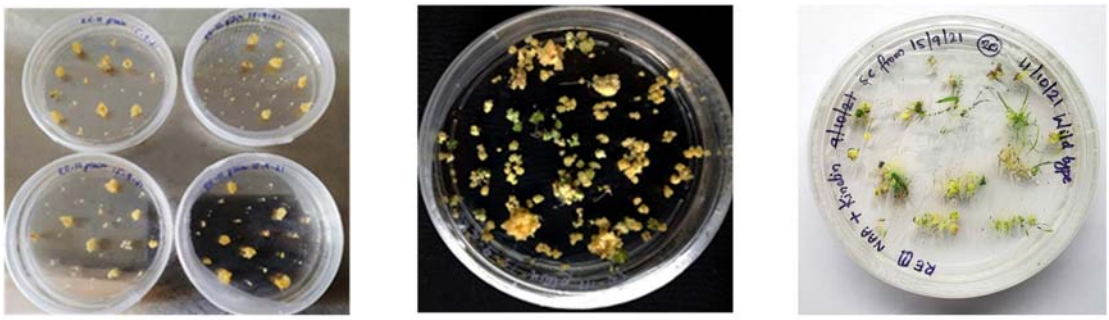
Plate 4.11. Calli on selection medium showing colour change during initial days A. Vector control calli B. OsMADS26#G1 + #G3

Plate 4.12. Calli sub cultured on selection medium



A **B**

Plate 4.13. A. Microcalli proliferation in selection medium - vector control B. Calli transferred regeneration medium



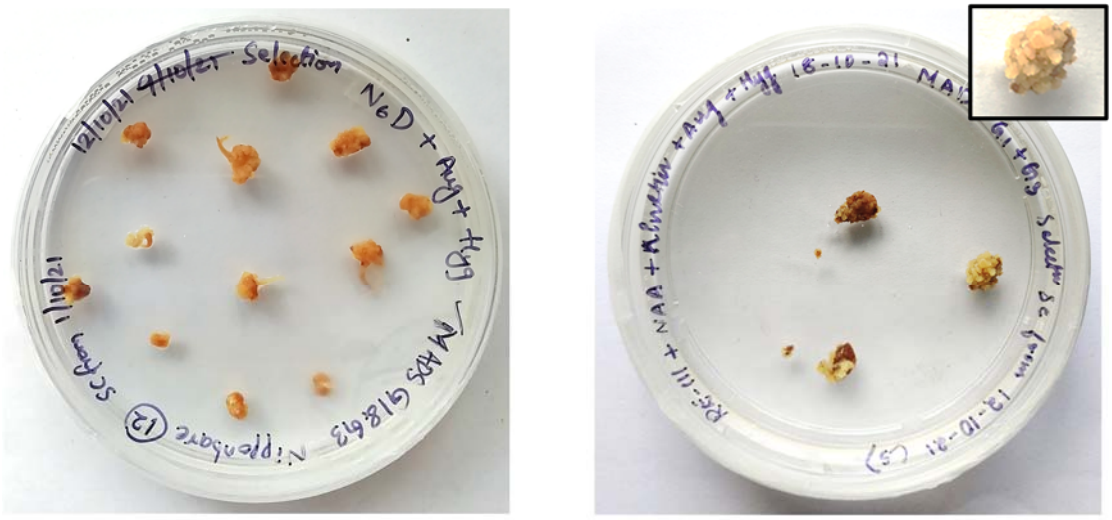
A B C

Plate 4.14. Calli cultured on regeneration medium

A - Regeneration of wild type Nipponbare

B - Untransformed calli proliferating on regeneration medium

C- Regenerated calli sub cultured after 14 days



A B

Plate 4.15. A. Proliferating calli of OsMADS26 #G1 + G3 in selection medium B. Calli of OsMADS26 #G1 + G3 transferred to regeneration medium

Table 23. Details of rice genetic transformation

Experiment No	Construct Name	Seeds inoculated	Calli infected	Calli on selection medium	Calli in regeneration medium	Current status
I	pRGEB32 vector control	75	60	40	20	Microcalli proliferation and regeneration seen
II	OsMADS26#G1	181	90	90	-	Vigorous *Agrobacterium* resurgence
III	OsMADS26#G3	115	92	90	-	Insufficient growth of calli
IV	OsMADS26#G3	105	85	42	-	No response and resurgence seen
V	OsMADS26#G1+G3	75	51	26	3	Microcalli are seen
VI	OsMADS26#G1 + G3	150	125	79	37	Proliferation of microcalli seen
VII	Wild type	173	89	88	29	144 microcalli seen, showed growth of leaf like structures and currently in rooting medium

DISCUSSION

5. DISCUSSION

India being the second largest producer and the largest exporter of rice in the world, it is very crucial to increase its productivity for ensuring global food security. Almost 45% of the global rice area is under rainfed conditions where the rice production is reduced due to various abiotic stresses (Lafitte *et al.*, 2004). Rice (*Oryza sativa* L.) is highly susceptible to water deficit than any other crops. Many stress responsive genes were identified and manipulated using various methods to develop abiotic stress tolerance in rice.

The existence of immense genetic variability in the germplasm for drought tolerance in rice was reported by Serraj *et al.*, (2011). *OsPYL* (Kim *et al.*, 2013), *OsGRP3* (Shim *et al.*, 2021), *DREB/CBF, DREB2* (Srivasta *et al.*, 2010), *ONAC022* (Hong *et al.*, 2016), *OsAKT1, OsNSCC2, OsSOS1* (Hwang *et al.*, 2016), *OsLEA3-1* (Xiao *et al.*, 2007, Yu *et al.*, 2016), *OsDRAP1, OsbZIP62* (Baillo *et al.*, 2019) and *OsHOX24* (Bhattacharjee *et al.*, 2017) were the few genes that were either overexpressed or down regulated via RNAi or ZFNs to induce stress tolerance in rice. The regulatory proteins like transcription factors were also found to have major role in enhancing the stress tolerance, since they bind to the promoters or other upstream elements to regulate the gene expression. One of the important transcription factors (TF) family found in rice is MADS-box TF family. Around 75 MADS-box genes were identified from rice genome (Arora *et al.*, 2007). Most of the TFs in this family have role in the various stages of plant growth and development (Saedler *et al.*, 2001). Transcription factors like *OsMADS26* were found to have role in multiple stress responses in rice (Khong *et al.*, 2015). Previous studies have demonstrated that the rice transcription factor *OsMADS26* is a negative regulator of abiotic and biotic stress tolerance (Khong *et al.*, 2015). Hence, the present study 'Editing of rice transcription factor *OsMADS26* for drought tolerance through CRISPR/Cas9 system' was taken up in Nipponbare rice cultivar during the period from 2019-2021 at the Centre for Plant Biotechnology and Molecular Biology, College of Agriculture, Kerala Agricultural University, Vellanikkara, Thrissur, Kerala. The details of the findings are discussed in this chapter.

5.1. Selection of CRISPR/Cas9 binary vector pRGEB32

pRGEB32 is one of the binary vector systems used for targeted genome editing using CRISPR/Cas9 system. It was used in the current study because of its high efficiency in targeted mutagenesis (Xie *et al.*, 2014). The vector system is highly specific to rice. The T-DNA region of the vector has Cas9, guide RNA cloning site and hygromycin resistance genes required for the targeted editing and subsequent selection of the edited events in rice. In pRGEB32, the gRNA cloning site is flanked by BsaI restriction sites. The vector consists of Cas9 from *Streptococcus pyogenes* driven by the rice ubiquitin promoter, a custom polycistronic gRNA assembly (precursor for one or more gRNAs) driven by the rice U3 snoRNA (Pol III) promoter and HPTII (hygromycin resistance) driven by the CaMV35S promoter (Hunter, 2021). Multiple knockouts were found to be possible by using pRGEB32 vector by multiplexing of gRNAs (Lacchini *et al.*, 2020).

There were many reports demonstrating the use of pRGEB32 vector in rice. Zafar *et al.*, (2020) developed resistance against *Xoo* strain of bacterial blight disease in super basmati rice by targeting effector binding elements (EBEs) of respective transcription activator like effectors (TALEs) through CRISPR/Cas9 approach. Xie *et al.* (2015) produced multiple gRNAs from a single synthetic *PTG* gene by hijacking the endogenous tRNA-processing system. Here, the plasmid vector pRGEB32 was used to transiently express *U3p:sgRNA* or *U3p:PTG* along with *UBIp:Cas9* constuct in rice protoplast and pRGEB32 was further used for the *Agrobacterium*-mediated rice transformation. Rice plants with reduced stature, to reduce lodging were produced by disrupting the *HTD1* gene using CRISPR/Cas9 system with pRGEB32 as vector (Lacchini *et al.*, 2020). Similarly, Hunter (2021) demonstrated the use of pRGEB32-bar vector for targeted mutagenesis in maize.

5.2. Guide RNA designing for gene editing

Guide RNAs for the *OsMADS26* gene were designed using the online tool CRISPR-P v2.0 (Xie and Yang, 2013) and the best three were selected based on their off-target sites, on-score values, GC content and location in genome. For efficient mutagenesis of a gene a minimum of two to three gRNAs should be selected since

mutation effects are unpredictable. Generally, gRNAs of 18-20nt long are considered, since they are more efficient in mutagenesis (Ren *et al.*, 2014). The features of guide RNAs given in CRISPR-P like on score value, off-target value, no. of off target sites, secondary structures if any and GC content were analyzed. The gRNAs with higher on-target and lower off-target values were preferred. On-score or on-target value greater than 0.5 were considered best to achieve higher editing efficiency. CRISPR/Cas9 system can lead to off-target editing in the genome as well. To reduce off-target effects avoiding sgRNAs with three or fewer nucleotide mismatches at potential off-targets had been recommended (Hsu *et al.*, 2013; Mali *et al.*, 2013). Selection of gRNAs with minimum off-target score can reduce off-target editing. sgRNAs with a 12 bp seed sequence (sequences closest to the PAM) that matches only with one site in the target genome can reduce off-target editing (Doench *et al.*, 2014). From the 20 nt targeting sequence of sgRNAs, mismatches in the seed region, the 5 to 12 nt closest to the PAM, have the largest impact on mutagenesis efficiency. Mismatches in the PAM-distal nucleotides were found to have less effect on disrupting the sgRNA-target DNA hybrid than those in the PAM proximal nucleotides (PAMPNs) (Jinek *et al.*, 2012; Cong *et al.*, 2013; Fu *et al.*, 2013). Literatures have suggested that, the gRNAs with GC content between 30-80% were found better for efficient mutagenesis (Ren *et al.*, 2014; Pan *et al.*, 2016). If GC content is high, the chances for formation of secondary structures in sgRNAs and at target DNA region will be less (Liang *et al.*, 2016). The gRNAs selected in the study had on-score value greater than 0.5 and the gRNAs were located upstream of the gene to ensure knock-out of the genes. Generally, gRNAs are selected from the coding regions (CDS) of the gene. If editing is targeted toward the 3' of the gene, there are chances of formation of truncated protein, which may still retain partial activity.

Since the vector used in the current study was pRGEB32, which has the BsaI restriction sites flanking the gRNA cloning site, we have added the BsaI restricted sites to either end of the gRNA sequences before synthesis.

