# Understanding the role of a novel miRNA:target module in DNA damage repair in rice

A Thesis

submitted to

Indian Institute of Science Education and Research Pune in partial fulfilment of the requirements for the BS-MS Dual Degree Programme

by

GITESH VILASRAO MASRAM


Indian Institute of Science Education and Research Pune

Dr. Homi Bhabha Road,

Pashan, Pune 411008, INDIA.

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Supervisor: Dr. P. V. SHIVAPRASAD

GITESH VILASRAO MASRAM

# Certificate

This is to certify that this dissertation entitled "Understanding the role of a novel miRNA: target module in DNA damage repair in rice" towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by GITESH VILASRAO MASRAM at the National Centre for Biological Sciences under the supervision of Dr P. V. SHIVAPRASAD, Associate Professor, National Centre for Biological Sciences, during the academic year 2022-2023.

Shivaprasad P.V.

Dr. P. V. SHIVAPRASAD

Committee:

Dr. P. V. SHIVAPRASAD

Dr. KALIKA PRASAD

This thesis is dedicated to my father, **Shree. Vilasrao Chindhuji Masram** for all the hard work he has done to educate his children. May the coming generations dedicate themselves to the upliftment of tribal people and the betterment of society.

दो बूँद हो मयस्सर क़यामत से पहले,

कहने को तो आसमान से किताब बरसी है।

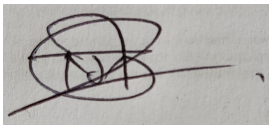
किसीकी जटा से निकल रही है गंगा,

कही छूने को पानी पूरी कौम तरसी है॥

- तुलसीदास खान

# Declaration

I hereby declare that the matter embodied in the report entitled “Understanding the role of a novel miRNA: target module in DNA damage repair in rice” is the result of the work carried out by me at the National Centre of Biological Sciences, Bangalore, under the supervision of Dr. P. V. SHIVAPRASAD the same has not been submitted elsewhere for any other degree.



GITESH VILASRAO MASRAM

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# Abstract

The characteristics of the plants and fruits we see today are far from similar to what they used to be. The change happened in due course of time with voluntary or involuntary actions. Favourable features have been added to the plants, so-called domestication and in some way, it proved to be a necessity in recent times. The factors affecting the process are little known, though we have some knowledge about it. One such factor is small RNA-mediated pathways. This thesis attempts to show one such small RNA that may be associated with the change in rice domestication. We have a micro RNA module which showed negative regulation in RNA sequencing. During the study, I generated overexpression and knockout lines of micro RNA and its target. The tools generated will be very helpful for establishing the relationship between miRNA, its target and its role in rice domestication.

# Acknowledgements

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न था कुछ तो ख़ुदा था कुछ न होता तो ख़ुदा होता

डुबोया मुझ को होने ने न होता मैं तो क्या होता

हुई मुद्दत कि 'ग़ालिब' मर गया पर याद आता है

वो हर इक बात पर कहना कि यूँ होता तो क्या होता

- मिर्ज़ा ग़ालिब

# Contributions

| Contributor name | Contributor role |
|---|---|
| Dr. P. V. Shivaprasad,<br>Dr. Swetha Chenna and Aravind M | Conceptualization Ideas |
| Gitesh Masram and Aravind M | Methodology |
| Gitesh Masram and Aravind M | Software |
| Gitesh Masram and Aravind M | Validation |
| Gitesh Masram and Aravind M | Formal analysis |
| Gitesh Masram, Aravind M and Dr. P. V. Shivaprasad | Investigation |
| Vivek Hari Sunder Gi, Dr. P. V. Shivaprasad, Dr. K. Veluthambi | Resources |
| Gitesh Masram and Aravind M | Data Curation |
| Gitesh Masram | Writing - original draft preparation |
| Aravind M and Dr. P. V. Shivaprasad | Writing - review and editing |
| Gitesh Masram and Aravind M | Visualization |
| Dr. P. V. Shivaprasad | Supervision |
| Dr. P. V. Shivaprasad and Dr. Kalika Prasad | Project administration |
| - | Funding acquisition |

## List of abbreviations

| | | | |
|---|---|---|---|
| 2-4-D | 2,4-Dichlorophenoxyacetic acid | MS | Murashige and Skoog |
| bp | Base pair | N | Normal |
| cm | centimetre | NAA | 1-Naphthaleneacetic acid |
| DMSO | Dimethyl sulphoxide | NaOH | Sodium hydroxide |
| DNA | Deoxyribonucleic acid | NEB | New England Biolabs |
| EDTA | Ethylenediaminetetraacetic acid | ng | nanogram |
| EtBr | Ethidium bromide | RH | Relative humidity |
| GFP | Green fluorescent protein | RNA | Ribonucleic acid |
| gm, g | gram | rpm | Rounds per minute |
| hr | hour | RT | Room temperature |
| IPTG | Isopropyl β-d-1-thiogalactopyranoside | SDS | Sodium Dodecyl Sulphate |
| k | thousand | sec, s | second |
| kb | Kilobase pair | TBE | Tris-Borate Ethylenediaminetetraacetic acid |
| L, l, ltr | litre | U | Unit |
| LB | Luria-Bertani | V/V | volume/volume |
| M | Molar | W/V | weight/volume |
| mg | milligram | X-gal | 5-bromo-4-chloro-3-indolyl-□-D- |

| | | | |
|---|---|---|---|
| | | | galactopyranoside |
| min | minute | µg | microgram |
| ml | millilitre | µl | microlitre |
| mM | millimolar | | |

# Chapter 1 Introduction

**Domestication** *noun*

/dəˌmestɪˈkeɪʃn/

/dəˌmestɪˈkeɪʃn/

[uncountable]

1. the process of making a wild animal used to living with or working for humans
    - *the domestication of cattle*
2. the process of making a plant or crop suitable to grow for human use
    - *the domestication of rice*

As the Oxford dictionary defines domestication, it is something that really played a crucial role in the journey of mankind and later proved to be the foundation stone for modern civilization as people started farming and settling down. That foundation stone is still there and hasn't been moved since placed, the effects of which we see in our day-to-day life to such an extent that we can't even imagine living life without it.

As a plant biologist, my interest is more leaned towards the latter part of the above definition. Plant domestication is a genetic modification of a wild species to create a new form of a plant altered to meet human needs (Doebley et al., 2006). For example, a weed which needs a lot of water to grow, can not stand erect, with very few seeds and that too gets shattered quickly is transformed into a crop which provides a staple diet which goes perfectly with everything, yes I am talking about rice. Here I will concentrate particularly on rice since that's the model plant I used for my study. Basically, rice is a perfect crop to feed billions of people every day which got boosted by the Haber-Bosch process and the green revolution. The evolution of domestication drastically changed the outputs from crops and we can see a constant evolution in the domestication process itself. The modern-day crops standing tall all alone in the anthropogenic open fields altogether have different problems to face as compared to their wild varieties and hence the strategies to tackle these problems also evolved. Though the products given

by domesticated varieties are far better from the human point of view, that's the case when the plants are grown in ideal conditions.

## Stress and its repair pathway

It is said that **“we mature with the damage, not with the years”,** and so does the cell. We are cells and as a matter of fact, excessive damage can be lethal. It is more noxious when the damage is upon the first and the main pillar of the central dogma of molecular biology, the DNA. DNA damage is defined as any modification of DNA that changes its coding properties or normal function in transcription or replication (Martin et al., 2008). External factors like ionising radiation, ultraviolet radiation, various chemical agents, etc can cause DNA damage, which can lead to errors in DNA replication, transcription block or cell death if not repaired. But mother nature has her way of tackling all these problems and presenting us with exquisite gifts, **“repair, regeneration and life.”**

Depending upon the type of damage and the factors associated with its repair, there are different types of DNA damage repair pathways like base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), homologous recombination (HR) and non-homologous end joining (NHEJ) (Chatterjee et al., 2017).

Here we are specifically focusing on the damage caused by ultraviolet radiation and various mechanisms that the process of evolution came up with to tackle these damages. With global warming and ozone layer depletion in mind, the findings on the topic can be advantageous for the betterment of the food we consume and ultimately for human well-being. Ultraviolet light (100-400 nm) starts its journey all the way from the sun, most of it is absorbed by the ozone layer which eventually breaks the ozone into an oxygen molecule and free oxygen.

Ozone in the stratosphere alone is insufficient to lower incident UV radiation to non-lethal levels and as a result of it every living being undergone UV damage on a regular basis. All thanks to Darwin or rather **“Evolution”** every being developed something to protect themselves from these radiations. All living things use UV-absorbing pigments and a range of DNA repair mechanisms in combination as a form of UV protection. There exists physical protection ranging from the skin layer, fur, feathers, scales, membranes, hairs and so on. On the other hand, there are chemical

protections which either scavenge the radiation or repair the damage done by these harmful radiations. These chemicals range from sinapate esters which defend against the sun by absorbing light (Dean et al., 2014) to flavonoids which act as a plant protective molecule under UV exposure (Ferreyra et al., 2021). After facing all these troubles there is some little amount of UV radiation which manages to reach the cell and this tiny number of marathon runners can make our life miserable.

All living organisms need to be able to protect their genetic material from any kind of threat either environmental or metabolic. Both prokaryotes and eukaryotes use almost the same repair process of nucleotide excision repair to reverse UV damage. Recent studies say that plants are further evolved and they have different layers of mechanisms to defend against the damage caused by UV radiation. This can be really seen by the diversification of most of the DNA damage repair proteins in different plant systems. When UV light reaches DNA it gets absorbed by thymine and cytosine bases and this added energy causes a photochemical reaction with a neighbouring base, causing a covalent bond to form between them. Two common DNA modifications done by these UV damages are **cyclobutane pyrimidine dimers** (CPDs) and **6–4 photoproducts**. Both of these modifications are generally called pyrimidine dimers, among which dimers formed between thymine bases are more common and dangerous because they usually cause a kink in the DNA which stalls polymerase or causes a misread (Errol et al., 2003). The case of DNA damage by ultraviolet light is handled by a highly conserved nucleotide excision repair pathway (NER). The steps of NER are similar in organisms from unicellular bacteria to complex mammals. They all involve, 1) recognition of a DNA lesion then 2) the helix is unwound and 3) incisions on both sides of the lesion are made so that the part of the DNA with the lesion is removed leaving behind empty space 4) This space is patched up using the undamaged complementary strand as a template then it's 5) ligated to restore the DNA to its original form.

## Wild to cultivated

With human intervention this world has seen many changes. Most of them proved to be problematic, but there are a few which are working towards the goal of a better tomorrow, especially domestication being the one proven to be the answer to world

hunger. Nearly half of the total population eats rice as their staple food and the journey itself is an outcome of substantial changes in both genotypes and phenotypes. Though we just don't like weeds growing in our gardens, rice was also a weed growing near water bodies consuming a lot of water to grow. On top of it, it had a very low seed setting rate and few secondary branches per panicle. With very poor lignification, the plant could not stand erect and the few seeds which it manages to grow were also used to shatter in no time. To facilitate proliferation it got long awns so that herbivory could be their driving force. It all changed with domestication, the same plant now gives millions of tons of grains, a much more seed-setting rate, no shattering, shorter awns, more secondary branches, erect standing, and comparatively less water consumption. These are all the phenotypic changes that were easily seen but what happens on the molecular level was not known for many centuries. It's still not understood in great depth but we have some knowledge of the process and a lot more effort is needed to fully understand domestication. In this scenario to exploit the process for the greater good, scientists have to dig deep into the subject. From the limited knowledge we have till now, most of the developments are associated with changes in gene expression or some changes in genes themselves. Like *PROG1* plays a profound role in regulating rice plant architecture (Jin et al., 2008), *OsLG1* regulates panicle architecture (Ishii et al., 2013), *RPAD* has a key role in increasing inflorescence size (Tan et al., 2008), *FZP* increases secondary branch number (Komatsu et al.,2003), etc to name a few. Till now there is a considerable lack of information regarding the molecular basis of some changes that happened during domestication. From recent studies, it is clear that some factors like epigenetic pathways and small RNA pathways also contributed towards domestication-associated phenotypes (Shivaprasad et al., 2019).