5.3. Cloning of the CRISPR/Cas9 construct to DH5α and its confirmation

The CRISPR/Cas9 construct was made by ligating the respective gRNAs to the vector pRGEB32 after BsaI digestion. The top and bottom strands of gRNAs were

annealed prior to this. pRGEB32-gRNA construct was then transformed into *E. coli* strain DH5α. The colonies were observed from the plates after 16-18 hours of incubation at 37°C. The transformation into DH5α was really a constraint in the experiment. The larger size of the vector pRGEB32 (15.9 kb) made it difficult to be taken up by the bacterial cells. Hence, the number of colonies observed for both guide RNAs were less. To overcome these constraints few modifications were made during gRNA annealing and ligation. Only OsMADS26#G3 (gRNA 3) got cloned easily into DH5α following the general protocol using NEB reaction components. Thereafter, gRNAs were annealed following PNK phosphorylation reaction, and then OsMADS26#G1 got cloned after multiple transformation reactions. Even though colonies were observed in all the three constructs, after Sanger sequencing only two of them were having expected inserts. Only empty vector was present in the colonies as evident from the sequencing results. Hence two constructs were further carried forward for rice genetic transformation.

Patigu *et al.*, (2021) very recently reported that while using pRGEB32 for cloning the transformation efficiency was found to be very low. They developed a protocol specifically for easy transformation of the large sized vectors like pRGEB32 using heat shock method. They identified that heat shock temperature of 55°C for 60 seconds was the best for efficient transformation of pRGEB32 compared to the conventional conditions of 42°C for one minute. In addition to this, ultra-high competent cells can be prepared for cloning or other strains of *E. coli* which can hold higher plasmid size like DH10B, Top10, Stbl3, etc. can be used.

After cloning, the clones observed were confirmed initially by streaking the colonies to fresh plate with antibiotics. This is to avoid the contaminants and false positives. Thereafter, colony PCR was done with vector and gRNA specific primers (Ratnayake and Hettiarachchi, 2010). Those colonies screened by these steps were further confirmed using Sanger sequencing (Pyott *et al.*, 2016; Zafar *et al.*, 2020). Positive clones were identified by multiple sequence alignment of the sequences retrieved after sequencing. They showed alignment almost perfectly except at the site of gRNA insertion (Hong *et al.*, 2016). Only one colony of OsMADS26#G1 and two

colonies of OsMADS26#G3 were found to be positive clones after ClustalW analysis. This is also may be due to the large size of the gRNA construct.

5.4. *Agrobacterium*-mediated rice genetic transformation

Agrobacterium mediated genetic transformation is accepted to be the most reliable method of genetic transformation in rice (Hiei *et al.*, 1994). Toki *et al.* (2006) have developed a highly efficient protocol for rice genetic transformation in which scutellum tissue of 1-5 days old pre-cultured seeds is competent to take up *Agrobacterium* leading to the establishment of a high-speed transformation system in rice. Hence in the current study *Agrobacterium* mediated genetic transformation was carried out in rice. For this, the positive clones obtained in *E. coli* were further mobilized in to *A. tumefaciens* strain EHA105 (Hood *et al.*, 1986). The genetic transformation of EHA105 was carried out by freeze-thaw method (Weigel and Glazebrook, 2006). About 13 and 12 colonies were observed in OsMADS26#G1 and OsMADS26#G3. Even though the method is less efficient compared to the electroporation (Toth *et al.*, 2018), it is more adaptable to our lab conditions. The transformation efficiency was found to be moderate.

In the current study genetic transformation of rice was carried out using the protocol developed by Toki *et al.* (2006) with minor modifications. The protocol is simpler and consumes lesser time compared to the other protocols. Initially another protocol developed by Sahoo *et al.* (2011) was tried. The protocol uses longer sub culture cycles and demands more labor due to frequent media changes.

The initiation of the calli was found to be slow in 2.0 mg/L 2,4-D, hence the concentration was enhanced to 3.0 mg/L for callusing at subsequent stages. Maltose is known as better carbon source compared to sucrose for rice *in-vitro* responses (Ming *et al.*, 2019). Hence in the current protocol sucrose was replaced with maltose. EHA105 is considered as a highly virulent strain of *Agrobacterium* (Gui *et al.*, 2016). Frequent re-growth of *Agrobacterium* was observed in selection medium even after thorough washing of the calli with the antibiotic cefotaxime (250 mg/L). Hence instead of cefotaxime, another broad-spectrum bacteriostatic agent Augmentin (Amoxycillin + Clavulanic Acid) was used at the concentration of 300 mg/L. This

gave better results compared to cefotaxime. However, the *Agrobacterium* could not be removed completely. Hence further, we have modified the washing step by increasing number of washes each of which was done for a duration of 15 min. The infection time was also limited to exactly one minute and the co-cultivation was reduced to one and a half day compared to 2-3 days in the protocol. This could reduce the frequency of *Agrobacterium* resurgence on the calli to a greater extent.

An empty vector control was kept along with the CRISPR/Cas9 constructs to evaluate whether the empty vector without the inserted gene cassette will affect the phenotype or cause any nonspecific effect after integration in to host genome (Sahoo *et al.*, 2014). Hence, pRGEB32 empty vector in *Agrobacterium* was also transformed to rice calli. Similarly, a set of untransformed calli was regenerated through tissue culture as a control for regeneration process. This will also ensure whether our transformation protocol is working properly. These two controls will be used along with the transgenic plants in future for all further analysis. About 123 calli were inoculated on selection medium of which about 37 calli were screened and sub-cultured for further proliferation. The untransformed calli turned dark brown and died on selection medium and only transformed calli were found proliferating in the selection medium (Toki *et al.*, 2006). However, the transformation protocol was found to be efficient as the vector control and untransformed calli showed greening and shoot primordia initiation in regeneration medium.

In the present study, for CRISPR/Cas9 mediated targeted editing of *OsMADS26* transcription factor, gRNAs were designed and successfully cloned in to pRGEB32 to generate gene editing cassette/construct. The construct is transformed successfully into the rice cultivar (Nipponbare). Rice transformation protocol was optimized to suit our lab and culture conditions. Plants with mutations in *OsMADS26* gene is expected, which confer drought tolerance in rice.

One of the greater advantages of CRISPR/Cas9 technology is evaluated as its potential to generate transgene free plants. The direct delivery of gRNA combined with Cas9 protein (Ribonucleoprotein complex-RNP) delivered to plant cells/ protoplast by biolistics or electroporation can give rise to transgene free plant development. However, this needs standardization of the procedure and demands an

efficient regeneration protocol (Bes *et al.*, 2021). The protoplast regenerated plant can also cause genetic instability. In rice unfortunately there is no efficient protoplast regeneration protocols reported till date. Hence *Agrobacterium* mediated plant transformation is more adaptable (Gao *et al.*, 2020). However, for elimination of transgenes after *Agrobacterium* mediated transformation, selfing is practiced in the segregating progeny of many plant species (Banfalvi *et al.*, 2020). Hence in the current study also transgene free edited plants can be generated in future.

SUMMARY

6. SUMMARY

The study entitled 'Editing of rice transcription factor *OsMADS26* for drought tolerance through CRISPR/Cas9 system' was carried out at the Centre for Plant Biotechnology and Molecular Biology, College of Agriculture, Kerala Agricultural University, Vellanikkara, Thrissur during the period from 2019 to 2021. The major objective of the study was to develop drought tolerant lines in rice using CRISPR/Cas9 mediated targeted editing of the *OsMADS26* gene. Salient findings of the study are summarized below:

- The *Oryza sativa* sub species japonica cultivar used Nipponbare was selected for the targeted editing of *OsMADS26* gene.
- The binary vector system, pRGEB32 was selected for the CRISPR/Cas9 mediated genome editing.
- The *OsMADS26* gene sequences were downloaded from 'Rice Genome Annotation Project' and guide RNAs (gRNAs) were designed using CRISPR-P v2.0 software.
- Three gRNAs were selected for the study based on features like on-score value, GC content, number of off-target sites, presence of secondary structures and location on genome etc.
- Two of the selected gRNAs were from the coding region (CDS) and the third one was located in the 5' untranslated region (UTR) of the gene.
- The gRNAs were synthesized as top and bottom strands after adding BsaI restriction sites to both strands.
- The sequences of MADS-box transcription factor gene *OsMADS26* target was confirmed in Nipponbare by amplifying the flanking region (~450bp) of the gRNA by Polymerase Chain Reaction (PCR).
- The PCR product of *OsMADS26* gene was confirmed further by Sanger sequencing and the sequencing results were analyzed using BLASTn and Clustal Omega software, which showed 100% similarity with the *OsMADS26* gene reported in MSU database (LOC_Os08g02070).
- The top and bottom strands of gRNAs were annealed following two protocols, i.e., with and without PNK phosphorylation.

- The BsaI digested vector pRGEB32 was ligated to the annealed gRNAs to develop the CRISPR/Cas9 construct for genome editing.
- The pRGEB32-gRNA construct was cloned into *E. coli* strain DH5α using the 'Bacterial Transformation kit GeneSure™' from Puregene life sciences.
- Colonies were observed in DH5α after 16-18 hours of transformation.
- Nine colonies were observed in OsMADS26 #G1, three in OsMADS26 #G2 and four in OsMADS26 #G3.
- The colonies in DH5α were confirmed by colony PCR and Sanger sequencing of the plasmids isolated from PCR positive colonies.
- The gRNA insertion in the vector backbone was confirmed using Clustal Omega software and the sequences showed alignment except at the gRNA cloning site.
- Positive clones obtained from OsMADS26 #G1 colony 1 and OsMADS26 #G3 colony 3 were mobilized into *Agrobacterium tumefaciens* strain EHA105 by freeze-thaw method.
- The colonies appeared for EHA105 were identified using colony PCR with hygromycin resistance gene specific primers (*hpt*).
- The plasmids isolated from EHA105 were also further confirmed by amplifying the hygromycin resistance gene.
- Rice genetic transformation was carried out following the protocol described by Toki *et al.* (2006) with minor modifications.
- The dehusked Nipponbare seeds were inoculated into callus induction medium (CIM) after proper surface sterilization.
- The growth of calli from scutellum was found better in medium containing 3 mg/L concentration of 2,4-D.
- The five-day old calli were co-cultivated with *Agrobacterium* strain harbouring OsMADS26 #G1-1, OsMADS26 #G3-3 and empty pRGEB32 (vector control).
- Untransformed calli were also cultured in CIM as wild type control.
- After 1.5 - 2 days of co-cultivation, the calli were washed with sterile distilled water supplemented with Augmentin (300 mg/L) to remove the *Agrobacterium* load.
- The washed calli were selected in the selection medium containing Augmentin (300 mg/L) and Hygromycin (50 mg/L).

- The calli initially turned slightly brownish in selection medium and many calli has to be discarded due to *Agrobacterium* resurgence.
- The calli showed proliferation of light creamish coloured microcalli on subculturing after 14 days.
- Microcalli proliferation were observed in vector control, wild type as well as in OsMADS26 #G1-1 and OsMADS26 #G3-3 transformed calli.
- These calli were then transferred to regeneration medium for development of shoot primordia.
- The wild type calli showed higher regeneration efficiency than transformed calli.
- From the 29 calli screened in selection medium, a total of 144 microcalli were obtained in wild type and about 20 calli were transferred for regeneration in vector control.
- In case of OsMADS26 #G1-1 and OsMADS26 #G3-3 co-transformed calli, 79 were screened in selection medium and from that about 35 calli showed proliferation of micro calli. These were further transferred to regeneration medium.
- In this study, rice transformation protocol was optimized to suit our laboratory and culture condition.
- Rice plants with mutations in *OsMADS26* gene are expected in future, which confer drought tolerance.

REFERENCES

REFERENCES

Alvarez-Buylla, E. R., Liljegren, S. J., Pelaz, S., Gold, S. E., Burgeff, C., Ditta, G. S., Vergara-Silva, F. and Yanofsky, M. F. 2000. MADS-box gene evolution beyond flowers: expression in pollen, endosperm, guard cells, roots and trichomes. *Plant J*.24: 457–466.

Anders, C., Niewoehner, O., Duerst, A. and Jinek, M. 2014. Structural basis of PAM-dependent target DNA recognition by the Cas9 endonuclease. *Nature*. 513: 569–573. doi: 10.1038/nature13579.

Arora, R., Agarwal, P., Ray, S., Singh, A. K., Singh, V. P., Tyagi, A. K. and Kapoor, S. 2007. MADS-box gene family in rice: genome- wide identification, organization and expression profiling during reproductive development and stress. *BMC Genomics* 8: 242p.

Baillo, E. H., Kimotho, R. N., Zhang, Z. and Xu, P. 2019. Transcription factors associated with abiotic and biotic stress tolerance and their potential for crops improvement. *Genes* 10: 771p.

Banfalvi, Z., Csakvari, E., Villanyl, V. and Kondrak, M. 2020. Generation of transgene-free PDS mutants in potato by Agrobacterium-mediated transformation. *BMC Biotechnol*. 20:25. https://doi.org/10.1186/s12896-020-00621-2 .

Barman, A., Deb, B. and Chakraborty, S. 2019. A glance at genome editing with CRISPR–Cas9 technology. *Current Genet*. https://doi.org/10.1007/s00294-019-01040-3

Barrangou, R. 2013. CRISPR-Cas systems and RNA-guided interference. *Wiley Interdiscip. Rev. RNA.* 4: 267–278. doi: 10.1002/wrna.1159.

Barrangou, R. and Horvath, P. 2017. A decade of discovery: CRISPR functions and applications. *Nature Microbiol*. 2: 17092. http://dx.doi.org/10.1038/nmicrobiol.2017.92 .

Bernier, J., Atlin, G. N., Serraj, R., Kumar, A. and Spaner, D. 2008. Breeding upland rice for drought resistance. *J. Sci. Food Agric*. 88(6): 927-939.

Bes M. et al. 2021. Efficient Genome Editing in Rice Protoplasts Using CRISPR/CAS9 Construct. In: Bandyopadhyay A. and Thilmony R. (eds.) Rice Genome Engineering and Gene Editing. Methods in Molecular Biology. 2238. Humana, New York, NY. https://doi.org/10.1007/978-1-0716-1068-8_11 .

Bhattacharjee, A., Sharma, R. & Jain, M. 2017. Over-expression of *OsHOX24* confers enhanced susceptibility to abiotic stresses in transgenic rice via modulating stress-responsive gene expression. *Front. Plant Sci*.

Bhattacharjee, A., Sharma, R. and Jain, M. 2017. Over-expression of *OsHOX24* confers enhanced susceptibility to abiotic stresses in transgenic rice via modulating stress-responsive gene expression. *Front.Plant Sci*.

Bouman, B. A. M., Peng, S., Castaneda, A. R. and Visperas, R. M. 2005. Yield and water use of irrigated tropical aerobic rice systems. *Agric. Water Manag*. 74(2): 87-105.

Butt, H., Eid, A., Ali, Z., Atia, M., Mokhtar, M., Hassan, N., Lee, C., Bao, G. and Mahfouz, M. 2017. Efficient CRISPR/Cas9-mediated genome editing using a chimeric single-guide RNA molecule. *Front. Plant Sci*.

Char, S.N., Neelakandan, A.K., Nahampun, H., Frame, B., Main, M., Spalding, M.H., Becraft, P.W.,Meyers, B.C., Walbot, V., Wang, K. and Yang, B. 2016. An Agrobacterium-delivered CRISPR/Cas9 system for high-frequency targeted mutagenesis in maize. *Plant Biotechnol. J.* 15, 257–268. doi: 10.1111/pbi.12611.

Chen, K., Du, K., Shi, Y., Yin, L., Shen, W. H., Yu, Y., Liu, B. and Dong, A. 2021. *H3K36* methyltransferase SDG708 enhances drought tolerance by promoting abscisic acid biosynthesis in rice. *New Physiologist*. 230: 1967-1984.

Chen, K., Li, G. J., Bressan, R. A., Song, C. P., Zhu, J. K. and Zhao, Y.2020. Abscisic acid dynamics, signaling and functions in plants. *J. Integr. Plant Biol*. 62: 25-54.

Chen, L., Song, Y., Li, S., Zhang, L., Zou, C. and Yu, D. 2012. The role of *WRKY* transcription factors in plant abiotic stresses. *Biochim. Biophys. Acta.* 1819: 120–128. doi: 10.1016/j.bbagrm.2011.09.002.

Cho, S., Jang, S., Chae, S., Chung, K. M., Moon, Y. H., An, G. and Jang, S. K. 1999. Analysis of the C-terminal region of Arabidopsis thaliana *APETALA1* as a transcription activation domain. *Plant Mol. Biol.* 40: 419-429.

Chukwu, S. C., Rafii, M. Y., Ramlee, S. I., Ismail, S. I., Oladosu, Y., Okporie, E. and Jalloh, M. 2019. Marker-assisted selection and gene pyramiding for resistance to bacterial leaf blight disease of rice (*Oryza sativa* L.). *Biotechnol. Biotechnol.* Equip.

Claes, B., Dekeyser, R., Villarroel, R., Van den Bulcke, M., Bauw, G., Van Montagu, M. and Caplan, A. 1990. Characterization of a rice gene showing organ-specific expression in response to salt stress and drought. *The Plant Cell*. 2(1): 19–27. https://doi.org/10.1105/tpc.2.1.19.

Cohen, S., Chang, A. and Hsu, L. 1972. Non-chromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. *Proc. Natl. Acad. Sci. USA* 69:2110-2114..

Colombo, L., Franken, J., Koetje, E., van Went, J., Dons, H.J., Angenent, G.C. and van Tunen, A.J. 1995. The petunia MADS box gene FBP11 determines ovule identity. *Plant Cell.* 7: 1859–1868.

Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W. and Marraffini, L.A. 2013. Multiplex genome engineering using CRISPR/Cas systems. *Science*. 339: 819-823.

Cutler, S. R., Rodriguez, P. L., Finkelstein, R. R. and Abrams, S. R. 2010. Abscisic acid: emergence of a core signaling network. *Ann. Rev. Plant Biol.* 61: 651–679.

de Framond, A. J., Barton, K. A. and Chilton, M.D. 1983. Mini Ti: a new vector strategy for plant genetic engineering. *BioTechnol*. 1:262-269.

Doench, J. G., Hartenian, E., Graham, D. B., Tothova, Z., Hegde, M., Smith, I., Sullender, M., Ebert, B. L., Xavier, R. J .and D.E. Root. 2014. Rational design of highly active sgRNAs for CRISPR-Cas9-mediated gene inactivation. *Nat. biotechnol*. 32:1262-1267.

Doudna, J. A. and Charpentier, E. 2014. The new frontier of genome engineering with CRISPR–Cas9. *Science*. 346(6213):1258.

Duan, Y., Li, J., Qin, R., Xu, R., Li, H., Yang, Y., Ma, H., Li, L., Wei, P. and Yang, J.2016. Identification of a regulatory element responsible for salt induction of rice *OsRAV2* through ex situ and in situ promoter analysis. *Plant Molecular Biol*.

Fang, S.C. and Fernandez, D. E. 2002. Effect of regulated overexpression of the MADS domain factor *AGL15* on flower senescence and fruit maturation. *Plant Physiol* 130: 78–89.

Fang, Y. J., You, J., Xie, K., Xie, W. B. and Xiong, L. Z. 2008. Systematic sequence analysis and identification of tissue-specific or stress-responsive genes of NAC transcription factor family in rice. *Mol. Genet. Genomics.* 280: 547–563. doi: 10.1007/s00438-008-0386-6.

Farhat, S., Jain, N., Singh, N., Sreevathsa, R., Dash, K. P., Rai, R., Yadav, S., Kumar, P., Sarkar, A. K., Jain, A., *et al*. 2019. CRISPR-Cas9 directed genome engineering for enhancing salt stress tolerance in rice. Seminars in Cell and Developmental Biology. *Elsevier Ltd.*

Fernandez, D. E., Heck, G. R., Perry, S. E., Patterson, S. E., Bleecker, A.B. and Fang, S. C. 2000. The embryo MADS domain factor *AGL15* acts post embryonically. Inhibition of perianth senescence and abscission via constitutive expression. *Plant Cell.* 12: 183–198.

Fu, Y., Foden, J. A., Khayter, C., Maeder, M. L., Reyon, D., Joung, J. K. and Sander, J.D. 2013. High-frequency off-target mutagenesis induced by CRISPR–Cas nucleases in human cells. *Nat. Biotechnol*. 10.1038/nbt.2623

Future implications. *Frontiers in Plant Sci*.9: 1361.

Gao, H., Xie, F., Zhang, W., Tian, J., Zou, C., Jia, C., Jin, M., Huang, J., Chang, Z., Yang, X. and Jiang, D. 2020. Characterization and improvement of curdlan produced by a high-yield mutant of Agrobacterium sp. ATCC 31749 based on whole-genome analysis. *Elsevier*. 245: 116486. https://doi.org/10.1016/j.carbpol.2020.116486 .

Golldack, D., Li, C., Mohan, H. and Probst, N. 2014. Tolerance to drought and salt stress in plants: unraveling the signaling networks. *Front. Plant Sci.* 5:151. doi: 10.3389/fpls.2014.00151.

Gong, Z., Xiong, L., Shi, H., Yang, S., Herrera-Estrella, L. R., Xu, G., Chao, D. Y., Li, J., Wang, P. Y., Qin, F., Li, J., Ding, Y., Shi, Y., Wang, Y., Yang, Y., Guo, Y. and Zhu, J. K. 2020. Plant abiotic stress response and nutrient use efficiency. *Sci. China Life Sci*. 63: 635-674.

Green, M. R. and Sambrook, J. 2016. Preparation of plasmid DNA by alkali lysis with sodium dodecyl sulfate: minipreps. *Cold Spring Harb Protoc*. doi:10.1101/pdb.prot093344

Grover, A. and Pental, D. 2003. Breeding objectives and requirements for producing transgenics for major field crops of India. *Current Sci*. 84(3): 310-320.

Gui, H., Li. X., Liu, Y. and Li, X. 2016. Evaluation of Factors Impacting Agrobacterium-Mediated Indica Rice Transformation of IR58025B-a Public Maintainer Line - *Rice Research*: Open Access

Guo, S., Xu, Y., Liu, H., Mao, Z., Zhang, C., Ma, Y., Zhang, Q., Meng, Z. and Chong, K. 2013. The interaction between OsMADS57 and OsTB1 modulates rice tillering via DWARF14. *Nat Commun* 4: 1566

Guo, S., Xu, Y., Liu, H., Mao, Z., Zhang, C., Ma, Y., Zhang, Q., Meng, Z. and Chong, K. 2013. The interaction between *OsMADS57* and *OsTB1* modulates rice tillering via *DWARF14*. *Nat Commun.* 4: 1566.

Gupta, A., Rico-Medina, A. and Cañ̃o-Delgado, A. I. 2020. The physiology of plant responses to drought. *Science.* 368: 266-269.