## Gene silencing

RNA silencing as a gene regulatory mechanism is conserved all across eukaryotes (Baulcombe et al., 2004). It plays a major role in the growth and development of an organism. The pathway operates through the production of small RNA of approximately 20 to 24 nucleotides (nt) in length. These small RNAs can regulate the gene via small interfering RNAs (siRNA) which inhibit the expression of specific targets or microRNAs

(miRNA) which regulate the expression of multiple messenger RNAs. The small RNAs are acted by Dicer which cuts the sRNA precursors into short segments (Bologna et al., 2014). These are then loaded into AGO proteins to form a ribonucleoprotein complex called RNA-induced silencing complex or RISC (Chen et al., 2009). miRNA cleaves the targeted messenger RNA to regulate its expression in a time and tissue-specific manner (Lam et al., 2015). In plants two distinct gene silencing phenomenon are observed. First is called transcriptional gene silencing (TGS) where addition of methylation on promoter results into decreased RNA synthesis. In this case the transcription of the gene from the chromatin itself is downregulated at specific loci. The other one is called post transcriptional gene silencing (PTGS) which leads to sequence specific RNA degradation. The silencing is promulgated through degradation of target RNA, or rarely through repression of translation, or even by amplification of small RNA population which in turn bring about silencing (Sijen et al., 2001).

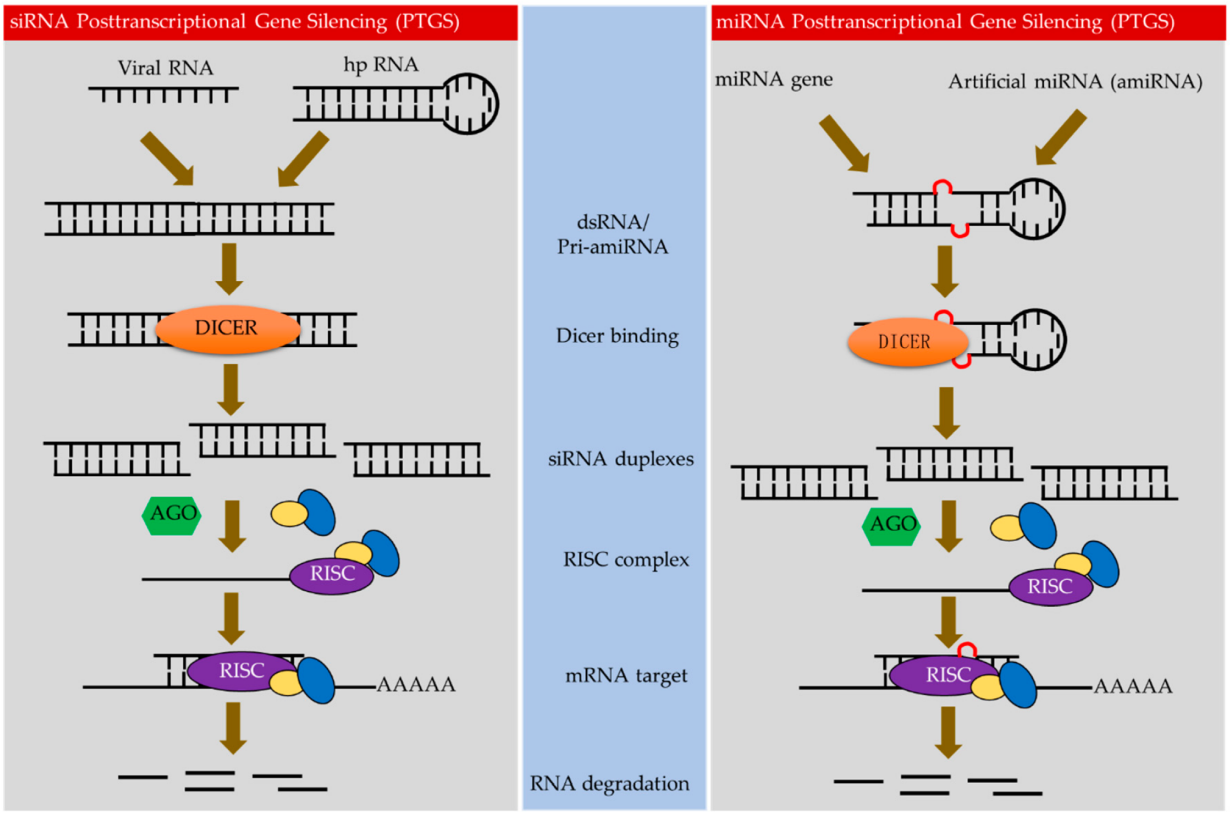



**Figure 1. RNA-mediated gene silencing in perennial plants (Singh et al., 2019).**

## miRNA regulates the cell wall lignification of rice

Opening some new doors to the existing knowledge, In the lab Chenna et al., has identified several miRNA target module that are differentially expressed between wild species of rice, landraces and cultivated lines. One of them which was thoroughly studied by Chenna et al is the miRNA397. In her outstanding work, she proved that miR397 targets the laccase genes which are required for the lignification of rice (Swetha et al., 2018). In her degradome analysis, it is clearly shown that miR397 expresses differentially among wild and cultivated varieties of rice. It's expressed high in the wild varieties prostrating the erect growth and low in cultivated varieties which stand tall and erect, securing their seeds for a longer span. Similarly Chenna et al., 2022 has identified several modules that are also differentially expressed. In such wide genome wide screening the host lab identified miRNA1880 as a differentially expressed miRNA between wild species and landraces and cultivated lines. In my thesis I am exploring the functions of this module.