Hiei, Y., Ohta, S., Komari, T. and Komashiro, T. 1994. Efficient transformation of rice (*Oryza sativa* L.) mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA. *The Plant J*. 6(2): 271-282pp.

Holsters, M., Waele, D., Depicker, A., Messens, E., Van Montagu, M. and Schell, J. 1978. Transfection and transformation of *A. tumefaciens*. *Mol. Gen. Genet*. 168: 181-187.

Hong, Y., Zhang, H., Huang, L., Li, D. and Song, F. 2016. Overexpression of a Stress-Responsive NAC transcription factor gene *ONAC022* improves drought and salt tolerance in rice. *Front. Plant Sci.* 7:4. doi: 10.3389/fpls.2016.00004.

Hood, E. E., Helmer, G. L., Fraley, R. T. and Chilton, M. D. 1986. The hypervirulence of Agrobacterium tumefaciens A281 is encoded in a region of pTiBo542 outside of T-DNA. *J. Bacteriol*. 168: 1291-1301.

Hsu, P. D., Scott, D. A., Weinstein, J. A., Ran, F. A., Konermann, S., Agarwala, V., Li, Y., Fine, E. J., Wu, X., Shalem, O., Cradick, T. J., Marraffini, L. A., Bao, G. and Zhang, F. 2013. DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat Biotechnol.* 31: 827–832.

Hu, H. and Xiong, L. Genetic engineering and breeding of drought-resistant crops.2014. *Annu. Rev. Plant Biol.*65: 715–741.

Hu, H. H., Dai, M. Q., Yao, J. L., Xiao, B. Z., Li, X. H., Zhang, Q. F., et al. 2006. Overexpressing a *NAM, ATAF*, and *CUC* (NAC) transcription factor enhances drought resistance and salt tolerance in rice. *Proc. Natl. Acad. Sci. U.S.A.* 103: 12987–12992. doi: 10.1073/pnas.0604882103.

Hu, L., Liang, W., Yin, C., Cui, X., Zong, J., Wang, X., Hu, J. and Zhang, D. 2011. Rice *MADS3* regulates ROS homeostasis during late anther development. *Plant Cell*. 23: 515–533.

Huang, Y., Guo, Y., Liu, Y., Zhang, F., Wang, Z., Wang, H., Wang, F., Li, D., Mao, D., Luan, S., *et al*. 2018. *9-cis- Epoxycarotenoid Dioxygenase 3* regulates the plant growth and enhances multi abiotic stress tolerance in rice. *Front. plant sci.* 9:162

Hunter, C. T. 2021. CRISPR/Cas9 Targeted Mutagenesis for Functional Genetics in Maize. *Plants*. 10(4):723. https://doi.org/10.3390/plants10040723.

Hwang, J. E., Jang, D-S., Lee, K. J., Ahn, J-W., Kim, S. H., Kang, S-Y., Kim, D. S. and Kim, J-B. 2016. Identification of gamma ray irradiation-induced mutations in membrane transport genes in a rice population by TILLING. *Genes and genet. sys*. 91(5): 245-256.

Ishino, Y., Krupovic, M. and Forterre, P. 2018. History of CRISPR-Cas from Encounter with a Mysterious Repeated Sequence to Genome Editing Technology. *J. Bacteriol*. 200:7

Ishino, Y., Shinagawam H., Makinom K., Amemura, M., Nakata, A. 1987. Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene product. *J Bacteriol*. 169:5429–5433.

Jagadish, S. V. K., Craufurd, P. Q. and Wheeler, T. R. 2008. Phenotyping parents of mapping populations of rice for heat tolerance during anthesis. *Crop Sci*., 48: 1140-1146.

Jaganathan, D., Ramasamy, K., Sellamuthu, G., Jayabalan, S. and Venkataraman, G. 2018. CRISPR for crop improvement: an update review. *Front. Plant Sci*. 9(985): 1-17. https://doi.org/10.3389/fpls.2018.00985 .

Jansen, R., van Emben, J. D. A., Gaastra, W. and Schouls L. M. 2002. Identification of genes that are associated with DNA repeats in prokaryotes. *Mol. Microbiol*. 43(6) : 1565–1575.

Jeon, J. S., Lee, S., Jung, K. H., et al. .2000. T-DNA insertional mutagenesis for functional genomics in rice. *Plant J*. 22: 561–570.

Jeong, J. S., Kim, Y. S., Redillas, M. F. R., Jang, G., Jung, H., Bang, S. W., et al. 2013. *OsNAC5* overexpression enlarges root diameter in rice plants leading to enhanced drought tolerance and increased grain yield in the field. *Plant Biotechnol. J*. 11: 101–114. doi: 10.1111/pbi.12011.

Jiang, D., Zhou, L., Chen, W., Ye, N., Xia, J. and Zhuang, C. 2019. Overexpression of a microRNA-targeted NAC transcription factor improves drought and salt tolerance in Rice via ABA-mediated pathways. *Rice*.12:76

Jiang, W., Bikard, D., Cox, D., Zhang, F. and Marraffini, L.A. 2013. RNA-guided editing of bacterial genomes using CRISPR-Cas systems. *Nat Biotechnol*. 31: 233–239.

Jin, Y., Yang, H., Wei, Z., Ma, H. and Ge, X. 2013. Rice male development under drought stress: phenotypic changes and stage-dependent transcriptomic reprogramming. *Molecular Plant.* 6(5): 1630-1645.

Jin, Y., Yang, H., Wei, Z., Ma, H. and Ge, X. 2013. Rice male development under drought stress: phenotypic changes and stage-dependant transcriptomic reprogramming. *Molecular Plant*. 6(5): 1630-1645.

Jinek, M et al. 2014. Structures of Cas9 endonucleases reveal RNA-mediated conformational activation. *Science*. 343(6176):1247997.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A. and Charpentier, E. 2012. A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity. *Science.* 337, 816–821. doi: 10.1126/science.1225829

Joshi, R., Wani, S. H., Singh, B., Bohra, A., Dar, Z. A., Lone, A. A., Pareek, A. and Singla-Pareek, S. L. 2016. Transcription factors and plants responses to drought stress: current understanding and future directions. *Front. Plant Sci*. 7: 1029.

Kámán-Tóth, E., Pogány, M., Dankó, T. *et al.* 2018. A simplified and efficient *Agrobacterium tumefaciens* electroporation method. *3 Biotech* **8,** 148. https://doi.org/10.1007/s13205-018-1171-9 .

Kaneda, T., Taga, Y., Takai, R., Iwano, M., Matsui, H., Takayama, S., et al. 2009. The transcription factor *OsNAC4* is a key positive regulator of plant hypersensitive cell death. *EMBO J*. 28: 926–936. doi: 10.1038/emboj.2009.39.

Kang, H.G., Jeon, J.S., Lee, S. and An, G. 1998. Identification of class B and class C floral organ identity genes from rice plants. *Plant Mol. Biol.* 38, 1021–1029.

Khong, G., Pati, P., Richaud, F., Parizot, B., Bidzinski, P., Mai, C., Bès, M., Bourrié, I., Meynard, D., Beeckman, T., Selvaraj, M., Manabu, I., Genga, A., Brugidou, C., Do, V., Guiderdoni, E., Morel, J. & Gantet, P. 2015. *OsMADS26* negatively regulates resistance to pathogens and drought tolerance in rice. *Plant Physiol.*

Kim, H. and Kim, JS. 2014. A guide to genome engineering with programmable nucleases. *Nat Rev Genet.* **15,** 321–334. https://doi.org/10.1038/nrg3686 .

Kim, H., Lee, K., Hwang, H., Bhatnagar, N., Kim, D. Y. Yoon, I. S. Byun, M. O., Kim, S.T., Ki-Hong, J. and Kim, B. G. 2014. Overexpression of *PYL5* in rice enhances drought tolerance, inhibits growth, and modulates gene expression, *Journal of Experimental Botany*, 65(2) :453–464, https://doi.org/10.1093/jxb/ert397.

Kim, H., Lee, K., Hwang, H., Bhatnagar, N., Kim, D., Yoon, I., Byun, M., Kim, S., Jung, K. & Kim, B. 2014. Overexpression of *PYL5* in rice enhances drought tolerance, inhibits growth, and modulates gene expression. *J. Experimental Botany*

Kim, H., Lee, K., Hwang, H., Bhatnagar, N., Kim, D., Yoon, I., Byun, M., Kim, S., Jung, K. and Kim, B.2014. Overexpression of *PYL5* in rice enhances drought tolerance, inhibits growth, and modulates gene expression. *J. Experimental Botany*.

Kofuji, R., Sumikawa, N., Yamasaki, M., Kondo, K., Ueda, K., Ito, M. and Hasebe, M. 2003. Evolution and Divergence of the MADS-Box Gene Family Based on Genome-Wide Expression Analyses. *Molecular Biology and Evolution*. 20(12):1963–1977, https://doi.org/10.1093/molbev/msg216.

Kumar, V. and Jain, M. 2015. The CRISPR-Cas system for plant genome editing: advances and opportunities. *J Exp Bot*. 66:47–57.

Kurata, N., Nonomura, K. I. and Ima, Y. I. H. 2002. Rice Genome Organization: the Centromere and Genome Interactions. *Ann. Bot*., 90: 427-435.

Lacchini, E., Kiegle, E., Castellani, M., Adam, H., Jouannic, S., Gregis, V., et al. 2020. CRISPR-mediated accelerated domestication of African rice landraces. *PLoS ONE* .15(3): e0229782. https://doi.org/10.1371/journal.pone.0229782.

Lafitte, H. R., Ismail, A., Bennett, J. Abiotic stress tolerance in rice for Asia: progress and the future. 2004.*Crop Sci*.

Lafitte, H. R., Li, Z. K., Vijayakumar, C. H. M., Gao, Y. M., Shi, Y., Xu, J. L. and Maghirang, R. 2006. Improvement of rice drought tolerance through backcross breeding: Evaluation of donors and selection in drought nurseries. *Field Crop Res*. 97: 77–86.

Lee, S., Kim, J., Son, J.S., Nam, J., Jeong, D.H., Lee, K., Jang, S., Yoo, J., Lee, J., Lee, D.Y., Kang, H.G. and An, G. 2003. Systematic reverse genetic screening of T-DNA tagged genes in rice for functional genomic analyses: MADS box genes as a test case. *Plant Cell Physiol*. 44, 1403–1411

Lee, S., Woo, Y. M., Ryu, S.I., Shin, Y. D., Kim, W. T., Park, K. Y., Lee, I. J., and An, G. 2008. Further characterization of a rice AGL12 group MADS-box gene, *OsMADS26*. *Plant Physiol*. 47: pp. 156-168.

Liang, C. Z., Wang, Y. Q., Zhu, Y., Tang, J. Y., Hu, B., Liu, L. C., et al. 2014. *OsNAP* connects abscisic acid and leaf senescence by fine-tuning abscisic acid biosynthesis and directly targeting senescence-associated genes in rice. *Proc. Natl. Acad. Sci. U.S.A.* 111: 10013–10018. doi: 10.1073/pnas.13215 68111.

Liang, G., Zhang, H., Lou, D. and D. Yu. 2016. Selection of highly efficient sgRNAs for CRISPR/Cas9-based plant genome editing. *Scientific reports*. 6.

Liang, P.P., Xu, Y.W., Zhang, X.Y., et al. 2015. CRISPR/Cas9-mediated gene editing in human tripronuclear zygotes. *Protein Cell*. 6(5):363– 372.