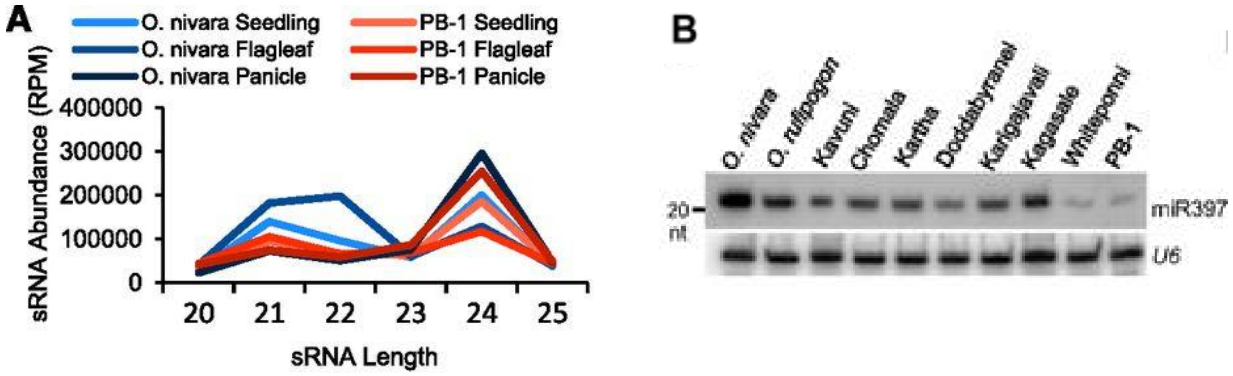



**Figure 2. Results from Chenna et al., PhD thesis**

A) Comparison between sRNA profiles of *O. nivara* and PB-1 indicating higher abundance of 22-nt reads in wild species.

B) Abundance of miR397 in flagleaf tissues among rice lines, including landraces. *U6* served as the control.

The same analysis revealed the presence of a 24 nt miRNA named miR1880 predicted to target a gene which is shown to be involved in DNA damage repair. According to sRNA sequencing miR1880 is highly expressed in wild varieties and less expressed in domesticated rice.

## miR1880 and its target

miRNA1880 is a 24 nucleotide sRNA which is highly expressed in the wild varieties of rice compared to cultivated varieties. Its targeted for psRNATarget is OsREX1 and OsREX1S. In degradome analysis carried out by Chenna et al., authentic target of this miRNA is found to be a gene called REX1S. Homologs of this gene in animals are shown to be important for DNA damage response.

Because it appears to be important in DNA damage response and very likely this contributed to domestication of rice, I am going to try and find how exactly this has contributed to domestication of Indica rice.

This gene was first identified in *Chlamydomonas reinhardtii* by insertional mutagenesis and it is proven to be active in nucleotide excision repair. Mutants of the gene were sensitive to UV damage and unable to perform excision of the pyrimidine dimers. As we discussed earlier, UV rays cause the formation of cyclobutane pyrimidine dimers which if not removed can be harmful to the cells (Kemp et al., 2012). Such UV-induced damage is taken care of by the NER, and later this proteins homologues are studied to be functioning in the NER pathway. As it is required for the excision of the pyrimidine dimers the protein is named **REX1** (Cenkci et al., 2003)**.** In *Chlamydomonas reinhardtii,* where it is studied first, REX1 has 2 homologues, one codes for a small protein (8.9 kDa) and the other makes a larger protein (31.8 kDa). The smaller protein has homologues in many organisms including *Saccharomyces cerevisiae*, arabidopsis, rice and humans. Rice also has two homologs one on chromosome 7 called OsREX1S which is 132 amino acids long and the other on chromosome 5 called OsREX1 which is 81 amino acids long (Kunihiro et al., 2014). miR1880 is capable of targeting both the homologues and the regulation arm of its targeting is not clear.

## XCT plays an important role in regulating proteins involved in DNA damage response

XAP5 CIRCADIAN TIMEKEEPER (XCT) is a gene which is highly conserved in eukaryotes**.** The name came from the highly conserved domain of unknown function found in the C-terminal of these genes. It was discovered in a random T-DNA insertion screen where a plant showed a short-period circadian phenotype in *Arabidopsis thaliana* (Martin-Tryon and Harmer, 2008). Thereafter different functions of the homologs of the same gene have been discovered in a variety of model organisms. The protein is studied to be involved in different processes including the circadian clock regulation, light responses, gene expression, ethylene signal transduction and the production of short RNAs in arabidopsis (MartinTryon et al., 2008; Ellison et al., 2011; Fang et al., 2015; Xu et al., 2017). The XCT homolog XAP5 has been found to function as a transcription factor to regulate flagellar assembly in the ciliate *Chlamydomonas reinhardtii* (Li et al., 2018). Downregulation of the XCT homolog in the worm *Caenorhabditis elegans* results in embryo mortality (Piano et al., 2002), while in *Schizosaccharomyces pombe* its absence hinders growth. Though it has a variety of functions our attention is grabbed by its role as a chromatin regulator in yeast (Anver et al., 2014). Modulation of chromatin is a key step in DNA double-strand breaks. DNA damage repair in most eukaryotes starts with the rapid phosphorylation of variant histone H2A.X at the site of damaged DNA. This initial signal acts as a signal to attract the repair complex to these damaged sites and initiate the DNA damage repair pathway (Kinner et al., 2008). A recent finding showed that XCT affects both DNA damage responses and immune signalling in Arabidopsis. When exposed to UV light, mutant plants of the gene showed defects in the early stage of DNA damage response and had inadequate phosphorylation of the H2AX (Kumimoto et al., 2021).