Liljegren, S. J., Ditta, G. S., Eshed, Y., Savidge, B., Bowman, J. L. and Yanofsky, M. F. 2000. *SHATTERPROOF* MADS-box genes control seed dispersal in Arabidopsis. *Nature*. 404: 766–770.

Liu, W., Reif, J. C., Ranc, N., Della, P. G. and Würschum, T. 2012. Comparison of biometrical approaches for QTL detection in multiple segregating families. *Theor. Appl. Genet*. 125: 987–998.

Lou, D., Wang, H. and Yu, D. 2018. The sucrose non-fermenting-1-related protein kinases *SAPK1* and *SAPK2* function collaboratively as positive regulators of salt stress tolerance in rice. *BMC Plant Biol*. 18:203.

Makarova, K. S., Haft, D.H., Barrangou, R., Brouns, S. J., Charpentier, E., Horvath, P., Moineau, S., Mojica, F. J., Terns, R. M., Terns, M. P., White, M. F, Yakunin, A. F., Garrett, R. A., van der Oost, J., Backofen, R. and Koonin, E. V. 2011. Evolution and classification of the CRISPR-Cas systems. *Nat Rev Microbiol.* 9:467– 477. https:// doi.org/10.1038/nrmicro2577.

Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M., Dicarlo, J.E., Norville, J.E. and Church, G.M. 2013. RNA-guided human genome engineering via Cas9. *Science*. 339: 823-826.

Mandel, M.A., Gustafson-Brown, C., Savidge, B. and Yanofsky, M.F. 1992. Molecular characterization of the Arabidopsis floral homeotic gene *APETALA1*. *Nature* .360: 273–277.

Mehrabi, R., Ding, S. and Xu, J. R. 2008. MADS-box transcription factor *mig1* is required for infectious growth in *Magnaporthe grisea*. *Eukaryot. Cell.* 7: 791–799.

Meng, X., Hu, X., Liu, Q., Song, X., Gao, C., Li, J. and Wang, K. 2018. Robust genome editing of CRISPR-Cas9 at NAG PAMs in rice. *Sci China Life Sci*. 61. https://doi.org/10.1007/s11427-017-9247-9

Miah, G., Rafii, M. Y., Ismail, M. R., Sahebi, M., Hashemi, F. S. G., Yusuff, O. and Usman, M. 2017. G. Blast disease intimidation towards rice cultivation: A review of pathogen and strategies to control. *J Anim. Plant Sci.* 27: 1058–1066.

Miao, J., Guo, D., Zhang, J., Huang, Q., Qin, G., Zhang, X., Wan, J., Gu, H. and Qu, L.J. 2013. Targeted mutagenesis in rice using CRISPR-Cas system. *Cell Res.* 23: 1233-1236.

Ming, N. J., Binte Mostafiz, S., Johon, N. S, Abdullah Zulkifli, N. S. and Wagiran, A. 2019. Combination of Plant Growth Regulators, Maltose, and Partial Desiccation Treatment Enhance Somatic Embryogenesis in Selected Malaysian Rice Cultivar. *Plants*. 8(6):144. https://doi.org/10.3390/plants8060144 .

Mishra, R., Joshi, R. and Zhao, K. 2018. Genome editing in rice: recent advances, challenges, and

Mohanty, B., Kitazumi, A., Cheung, C.Y., Lakshmanan, M., de Los Reyes, B.G., Jang, I.C. and Lee, D.Y., 2016. Identification of candidate network hubs involved in metabolic adjustments of rice under drought stress by integrating transcriptome data and genome-scale metabolic network. *Plant Sci*. 242: 224–239.

Mojica, M. J., Juez, G. and Rodríguez-Valera, F. 1993. Transcription at different salinities of *Haloferax mediterranei* sequences adjacent to partially modified *PstI* sites. *Mol Microbiol.* 9:613–621.

Muthayya, S., Sugimoto, J. D., Montgomery, S. and Marberly, G. F. 2014. An overview of global rice production, supply, trade and consumption. *Ann. New York Acad. Sci*. 1324(1): 7-14.

Nakashima, K., Tran, L. P., Nguyen, D. V., Fujita, M., Maruyama, K., Todaka, D., et al. 2007. Functional analysis of a NAC-type transcription factor *OsNAC6* involved in abiotic and biotic stress-responsive gene expression in rice. *Plant J.* 51: 617–630. doi: 10.1111/j.1365-313X.2007.03168.x.

Nakashima, K., Yamaguchi-Shinozaki, K. and Shinozaki, K. 2014. The transcriptional regulatory network in the drought response and its crosstalk in abiotic stress responses including drought, cold, and heat. *Front. Plant Sci.* 5:170. doi: 10.3389/fpls.2014.00170.

Nuruzzaman, M., Sharoni, A. M., Satoh, K., Moumeni, A., Venuprasad, R., Serraj, R., Kumar, A., Leung, H., Attia, K. and Kikuchi, S. 2012. Comprehensive gene expression analysis of the NAC gene family under normal growth conditions, hormone treatment, and drought stress conditions in rice using near-isogenic

lines (NILs) generated from crossing Aday Selection (drought tolerant) and IR64. *Mol Genet Genomics*. 287: 389–410.

Oh, S.J.; Kim, Y.S.; Kwon, C.W.; Park, H.K.; Jeong, J.S.; Kim, J.K. 2009. Overexpression of the transcription factor AP37 in rice improves grain yield under drought conditions. *Plant Physiol*. 150: 1368–1379.

Oladosu, Y., Rafii, M. Y., Samuel, C., Fatai, A., Magaji, U., Kareem, I., Kamarudin, Z.S., Muhammad, I., and Kolapo, K. 2019. Drought resistance in rice from conventional to molecular breeding: A Review. *Int. J. Mol. Sci.* 20(14): pp. 3519 – 3540.

Pan, C., Ye, L., Qin, L., Liu, X., He, Y., Wang, J., et al. 2016. CRISPR/Cas9- mediated efficient and heritable targeted mutagenesis in tomato plants in the first and later generations. *Sci. Rep*. 6:24765. doi: 10.1038/srep24765.

Parenicová, L., de Folter, S., Kieffer, M., Horner, D. S., Favalli, C., Busscher, J., Cook, H. E., Ingram, R. M, Kater, M. M., Davies, B., et al. 2003. Molecular and phylogenetic analyses of the complete MADS-box transcription factor family in Arabidopsis: new openings to the MADS world. *Plant Cell.* 15: 1538–1551.

Patigu, R. F., Wijayanti, P., Sebastian, A. and Purwestri, Y. A. 2021. Optimization of heat shock temperature and time on the transformation of pRGEB32 into *Escherichia coli* DH5α. *J. Biologi Tropis*. 21(3): 632-640. http://dx.doi.org/10.29303/jbt.v21i3.2811 .

Pelucchi, N., Fornara, F., Favalli, C., Masiero, S., Lago, C., Colombo, L. and Kater, M. M. 2002. Comparative analysis of rice MADS-box genes expressed during flower development. *Sex Plant Reprod*. 15: 113–122.

Puranik, S., Sahu, P. P., Srivastava, P. S. and Prasad, M. 2012. NAC proteins: regulation and role in stress tolerance. *Trends Plant Sci*. 17: 369–381. doi: 10.1016/j.tplants.2012.02.004.

Pyott, D. E., Sheehan, E. and Molnar, A. 2016. Engineering of CRISPR/Cas9- mediated potyvirus resistance in transgene-free Arabidopsis plants. *Mol. Plant Pathol*. 17, 1276–1288. doi: 10.1111/mpp.12417.

Qi, J., Song, C. P., Wang, B., Zhou, J., Kangasjarvi, J., Zhu, J. K. and Gong, Z. 2018. Reactive oxygen species signaling and stomatal movement in plant responses to drought stress and pathogen attack. Journal of Integrative. *Plant Biol.* 60: 805–826.

Ratnayake, R. M. L. K. and Hettiarachchi. 2010. Development of an efficient Agrobacterium mediated transformation protocol for Sri Lankan rice variety-Bg 250. *Tropical. Agricul. Res.* 22(1): 45-53.

Ren, X., Yang, Z., Xu, J., Sun, J., Mao, D., Hu, Y., Yang, S-J., Qiao, H-H., Wang, X., Hu, Q., Deng, P., Liu, L-P., Ji, J-Y., Li, J. B. Ni, J-Q. 2014. Enhanced Specificity and Efficiency of the CRISPR/Cas9 System with Optimized sgRNA Parameters in Drosophila. *Cell Reports*. 9(3): 1151-1162.

Ricroch, A., Clairand, P. and Harwood, W. 2017. Use of CRISPR systems in plant genome editing: toward new opportunities in agriculture. *Emerg. Top. Life Sci*. 1: 169–182.

Rogers, S. O. and Bendich, A. J. 1994. Extraction of total cellular DNA from plants, algae and fungi. In: Gelvin S.B., Schilperoort R.A. (eds.) *Plant Molecular Biol. Manual*. 183-190. https://doi.org/10.1007/978-94-011-0511-8_12 .

Sadanandom, A., Srivastava, A. & Zhang, C. 2019. Targeted mutagenesis of the *SUMO* protease, Overly Tolerant to *Salt1* in rice through CRISPR/Cas9-mediated genome editing reveals a major role of this *SUMO* protease in salt tolerance.

Saedler, H., Becker, A., Winter, K. U., Kirchner, C. and Theiβen, G. 2001. MADS-box genes are involved in floral development and evolution. *Acta Biochimica Polonica*. 48(2): 351-358.

Sahebi, M., Hanafi, M. M., Rafii, M. Y., Mahmud, T. M. M., Azizi, P., Osman, M. and Miah, G.2018. Improvement of drought tolerance in rice (*Oryza sativa* L.): Genetics, genomic tools, and the WRKY gene family. *BioMed Res. Int.* 2018: 3158474.

Sahoo, D. K., Dey, N. and Maiti, I. B. 2014. pSiM24 Is a Novel Versatile Gene Expression Vector for Transient Assays As Well As Stable Expression of

Foreign Genes in Plants. *PLOS ONE*. https://doi.org/10.1371/journal.pone.0098988

Sahoo, K. K., Tripathi, A. K., Pareek, A., Sopory, S. K. and Singla-Pareek, S. L. 2011. An improved protocol for efficient transformation and regeneration of diverse indica rice cultivars. *Plant Methods*. 7: 49

Salam, S. A., Sindhumole, P., Waghmare, S. G. and Sajini, S. 2017. Molecular characterization of rice (*Oryza sativa* L.) genotypes for drought tolerance using two SSR markers. *Electronic J. Plant. Breed*. 8(2): 474-479.

Sallaud, C., Gay, C., Larmande, P., et al. .2004. High throughput T-DNA insertion mutagenesis in rice: a first step towards in silico reverse genetics. *Plant J.* 39: 450–464.

Sambrook, J., Fritsch, E. F. and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual 2nd Edn. Cold Spring Harbor, NY: *Cold Spring Harbor Laboratory Press*.

Sander, J. D. and Joung, J. K. 2014. CRISPR-Cas systems for editing, regulating and targeting genomes. *Nat Biotechnol.* 32(4):347–355.

Sato, H., Takasaki, H., Takahashi, F., Suzuki, T., Iuchi, S., Mitsuda, N., Ohme-Takagi, M., Ikeda, M., Seo, M., Yamaguchi-Shinozaki, K., et al. 2018. Arabidopsis thaliana NGATHA1 transcription factor induces ABA biosynthesis by activating *NCED3* gene during dehydration stress. Proceedings of the National Academy of Sciences, USA 115: E11178–E11187.