## The goal of the project

Taking accountability for the rice we consume and assume that this will be the case in the coming years unless something catastrophic happens. The following study will create new pathways towards the upgradation of our staple food in unfavourable

environmental conditions taking global warming into account. Here the goal is to see the role of small RNA in DNA Damage repair, which can be a possibility in other model organisms too. This work will manifest the correspondence between miR1880 and OsREX1S ultimately leading to their role in DNA Damage repair. Though the exact mechanism of action of OsREX1S will be unclear. The work will try to exhibit one more piece of information underlying the decoupling of wild and cultivated varieties of rice. This study will provide the early characterization of the protein which will be of great help for further studies. Also, we will be trying to generate tools in the form of transgenic plants which will be helpful for understanding the pathway in depth.

1) **To Understand the role of protein REX1 in DNA damage repair.**
2) **To understand the role of miR1880 in regulating DNA damage repair.**
3) **To elucidate the difference in DNA damage repair and its regulation in the wild and domesticated varieties of rice.**

To achieve this goal we will be:-

1) Constructing clones with overexpression and knockout of OsREX1S, OsREX1 and miR1880.
2) Infecting them in the model plant i.e. rice, in order to engender transgenic lines.
3) Performing different types of assay and carrying out phenotyping with a view of the project's objective.

# Chapter 2 Methods and Protocols

**Table 1. List of primers used**

| Primer name | Lab's stock number | Sequence | Manufacturer |
|---|---|---|---|
| AM_OsREX1SF_cCL | 4327 | ATGCCTGACCTCCGCCT | Eurofins |
| AM_OsREX1&SRcCL | 4328 | TCATGTTGGCTTCTCGTAGCTG | Eurofins |
| AM_BamH1_OsREX1S_OE_F | 4390 | GACGGATCCGACATGCCTGACCTCCGC | Eurofins |
| AM_Hind3_OsREX1S_OE_R | 4391 | GCCAAGCTTCTATCATGTTGGCTTCTCGTAG | Eurofins |
| AM_OsREX1&S_OE_2XMyc_R_Hind3 | 4392 | GCCAAGCTTCTACAAATCTTCTTCAGAAATCAACTTTTGTTCCAAATCTTCTTCAGAAATCAACTTTTGTTCTGTTGGCTTCTCGTAGC | Eurofins |
| AM_OsREX1_OE_F_BamH1 | 4388 | GAGACGGATCCGACATGGTGGCATTCTGT | Eurofins |
| pRGEB32_Seq_F | 1435 | CCGGCTCGTATGTTGTGTGG | Eurofins |

| | | | |
|---|---|---|---|
| AM_miR1880_gRNA_1_pRGEB32 | 4639 | ATTAGGTCTCCAAACAGCGGGCCACTTAAGCATTCTGCCACGGATCATCTGCACAAC | Eurofins |
| AM_OsXCT_gRNA_1_pRGEB32 | 4640 | ATTAGGTCTCCAAACGGCCTGCTCCAGTTCGGCTCTGCCACGGATCATCTGCACAAC | Eurofins |
| AM_OsREX1S fwd | 4291 | TAGAGAAGACAACAGCACAGG | Eurofins |
| AM_OsREX1S rev | 4292 | GCTGTTTTGGTCTCTGAACTCTGAA | Eurofins |
| AM_OsREX1 FWD | 4293 | TCTGTTCTCCAAAGAGCAGAAAC | Eurofins |
| AM_OsREX1 REV | 4294 | GCTGTTTTGGTCTCTGAACTCTGAT | Eurofins |
| STN_OsGAPDH_qPCR_F | 3873 | GGGTATTCTGGGTTACGTTGAGGAG | Eurofins |
| STN_OsGAPDH_qPCR_R | 3874 | ACGGATCAGGTCAACAACGCGAGAG | Eurofins |

## Table 2. List of enzymes used for restriction digestion

| Enzymes | Stock concentr-ation | Buffer | Tempera-ture | Manufacturer |
|---|---|---|---|---|
| BamHI | 20k Units/ml | Cutsmart | 37°C | NEB, USA |
| HindIII | 20k Units/ml | Cutsmart | 37°C | NEB, USA |
| EcoRI | 20k Units/ml | Cutsmart | 37°C | NEB, USA |
| SmaI | 20k Units/ml | Cutsmart | 25°C | NEB, USA |
| PstI | 20k Units/ml | Cutsmart | 37°C | NEB, USA |
| NotI | 20k Units/ml | Cutsmart | 37°C | NEB, USA |
| EcoRV | 20k Units/ml | Cutsmart | 37°C | NEB, USA |
| BsaI | 20k Units/ml | Cutsmart | 37°C | NEB, USA |
| XhoI | 1 lac Units/ml | Cutsmart | 37°C | NEB, USA |
| NdeI | 20k Units/ml | Cutsmart | 37°C | NEB, USA |

## 2.1 Plasmid isolation (Alkaline lysis method)

| Solutions | Components | Stock | Working |
|---|---|---|---|
| Solution I (Resuspension Solution) | D-Glucose | 500 mM | 50 mM |
| | Tris-Cl (pH-8) | 250 mM | 25 mM |
| | EDTA (pH-8) | 250 mM | 10 mM |
| Solution II (Lysis Solution) | NaOH | 0.4 N | 0.2 N |
| | SDS | 2% W/V | 1% W/V |
| Solution III (Neutralisation Solution) | Potassium Acetate | 5 M | 3 M |
| | Glacial Acetic Acid | 17.4 M | 2 M |

1) Take the overnight grown culture, and transfer it to 2 ml Eppendorf tubes.

2) Centrifuge it at 13k rpm for two minutes at room temperature. Discard the supernatant, and keep the pellet.

3) Repeat the above two steps.

4) Add 150 µl of solution I to this pellet and vortex nicely till the whole pellet is dissolved. Some milky solution will form then it's ready.

5) Add 300 µl of freshly prepared solution II. Gently invert the mix and keep to the ice for 6-8 min. Now the solution will be transparent. Everything onwards this step will be on the ice.

6) Add 225 µl of solution III to this transport solution. Invert 10-12 times. Supercoiled DNA can be seen after this step.

7) Centrifuge at 13k for 10 minutes at 4℃.

8) Collect supernatant from these tubes and transfer it to new 1.5 ml Eppendorf tubes. Any volume is permitted as long as the upper phase is collected.

9) Treat this solution with RNase (100 ng/ml), and incubate for 20-30 min at 40 ℃.

10) Add the same volume of chloroform to this solution as that of the supernatant taken. Shake vigorously till the phase separation is not seen. (Take chloroform once or twice in a pipette and release. After that it won't dip)

11) After this centrifuge At 13k rpm for 6 minutes at 4℃.

12) Transfer the supernatant to new 1.5 ml Eppendorf tubes.

13) Add chilled Isopropanol to the solution (3:2 ratio, 3 parts of Isopropanol and 2 parts of the supernatant).

14) Centrifuge at 13k rpm for 20 minutes at 4℃. Discard the supernatant. One small pellet can be seen.

15) Add 1 ml of chilled 70% ethanol to this pellet for ethanol wash.

16) Centrifuge at 13k rpm for 2 minutes at 4℃.

17) Discard the supernatant and repeat the above two processes.

18) Give one dry spin at 13k rpm for 2 minutes at 4℃.

17) Keep the tubes open for 10-15 minutes. Let the extra ethanol evaporate.

18) Add 20-30 µl of ultrapure water to this pellet. Tap and give a short spin.

19) The plasmid is isolated. Load on the gel and see the results.

## 2.2 Primer Reconstitution

1) Spin Down the ordered primers for 2 min at 13k rpm.

2) Add ultrapure water as mentioned in the primer sheet, to make up the final concentration for stock.

3) Keep it on the thermomixer for 15 min at 55℃ at 550 rpm.

4) Label it correctly and keep it in -20℃ storage.

## 2.3 Polymerase Chain Reaction

PCR with Phusion (DNA Polymerase)

| Components | Stock | Working | 50 µl Reaction |
|---|---|---|---|
| Ultrapure Water | _ | _ | 36 µl |
| 5X Phusion buffer HF | 5X | 1X | 10 µl |
| DMSO | 100% | 3% | 1.5 µl |
| dNTP | 10 mM | 200 µM | 1 µl |
| Forward Primer | 100 µM | 0.5 µM | 0.25 µl |
| Reverse Primer | 100 µM | 0.5 µM | 0.25 µl |
| DNA polymerase (Phusion) | 3 U/µl | 1 U | 0.5 µl |
| Template | 10 ng/µl | 5 ng | 0.5 µl |

General program set in thermocycler

| Step | Temperature | Time |
|---|---|---|
| Initial denaturation | 98℃ | 2 min |
| Denaturation | 98℃ | 30 sec |
| Annealing | 66* | 30 sec |
| Extension | 72℃ | 40 sec |
| Final extension | 72℃ | 5 min |
| Hold | 14℃ | 15 min |

* The annealing temperature varies according to the primer length. Similarly with that time for other steps also may vary.

PCR with Taq (DNA Polymerase)

| Components | Stock | Working | 50µl Reaction |
|---|---|---|---|
| Ultrapure Water | _ | _ | 36 µl |
| 5X GoTaq buffer | 5X | 1X | 10 µl |
| DMSO | 100% | 3% | 1.5 µl |
| dNTP | 10 mM | 200 µM | 1 µl |
| Forward Primer | 100 µM | 0.2 µM | 0.1 µl |
| Reverse Primer | 100 µM | 0.2 µM | 0.1 µl |
| DNA polymerase (Brit Taq) | 5 U/µl | 1 U | 0.2 µl |
| Template | 10 ng/µl | 5 ng | 0.5 µl |

General program set in thermocycler

| Step | Temperature | Time |
|---|---|---|
| Initial denaturation | 95℃ | 2 min |
| Denaturation | 95℃ | 30 sec |
| Annealing | 66* | 30 sec |
| Extension | 72℃ | 40 sec |
| Final extension | 72℃ | 5 min |

| | | |
|---|---|---|
| Hold | 14℃ | 15 min |

* The annealing temperature varies according to the primer length. Similarly with that time for other steps also may vary.

## 2.4 Restriction Digestion

| Components | Stock | Working | 50µl Reaction |
|---|---|---|---|
| Ultrapure Water | _ | _ | 39 µl |
| Cutsmart Buffer | 10X | 1X | 5 µl |
| Enzyme | 20K U/ml | 20 U | 1 µl |
| DNA | 200 ng/µl* | 1 µg | 5 µl |

* DNA concentration may vary according to the experiments.

## 2.5 Gel Electrophoresis

1) 0.8% to 1.2% agarose gels were used along with the TBE buffer. EtBr (0.5 µg / ml) is added to visualise the gel under a UV light source.
2) For PCR 1.2% gel is used otherwise for every other purpose like gel extraction, to visualise digestion, etc. 0.8% is used.
3) The gels are visualised in the gel doc machine.

To make 1 litre of TBE buffer (10X)

| | |
|---|---|
| Tris base | 54.02 g |
| Boric acid | 27.51 g |
| EDTA | 3.75 g |

Autoclave and should be diluted to 1X before use.