Serraj, R., McNally, K., Slamet-Loedin, I., Kohli, A., Haefele, S., Atlin, G. & Kumar, A. 2011. Drought resistance improvement in rice: An integrated genetic and resource management strategy. *Plant Production Sci*.

Shan Q, Wang Y, Li J, Zhang Y, Chen K, Liang Z, et al. 2013. Targeted genome modification of crop plants using a CRISPR-Cas system. *Nat Biotech*. 31(8): 686–688.

Shan, Q., Wang, Y., Li, J. and Gao, C. 2014. Genome editing in rice and wheat using the CRISPR/Cas system. *Nature protocols*. 9(10): 2395-2410pp.

Shan, Q., Wang, Y., Li, J. and Gao, C. 2014. Genome editing in rice and wheat using the CRISPR/Cas system. *Nature protocols*. 9(10):2395-2410pp.

Shao, H., Wang, H. and Tang, X. 2015. NAC transcription factors in plant multiple abiotic stress responses: progress and prospects. *Front. Plant Sci.* 6:902. doi: 10.3389/fpls.2015.00902.

Shen, C., Que, Z., Xia, Y., Tang, N., Li, D., He, R. and Cao, M. 2017. Knock Out of the Annexin Gene *OsAnn3* via CRISPR/Cas9-mediated Genome Editing Decreased Cold Tolerance in Rice. *J. Plant Biol*. 60:539-547pp

Shim, J. S., Park, S-H., Lee, D-K., Kim, Y. S., Park, S-C., Redillas, M. C. F. R., Seo, J. S. and Kim, J-K. 2021. The rice *GLYCINE RICH PROTEIN 3* confers drought tolerance by regulating mRNA stability of ROS scavenging-related genes. *Rice*. 14: 31.

Shore, P. and Sharrocks A. D. 1995. The MADS-box family of transcription factors. *Eur J Biochem* 229: 1–13.

Singhal, P., Jan, A. T., Azam, M. and Haq, Q. M. R.2016. Plant abiotic stress: A prospective strategy of exploiting promoters as alternative to overcome the escalating burden. *Front. Life Sci.* 9: 52–63.

Skamnioti, P. and Gurr, S. J. 2009. Against the grain: Safeguarding rice from rice blast disease. *Trends in Biotechnol.* 27: 141-150.

Song, S. Y., Chen, Y., Chen, J., Dai, X. Y. and Zhang, W. H. 2011. Physiological mechanisms underlying OsNAC5-dependent tolerance of rice plants to abiotic stress. *Planta* 234: 331–345. doi: 10.1007/s00425-011-1403-2.

Srivasta, A., Mehta, S., Lindlof, A. and Bhargava, S. 2010. Over-represented promoter motifs in abiotic stress-induced *DREB* genes of rice and sorghum and their probable role in regulation of gene expression. *Plant signalling and behaviour*. 5(7): 775-784.

Sun, L. J., Zhang, H., Li, D., Huang, L., Hong, Y., Ding, X. S., et al. 2013. Functions of rice NAC transcriptional factors, *ONAC122* and *ONAC131*, in defense responses against Magnaporthe grisea. *Plant Mol. Biol*. 81: 41–56. doi: 10.1007/s11103-012-9981-3.

Tahir, T., Ali, Q., Rashid, M. S. and Malik, A. 2020. The journey of crispr-cas9 from bacterial defense mechanism to a gene editing tool in both animals and plants. *Biol. Clin. Sci. Res. J*., Volume,: e017.

Tang, Y., Bao, X., Zhi, Y., Wu, Q., Guo, Y., Yin, X., Zeng, L., Li, J., *et al*. 2019. Overexpression of a MYB family gene, *OsMYB6*, increases drought and salinity stress tolerance in transgenic rice. *Front. Plant Sci.* 10: pp. 1-12.

Todaka, D., Shinozaki, K., Yamaguchi-Shinozaki, K., 2015. Recent advances in the dissection of drought-stress regulatory networks and strategies for development of drought-tolerant transgenic rice plants. *Front. Plant Sci*. 6, 84.

Toki, S., Hara, N., Ono, K., Onodera, H., Tagiri, A., Oka, S. and Tanaka, H. 2006. Early infection of scutellum tissue with *Agrobacterium* allows high-speed transformation of rice. *The Plant J.* 47: pp. 969-976

Toki, S., Hara, N., Ono, K., Onodera, H., Tagiri, A., Oka, S. and Tanaka, H. 2006. Early infection of scutellum tissue with *Agrobacterium* allows high-speed transformation of rice. *The Plant J.* 47: pp. 969-976

Uga, Y., Sugimoto, K., Ogawa, S., Rane, J., Ishitani, M., Hara, N., Kitomi, Y., Inukai, Y., Ono, K., Kanno, N. et al. 2013. Control of root system architecture by *DEEPER ROOTING 1* increases rice yield under drought conditions. *Nature Genetics* 45: 1097–1102.

USDA [United States Department of Agriculture]. 2016. Economic Research Service. Available at https:// www.ers.usda.gov/topics/crops/rice.aspx.

Waghmare, S. G., Sindhumole, P., Mathew, D., Shylaja, M. R., Francies, R. M., Abida, P. S. and Narayanankutty, M. C. 2020. Identification of QTL linked to heat tolerance in rice (*Oryza sativa* L.) using SSR markers through bulked segregant analysis. *Electronic J. Plant. Breed.* 12(1): 46-53.

Weigel, D. and Glazebrook, J. 2006. Transformation of agrobacterium using the freeze-thaw method. *CSH Protocols*. 2006:(7). DOI: 10.1101/pdb.prot4666. PMID: 22484682.

Wing, R. A., Purugganan, M. D. and Zhang, Q. 2018. The rice genome revolution: From an ancient grain to Green Super Rice. *Nature Reviews Genetics* 19:505-17.

Xiang, J., Chen, X., Hu, W., Xiang, Y., Yan, M. and Wang, J. 2018. Overexpressing heat-shock protein OsHSP50 .2 improves drought tolerance in rice. *Plant Cell Reports*. 37(11): 1585-1595.

Xiao, B. Z., Huang, Y. M., Tang, N. and Xiong, L. Z. 2007. Over-expression of a *LEA* gene in rice improves drought resistance under the field conditions. *Theor. Appl. Genet*. 115: 35–46. doi: 10.1007/s00122-007-0538-9.

Xie, K. and Yang, Y. 2013. RNA-guided genome editing in plants using a CRISPR-Cas system. *Mol Plant*. 6(6):1975–1983.

Xie, K. and Yang, Y. 2013. RNA-guided genome editing in plants using a CRISPR–Cas system. *Mol Plant*. 6(6):1975–1983.

Xie, K., Minkenberg, B. and Yang, Y. 2014. Targeted gene mutation in rice using CRISPR-Cas9 system. *bio-protocol*. 17:4.

Xie, K., Minkenberg, B. and Yang, Y. 2015. Boosting CRISPR/Cas9 multiplex editing capability with the endogenous tRNA-processing system. *Proc. Natl. Acad. Sci. U. S. A.* 112: 3570-3575.

Xie, Z., Zhang, Z. L., Zou, X., Huang, J., Ruas, P., Thompson, D. and Shen, Q. J. 2005. Annotations and functional analyses of the rice WRKY gene superfamily reveal positive and negative regulators of abscisic acid signaling in aleurone cells. *Plant Physiol.* 137: 176–189.

Yamaguchi, T. and Hirano, H-Y. 2006. Functions and diversification of MADS-box genes in rice. *The Scientific World J.* 6: 1923-1932. DOI 10.1100/tsw.2006.320.

Yang, Y. C., Argayoso, M., Redoña, E., Sierra, S., Laza, M., Dilla, C., Mo, Y. J., Thomson, M. J., Chin, J. H., Delaviña, C. B., Diaz, G. Q. and Hernandez, J. 2011. Mapping QTL for heat tolerance at flowering stage in rice using SNP markers. *Plant Breed*., 131: 33- 41.

Yang, Y., Fanning, L. and Jack, T. 2003. The K domain mediates heterodimerization of the *Arabidopsis* floral organ identity proteins, *APETALA3* and *PISTILLATA*. *The Plant J.* 33: 47-59. https://doi.org/10.1046/j.0960-7412.2003.01473.x.

Yokotani, N., Tsuchida-Mayama, T., Ichikawa, H., Mitsuda, N., Ohme-Takagi, M., Kaku, H., et al. 2014. *OsNAC111*, a blast disease-response transcription factor in rice, positively regulates the expression of defense-related genes. *Mol. Plant Microbe Interact*. 27: 1027–1034. doi: 10.1094/MPMI-03-14- 0065-R.

Yoshii, M., Shimizu, T., Yamazaki, M., Higashi, T., Miyao, A., Hirochika, H., et al. 2009. Disruption of novel gene for a NAC-domain protein in rice confers resistance to Rice dwarf virus. *Plant J.* 57: 615–625. doi: 10.1111/j.1365-313X.2008.03712.x.

Yu, J., Lai, Y., Wu, X., Wu, G. and Guo, C. 2016. Overexpression of *OsEm1* encoding a group I LEA protein confers enhanced drought tolerance in rice. *Biochem. Biophys. Res. Commun*. 478: pp.703-709.

Zafar, K., Khan, M. Z., Amin, I., Mukhtar, Z., Yasmin, S., Arif, M., Ejaz, K. and Mansoor, S. 2020. Precise CRISPR-Cas9 Mediated Genome Editing in Super Basmati Rice for Resistance Against Bacterial Blight by Targeting the Major Susceptibility Gene. *Front. Plant Sci*. https://doi.org/10.3389/fpls.2020.00575 .

Zeng, D., Hou, P., Xiao, F. & Liu, Y. Overexpression of Arabidopsis *XERICO* gene confers enhanced drought and salt stress tolerance in rice (*Oryza Sativa L.*) 2013. *J. Plant Biochemistry and Biotechnol*.

Zhang, A., Liu, Y., Wang, F., Li, T., Chen, Z., Kong, D., Bi, J., Zhang, F., Luo, X., Wang, J., Tang, J., Yu, X., Liu, G. & Luo, L. 2019. Enhanced rice salinity tolerance via CRISPR/Cas9-targeted mutagenesis of the *OsRR22* gene. *Molecular Breeding*

Zhang, H., Zhang, J., Wei, P., Zhang, B., Gou, F., Feng, Z., Mao, Y., Yang, L., Zhang, H., Xu, N., *et al.* 2014. The CRISPR/Cas9 system produces specific and homozygous targeted gene editing in rice in one generation. *Plant Biotechnol. J.* 12:797–807pp

Zhang, H., Zhang, J., Wei, P., Zhang, B., Gou, F., Feng, Z., Mao, Y., Yang, L., Zhang, H., Xu, N., *et al.* 2014. The CRISPR/Cas9 system produces specific and homozygous targeted gene editing in rice in one generation. *Plant Biotechnol. J.* 12:797–807pp

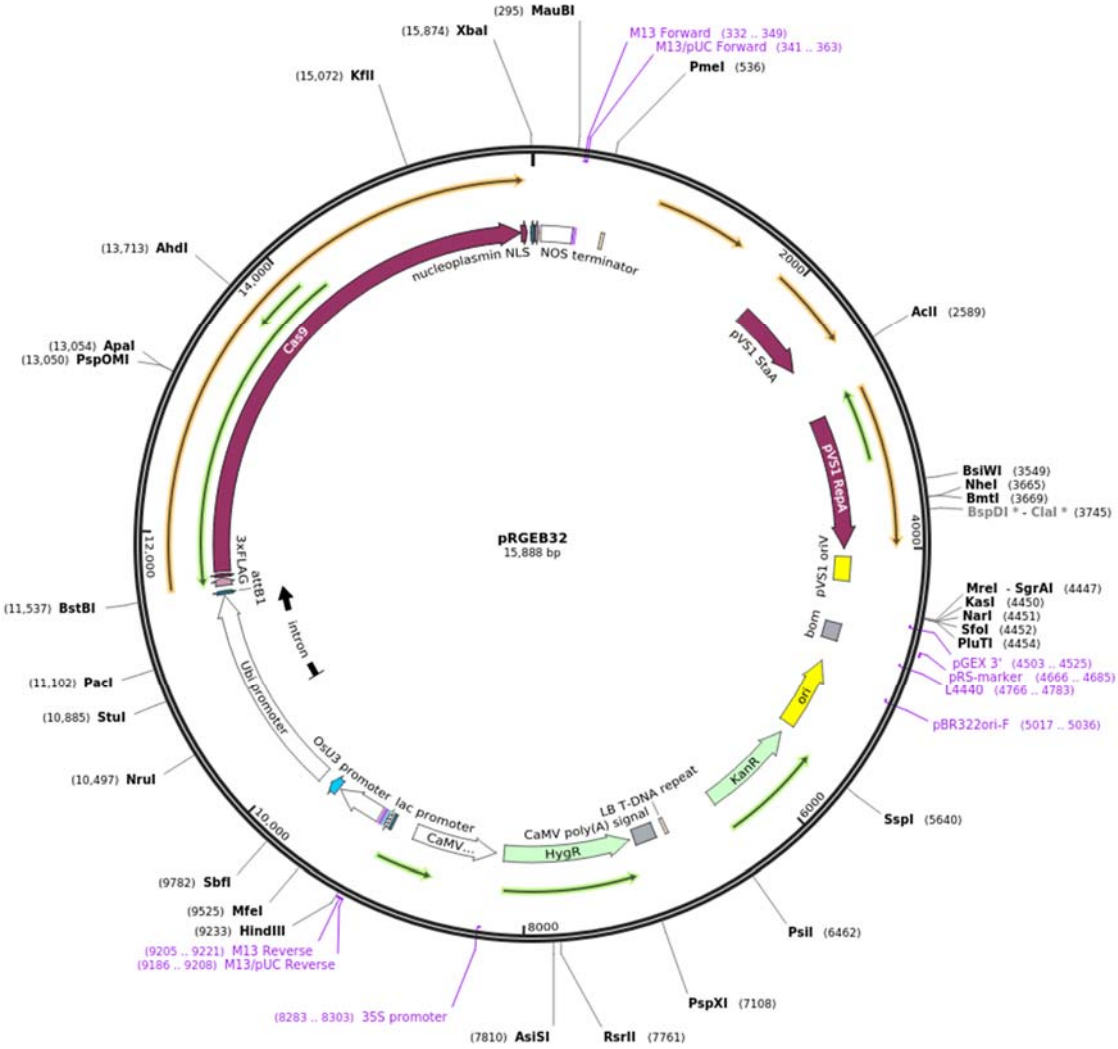
Zheng, X. N., Zhen, B., Lu, G. J. and Han, B. 2009. Overexpression of a NAC transcription factor enhances rice drought and salt tolerance. *Biochem. Biophys. Res. Commun*. 379: 985–989. doi: 10.1016/j.bbrc.2008. 12.163.

Zhou, J., Peng, Z., Long, J., Sosso, D., Liu, B., Eom, J. S., Huang, S., Liu, S., Cruz, C. V., Frommer, W. B., White, F. F. and Yang, B. 2015. *Plant J*. 82, 632.

ANNEXURES

ANNEXURE I

Vector map of pRGEB32 procured from Addgene (CAT#63142)



ANNEXURE II

Genomic sequence of *OsMADS26* gene sequence from 'Rice Genome Annotation Project'

LOC_Os08g02070 sequence information Genomic sequence length: 2382 nucleotides

CDS length: 669 nucleotides

Protein length: 222 amino acids

Putative Function: OsMADS26 - MADS-box family gene with MIKCc type-box, expressed

Genomic Sequence

>LOC_Os08g02070

AAGCAAGAGATAGGGATAAGGGGAAGAGGAGGAAGAAGGAGGAGGTGT
AGGGAGAAACCGGAGCAACCTCGAAGCTAGTCCAAACTAGTGGGAGGTT
GTCTTTCCGGCAAGCCGGAGCCCGGAGCTATCGATCATCAAGCTTTCTACC
CCGACCGACGAGGAAGAAGACGACTGATCAATTGATCAAACCGATCTCTC
CATAGCTAGGTAGACAGGAGGAGAGGAGGAAGAAGAGGGGGAGAGGAG
ACTTATCTTGATCGATGGCGCGAGGCAAGGTGCAGCTCCGTCGCATCGAG
AACCCGGTTCACCGTCAGGTCACCTTCTGCAAGCGCCGTGCCGGCCTGCTG
AAGAAGGCCAGGGAGCTCTCCATCCTCTGCGAGGCCGACATCGGCATCAT
CATCTTCTCCGCCCACGGCAAGCTCTACGACCTCGCCACCACCGGGTACCT
TCAGCTTCTTCTCCATCAAATCAAATTAAAAAAGGTTTTGAGCTTAGCTCT
GATATACCATGGAGGTTAATTGATTTCTTTGAACAGAACCATGGAGGAGC
TGATCGAGAGGTACAAGAGTGCTAGTGGCGAACAGGCCAACGCCTGCGG
CGACCAGAGAATGGTTAGTGATTATAACTCTTTCCTTTTGTTTCAGTTCTTT
TTCTGTTTCTGTTTCAGTTGTTATCCTTTACTGTTTCCATTTCAGTTGTTAGC
TAGCCCCCACTCCAATTTCTAATCAGTGCCTAGTTTCATATGTAAATTAAG
CATAGGGAGTTGCTAGTGATGAAAAAAAAGGTCTGGAAATAGAGCTAGTT
GTATCCGAAAAATCTGAGCTGCTATTATGTTACAGATGATTAAACCTGGA

ACTGAAAACTCTAGGCTCTAGCCCAAAACTAGTTGGAATACATCAAATAG
ACAATAGTATAAAGTATAAACACCTGATCTACACATCTTAGGTGGCAAGT
AGTTGCAACTTCGTAATATTTCCTCCATTTTACCTCATGCTCTCCTATCACT
TAAAACTGGTGCATGTATGTCATCAGGACCCAAAACAGGAGGCAATGGTG
CTCAAACAAGAAATCAATCTACTGCAGAAGGGCCTGAGGTAAAAACTATT
CACAGATTGCATTACAGATTGGTATAATTCCTTTCTAGAAGAAAAAATAG
ATAAAATCATACAATCTTCCAGCAAAAATATCCAAGTTTGATTTCATAAAC
AAACATAGCTTTCTATGTCCAATTAGTAAGTTCACATCGCAACAAGTATGC
AATGCTATCCAGACTTGGTCCACACTCAGTATCATTCACTAGATCACTGCA
AGTAACATAACCATACATGGAAGTGTTACAAAAAAATTGTTGAACTCACA
CTAAGTAAAAGAGCTTCATTCACCAAACCGAACTGTTTACTCGAGAAATT
GATGTAATAAGAATACATCATATATTATTTACATTATAATGAGTTCAGACA
CTTAACTCTTGACAAAATTAAAACCCATGGCAGGTACATCTATGGGAACA
GGGCAAATGAACACATGACTGTTGAAGAGCTGAATGCCCTAGAGAGGTAC
TTAGAGATATGGATGTACAACATTCGCTCCGCAAAGGTAACTCATTGAAA
GTCTTCAGATTTCATAAAGAACAAATTGTTCAAGAGATCTAAAATCTTTAA
TCTAACAGATGCAGATAATGATCCAAGAGATCCAAGCACTAAAGAGCAA
GGCAAGCCAAACTATCTTGTTTAACTCTATAAATATCCAATAAAATGATTA
ATAACTAACTAAAAGCATAGTTTATCTCTAATCGTGCAGGAAGGCATGTT
GAAAGCTGCTAACGAAATTCTCCAAGAAAAGGTACTGGCAGTCCATTGAC
ATAGAACTGTGCACTTGCCAGTTTTAAAAATGAAGATAAAATGTTGCAGC
GTGCCGTAAACTGATGTCAGATATGTGCTTTTGCTTGCAGATAGTAGAACA
GAATGGTCTGATCGACGTAGGCATGATGGTAGCAGATCAACAGAATGGGC
ATTTTAGTACAGTCCCACTGTTAGAAGAGATCACTAACCCACTGACTATAC
TGAGTGGCTATTCTACTTGTAGGGGCTCGGAGATGGGCTATTCCTTCTAAC
ACTAATAATGGCCTGGGGGATACTTGTGTTCATTACTAGTGTGTAATATGG
TTAATAATGCTTGTGTTGCTGTTTGCTTTGCTATTCTGATGTACCTTATTTA
GACAAGTTCCCGCAGGAAGTGTCTTTTAGTATTGTATTTGTCTTGGGCTGT
GGTGCTTTGTTTTTCCCTAAAGAACTCTTGAGGAGCTCTGTTGTTGAACCA
TTTCAAGTAATTGAGACTATTGTTTCCTGGAACGTTTATTACATTTGTAGA
ATTAAGCATTTT

CDS

>LOC_Os08g02070.1

ATGGCGCGAGGCAAGGTGCAGCTCCGTCGCATCGAGAACCCGGTTCACCG
TCAGGTCACCTTCTGCAAGCGCCGTGCCGGCCTGCTGAAGAAGGCCAGGG
AGCTCTCCATCCTCTGCGAGGCCGACATCGGCATCATCATCTTCTCCGCCC
ACGGCAAGCTCTACGACCTCGCCACCACCGGAACCATGGAGGAGCTGATC
GAGAGGTACAAGAGTGCTAGTGGCGAACAGGCCAACGCCTGCGGCGACC
AGAGAATGGACCCAAAACAGGAGGCAATGGTGCTCAAACAAGAAATCAA
TCTACTGCAGAAGGGCCTGAGGTACATCTATGGGAACAGGGCAAATGAAC
ACATGACTGTTGAAGAGCTGAATGCCCTAGAGAGGTACTTAGAGATATGG
ATGTACAACATTCGCTCCGCAAAGATGCAGATAATGATCCAAGAGATCCA
AGCACTAAAGAGCAAGGAAGGCATGTTGAAAGCTGCTAACGAAATTCTCC
AAGAAAAGATAGTAGAACAGAATGGTCTGATCGACGTAGGCATGATGGT
AGCAGATCAACAGAATGGGCATTTTAGTACAGTCCCACTGTTAGAAGAGA
TCACTAACCCACTGACTATACTGAGTGGCTATTCTACTTGTAGGGGCTCGG
AGATGGGCTATTCCTTCTAA