## 2.6 Gel purification - by PCR purification kit (QIAGEN)

1) Weigh the product (gel).
2) Add 3 volumes of QG buffer (weight in mg × 3 in µl).
3) Keep on the thermomixer at 500 rpm for 10 min at 50°C. Till the gel gets completely dissolved.
4) Add one volume of isopropanol to the above solution and gently invert two-three times.
5) Add to the column provided in the kit.
6) Centrifuge at 13k rpm for 2 min at room temperature and discard the flow-through.
7) Add 750 µl of PE buffer to the column.
8) Centrifuge at 13k rpm at room temperature for 2 min and discard the flow-through.
9) Give it one dry spin at 13k rpm for 2 minutes at room temperature.
10) Transfer to 1.5 ml Eppendorf tubes.
11) Keep it open for 8-10 minutes so that ethanol can evaporate.
12) Add 25 µl of ultrapure water to the column and set it for 1-2 min.
13) Centrifuge at 13k rpm for 2 min at room temperature to elute purified DNA.

## 2.7 Digestion purification - by PCR purification kit (QIAGEN)

1) Add the same amount of ultrapure water as the digestion reaction volume.
2) Add 5 volumes of coloured buffer PB of the above volume.
3) Invert 5-10 times.
4) Add to the column provided in the kit.
5) Centrifuge at 13k rpm for 2 min at room temperature and discard the flow-through
6) Add 750 µl of PE buffer to the column.
7) Centrifuge at 13k rpm at room temperature for 2 min and discard the flow-through.
8) Give it one dry spin at 13k rpm for 2 minutes at room temperature.
9) Transfer to 1.5 ml Eppendorf tubes.
10) Keep it open for 8-10 minutes so that ethanol can evaporate.
11) Add 25 µl of ultrapure water to the column and let it set for 2-3min
12) Centrifuge at 13k rpm for 2 min at room temperature. And we will get the purified product.

## 2.8 DNA Ligation

| Components | Stock | Working | 20 µl Reaction |
|---|---|---|---|
| Ultrapure Water | _ | _ | 12 µl |
| Vector | 10 ng/µl | 1 part of insert | 2 µl |
| Insert | 20 ng/µl | 3 parts of Vector | 3 µl |
| 10X Ligation buffer for T4 DNA LIgase | 10X | 1X | 2 µl |
| T4 DNA Ligase | 4 lacU/ml | 400 U | 1 µl |

The Ligation mixture is kept at 22℃ for 1.5 hours. Generally, Vector: Insert is taken 1:3. For base plasmid-based transformations with blunt end ligations 20-30 ng of the vector was taken and for sticky end ligation 50-100 ng of the vector was taken.

## 2.9 *E.coli* Transformation

1) Take competent cells (DH5α) in the ice bucket from glycerol stock stored at -80℃.
2) Place it in the ice bucket for five minutes only so it thaws.
3) Add the desired amount of DNA (Ligation mix or 20-30 ng DNA in case of retransformation).
4) Place it in the ice bucket for 15 minutes.
5) Give precisely two minutes of heat shock at 42℃.
6) Again place it on ice for 5 minutes.
7) Add 800 µl of liquid LB broth (Already kept at 37℃ )
8) Incubate for 1 hour at 37℃. Plate on the appropriate plate according to antibiotic resistance.

## 2.10 *Agrobacterium tumefaciens* Transformation

1) Take 30 ng plasmid in 10 µl ultrapure water.
2) Take cuvettes, tip boxes new, 10P and 200P pipettes, tissue paper, YEB broth and 70% ethanol. LBA 4404 (with pSB1) comp cells in ice.
3) Add plasmid to the comp cell tube and label
4) Pipette out all the comp cells to the cuvette. Give heat shock one pulse in the electroporation machine.
5) Take fresh YEB broth 200 µl three times. (One at a time)

6) After taking 200 µl YEB in a 200P pipette add it to the cuvette and mix gently. Add this to the same tube where you take comp cells from. (Don't keep it in ice after this)
7) Repeat this process two more times. (Final volume will be 600 µl)
8) Give 2 hr recovery in 28℃.
9) Plate 150 µl in RTK plate
10) Keep the plates at 28℃ for two days.

## 2.11 Plates Making

LB broth - 2% of LB media

LB agar - 1.5% of bacterial agar into the broth mentioned above

| | Liquid | | Solid | |
|---|---|---|---|---|
| **Antibiotics** | **Stock** | **Working** | **Stock** | **Working** |
| Carbenicillin | 100 mg/ml | 100 mg/ltr | 100 mg/ml | 100 mg/ltr |
| kanamycin | 100 mg/ml | 50 mg/ltr | 100 mg/ml | 50 mg/ltr |
| X-gal | _ | _ | 25 mg/ml | 25 mg/ltr |
| IPTG | _ | _ | 1 M | 0.5 mM |

**Making Yeast Extract Broth (Solid and Liquid)**

| Components | For 1 litre |
|---|---|

| | |
|---|---|
| Yeast extract powder | 1 g |
| Peptone, bacteriological | 5 g |
| Sucrose | 5 g |
| $MgCl_2$.6H2O | 500 mg |
| Meat/Beef extract | 5 g |

Initial pH should be between 6.7 - 6.8. Adjust it to 7.2 with 1N NaOH.
For Solid - We take 1.5%  Agar powder, bacteriological grade. Give it for autoclaving.

**Making RTK Plates (Rifampin-Tetracycline-Kanamycin)**

| | **Liquid** | | **Solid** | |
|---|---|---|---|---|
| **Antibiotics** | **Stock** | **Working** | **Stock** | **Working** |
| Rifampicin | 10 mg/ml | _ | 10 mg/ml | 10 mg/ltr |
| Tetracycline | 12.5 mg/ml | 2.5 mg/ltr | 12.5 mg/ml | 5 mg/ltr |
| kanamycin | 100 mg/ml | 50 mg/ltr | 100 mg/ml | 100 mg/ltr |

Add these to Solid YEB according to volume.

## 2.12 Inoculation

1) The grown colonies were streaked on LB plates with appropriate antibiotics and were inoculated for screening.