ANNEXURE III

Guide RNA targeting regions in *OsMADS26* gene sequence

Genomic Sequence

>LOC_Os08g02070

AAGCAAGAGATAGGGATAAGGGGAAGAGGAGGAAGAAGGAGGAGGTGT
AGGAGAAACCGGAGCAACCTCGAAGCTAGTCCAAACTAGTGGGAGGTTG
TCTTTCCGGCAAGCCGGAGCCCGGAGCTATCGATCATCAAGCTTTCTACCC
CGACCGACGAGGAAGAAGACGACTGATCAATTGATCAAACCGATCTCTCC
ATAGCTAGGTAGACAGGAGGAGAGGAGGAAGAAGAGGGGGAGAGGAGA
CTTATCTTGATCGATGGCGCGAGGCAAGGTGCAGCTCCGTCGCATCGAGA
ACCCGGTTCACCGTCAGGTCACCTTCTGCAAGCGCCGTGCCGGCCTGCTGA
AGAAGGCCAGGGAGCTCTCCATCCTCTGCGAGGCCGACATCGGCATCATC
ATCTTCTCCGCCCACGGCAAGCTCTACGACCTCGCCACCACCGGGTA
CCTTCAGCTTCTTCTCCATCAAATCAAATTAAAAAAGGTTTTGAGCTTAGC
TCTGATATACCATGGAGGTTAATTGATTTCTTTGAACAGAACCATGGAGG
AGCTGATCGAGAGGTACAAGAGTGCTAGTGGCGAACAGGCCAACGCCTG
CGGCGACCAGAGAATGGTTAGTGATTATAACTCTTTCCTTTTGTTTCAGTT
CTTTTTCTGTTTCTGTTTCAGTTGTTATCCTTTACTGTTTCCATTTCAGTTGT
TAGCTAGCCCCCACTCCAATTTCTAATCAGTGCCTAGTTTCATATGTAAAT
TAAGCATAGGGAGTTGCTAGTGATGAAAAAAAAGGTCTGGAAATAGAGC
TAGTTGTATCCGAAAAATCTGAGCTGCTATTATGTTACAGATGATTAAACC
TGGAACTGAAAACTCTAGGCTCTAGCCCAAAACTAGTTGGAATACATCAA
ATAGACAATAGTATAAAGTATAAACACCTGATCTACACATCTTAGGTGGC
AAGTAGTTGCAACTTCGTAATATTTCCTCCATTTTACCTCATGCTCTCCTAT
CACTTAAAACTGGTGCATGTATGTCATCAGGACCCAAAACAGGAGGCAAT
GGTGCTCAAACAAGAAATCAATCTACTGCAGAAGGGCCTGAGGTAAAAA
CTATTCACAGATTGCATTACAGATTGGTATAATTCCTTTCTAGAAGAAAAA
ATAGATAAAATCATACAATCTTCCAGCAAAAATATCCAAGTTTGATTTCAT

AAACAAACATAGCTTTCTATGTCCAATTAGTAAGTTCACATCGCAACAAG
TATGCAATGCTATCCAGACTTGGTCCACACTCAGTATCATTCACTAGATCA
CTGCAAGTAACATAACCATACATGGAAGTGTTACAAAAAAATTGTTGAAC
TCACACTAAGTAAAAGAGCTTCATTCACCAAACCGAACTGTTTACTCGAG
AAATTGATGTAATAAGAATACATCATATATTATTTACATTATAATGAGTTC
AGACACTTAACTCTTGACAAAATTAAAACCCATGGCAGGTACATCTATGG
GAACAGGGCAAATGAACACATGACTGTTGAAGAGCTGAATGCCCTAGAG
AGGTACTTAGAGATATGGATGTACAACATTCGCTCCGCAAAGGTAACTCA
TTGAAAGTCTTCAGATTTCATAAAGAACAAATTGTTCAAGAGATCTAAAA
TCTTTAATCTAACAGATGCAGATAATGATCCAAGAGATCCAAGCACTAAA
GAGCAAGGCAAGCCAAACTATCTTGTTTAACTCTATAAATATCCAATAAA
ATGATTAATAACTAACTAAAAGCATAGTTTATCTCTAATCGTGCAGGAAG
GCATGTTGAAAGCTGCTAACGAAATTCTCCAAGAAAAGGTACTGGCAGTC
CATTGACATAGAACTGTGCACTTGCCAGTTTTAAAAATGAAGATAAAATG
TTGCAGCGTGCCGTAAACTGATGTCAGATATGTGCTTTTGCTTGCAGATAG
TAGAACAGAATGGTCTGATCGACGTAGGCATGATGGTAGCAGATCAACAG
AATGGGCATTTTAGTACAGTCCCACTGTTAGAAGAGATCACTAACCCACT
GACTATACTGAGTGGCTATTCTACTTGTAGGGGCTCGGAGATGGGCTATTC
CTTCTAACACTAATAATGGCCTGGGGGATACTTGTGTTCATTACTAGTGTG
TAATATGGTTAATAATGCTTGTGTTGCTGTTTGCTTTGCTATTCTGATGTAC
CTTATTTAGACAAGTTCCCGCAGGAAGTGTCTTTTAGTATTGTATTTGTCTT
GGGCTGTGGTGCTTTGTTTTTCCCTAAAGAACTCTTGAGGAGCTCTGTTGT
TGAACCATTTCAAGTAATTGAGACTATTGTTTCCTGGAACGTTTATTACAT
TTGTAGAATTAAGCATTTT

GGAGCTCTCCATCCTCTGCG : gRNA 1

CCGGCCTGCTGAAGAAGGCC : gRNA 2

CGAAGCTAGTCCAAACTAGT : gRNA 3

OsMADS26 FP: GGATAAGGGGAAGAGGAGGA

OsMADS26 RP: CCTTCAGCTTCTTCTCCATCA

ANNEXURE IV

Primers designed for the study

Sl. No.	Primer Name	Sequence 5' – 3'	Length	Tm	Remarks
1	RA042	GGATAAGGGGAAGAGGAGGA	20	63.2	FP *OsMADS 26*
2	RA043	TGATGGAGAAGAAGCTGAAGG	21	63.4	RP *OsMADS 26*
3	RA048	ATGAAAAAGCCTGAACTCACCGC	23	69.2	FP Hyg
4	RA049	CTTTGCCCTCGGACGAGTGCTG	22	74.1	RP Hyg
5	RA050	CGGCGAGTACTTCTACACAGC	21	63.8	FP Hyg
6	RA051	GCGAAGAATCTCGTGCTTTC	20	63.9	RP Hyg
7	RA052	GACGAGTACAAGGTGCCCAG	20	64.7	FP Cas9
8	RA053	TTTTCCAGGATGGGCTTGAT	20	65.5	RP Cas9
9	RA054	AGCGGATAACAATT TCACACAGG	23	65.8	M13 Puc REV GUIDE SEQ FP
10	RA055	GAATTTGTGGACCTGCAGGC	20	66.8	GUIDE SEQ REV PRIMER

ABSTRACT

EDITING OF RICE TRANSCRIPTION FACTOR *OsMADS26* FOR DROUGHT TOLERANCE THROUGH CRISPR/Cas9 SYSTEM

By

ANJALA K.

(2019-11-004)

ABSTRACT OF THE THESIS

Submitted in partial fulfilment of the requirement for the degree of

Master of Science in Agriculture

(PLANT BIOTECHNOLOGY)

Faculty of Agriculture

Kerala Agricultural University, Thrissur

DEPARTMENT OF PLANT BIOTECHNOLOGY

CENTRE FOR PLANT BIOTECHNOLOGY AND MOLECULAR BIOLOGY

COLLEGE OF AGRICULTURE

VELLANIKKARA, THRISSUR-680656

KERALA, INDIA

2021

ABSTRACT

Rice (*Oryza sativa* L.) is the most widely consumed staple food of world's human population belonging to Asia and Africa. Being a semi-aquatic annual plant, rice is highly prone to losses due to various environmental stresses. Many studies regarding this had revealed the need for developing varieties tolerant to abiotic and biotic stresses. Various methods like Marker Assisted Breeding, mutation breeding, RNAi, Antisense technology, ZFNs and TALENs were in use to develop elite traits for abiotic stress tolerance in crops like rice. But very recently, CRISPR/Cas9 system had come into the limelight as an efficient tool for the genetic manipulations of crops. Studies have identified *OsMADS26* transcription factor as a negative regulator of drought tolerance in rice. Hence the current study, 'Editing of rice transcription factor *OsMADS26* for drought tolerance through CRISPR/Cas9 system' was undertaken during the period from 2019 to 2021 at the Centre for Plant Biotechnology and Molecular Biology, CoA, Vellanikkara, Thrissur with an objective to develop drought tolerance in rice.

The rice cultivar Nipponbare was selected for the study due to its competence in genetic transformation and regeneration. For CRISPR/Cas9 mediated targeted editing of *OsMADS26* gene, guide RNAs (gRNAs) were designed using online software CRISPR-P v2. Genome sequence information of *OsMADS26* gene available from rice genome annotation project was used for the study. Genomic region of *OsMADS26* gene, flanking the gRNA target (~ 450 bp) was amplified using gene specific primers and sequence of the target region was confirmed using BLASTn and ClustalW analysis. The CRISPR/Cas9 binary vector pRGEB32 was used to clone the guide RNAs using BsaI restriction sites. Three gRNAs were selected for cloning based on features like on score value (higher the value better the editing efficiency), GC content, (40-60%), no. of off-target sites (Minimum number of off-target sites preferred), presence of secondary structure, location on the genome (towards 5' end of gene in exonic region is preferred) etc.

The CRISPR/Cas9 construct for cloning was developed by annealing and ligating the gRNAs to the pRGEB32 vector followed by cloning in *E. coli* strain DH5α. The putative positive clones were identified by colony PCR and further confirmed by Sanger sequencing. The plasmids isolated from PCR positive colonies were sequenced

using universal M13 Reverse primer which is present on pRGEB32 vector. The sequences of the clones were confirmed using multiple sequence alignment tool ClustalW. One colony of gRNA 1 construct (OsMADS26 #G1-1) and two colonies of gRNA 3 (OsMADS26 #G3-3 and OsMADS26 #G3-4) were found positive.

The CRISPR/Cas9 constructs of *OsMADS26* were then mobilized into *Agrobacterium tumefaciens* strain EHA105 following the Freeze-thaw method. The positive clones were identified using plasmid PCR using hygromycin gene specific primers. Positive colonies of OsMADS26 #G1-1 and OsMADS26 #G3-3 constructs in EHA105 were then used for rice genetic transformation.

The seeds of *Oryza sativa* sub species japonica cultivar Nipponbare were inoculated into N6 medium supplemented with 3.0 mgL^{-1} 2,4-D for callus induction. After five days, the calli were infected with *Agrobacterium* cultures harboring desired gRNA constructs for 1.5-2 min. Along with the gRNA constructs, an empty vector was also transformed to rice as vector control and a set of untransformed culture were also maintained. After around two days of co-cultivation, the excess *Agrobacterium* growth was washed-off thoroughly from the calli using the bacteriostatic agent Augmentin. The calli were then placed on selection medium containing Augmentin and Hygromycin. The hygromycin resistant calli showed proliferation after 14 days of incubation. The proliferating microcalli were then transferred to regeneration medium after 21 days. Proliferation of microcalli was observed in vector control, wild type as well as OsMADS26 #G1-1 and OsMADS26 #G3-3 co-transformed plates. The vector control and untransformed calli showed greening and shoot primordia initiation in regeneration medium. The regenerated shoots will be analyzed for mutation in future. Hence, in the current study, gRNA constructs for targeted editing of *OsMADS26* gene was successfully developed and transformed in to rice cultivar Nipponbare. Rice genetic transformation suitable to our lab conditions were also optimized. Rice plants with mutations in the *OsMADS26* gene is expected in future which can confer drought tolerance.