2) Colonies were inoculated in LB broth (5 ml per colony) with appropriate antibiotics at the concentrations mentioned above.
3) *Agrobacterium tumefaciens* colonies were streaked on RTK plates and inoculated in liquid YEB with appropriate antibiotics.
4) Cultures were grown overnight (~ 14 hours) at 37℃ and 28℃ for *E.coli* and *A.tumefaciens* respectively, rotating at 180 rpm.

## 2.13 Plasmid isolation (Kitprep, QIAGEN)

1) Take the overnight grown culture, and transfer it to 2 ml Eppendorf tubes.
2) Centrifuge it at 13k rpm for two minutes at room temperature. Discard the supernatant, and keep the pellet. Use a tissue to remove the extra LB.
3) Repeat the above two steps.
4) Resuspend pelleted bacterial cells in 250 µl buffer P1.
5) Add 250 µl buffer P2, and invert gently 2-3 times. Keep for two minutes.
6) Add 350 µl buffer N3, and invert 5-6 times.
7) Centrifuge for 10 minutes at 13k rpm.
8) Take 750 µl supernatant and transfer it into the spin column.
9) Centrifuge for 2 minutes at 13k rpm. Discard the flowthrough.
10) Wash it by adding 750 µl buffer PE.
11) Centrifuge for 2 minutes at 13k rpm. Discard the flowthrough.
12) Give a dry spin for 2 minutes at 13k rpm.
13) Place the column into a 1.5 ml Eppendorf tube and let extra ethanol evaporate for 8-10 minutes.
14) Elute the DNA into 25-30 µl preheated lukewarm ultrapure water.
15) Spin for 2 minutes at 13k rpm.

## 2.14 Glycerol stock preparation

1) Nicely grown culture was taken.
2) In a cryo tube 800 µl autoclaved glycerol and 1200 µl of culture were taken.
3) After mixing properly the tube was put in liquid nitrogen(snap chill) and stored at -80°C.

## 2.15 Infection protocol

**Overview**

Day 1:- Surface sterilisation of freshly dehusked seeds and put them into a callus-inducing medium. (Keep watch on fungus, if contaminated throw)

Day 22:- Cut the calli and subculture. (Four days in the dark)

Day 26:- Infection with *Agrobacterium tumefaciens* carrying the binary vector, kept for co-cultivation. (Three days in the dark)*

Day 29:- Wash and transfer to selection medium (Hygromycin).

Day 44:- Subculture and keep for the second selection. (21 days in the dark)

Day 65:- Subculture and keep for the third selection. (21 days in the dark)

Day 86:- Subculture and keep in regeneration medium (Place in the dark for 2 weeks and in the light for 1 week)

Day 107:- Subculture in new regeneration medium. (3 weeks)

Day 128:- Repeat the above step until plants are regenerated. Once grown, transfer into the greenhouse.

**Agrobacterium*-mediated rice transformation was carried out as described previously (Hiei et al., 1997).

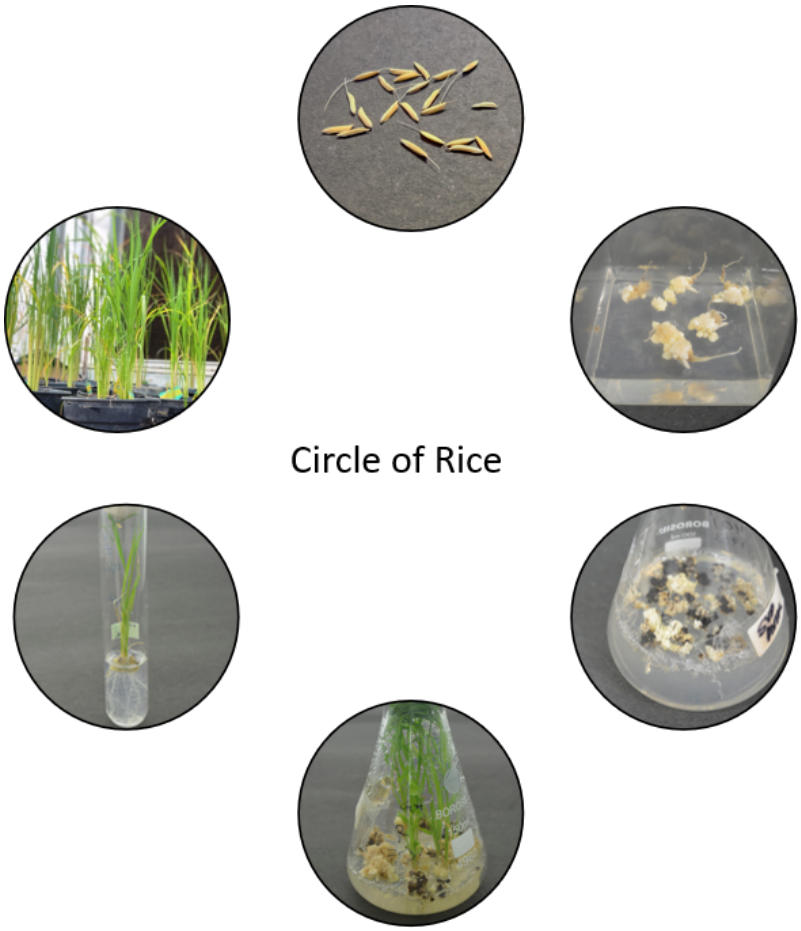



**Figure 3. Different stages of rice transgenic germination**

**Seed surface sterilisation of rice**

1) Dehusk the required number of seeds.
2) Water wash 5-8 times.
3) Ethanol wash for 2 minutes.
4) Water wash 5-6 times.

5) Bleach wash for 8 minutes.
6) Water wash 8-20 times.
7) $HgCl_2$ wash for 2 minutes.
8) Water wash 12-15 times.

Here one water wash means, wash given to one set of seeds (200-300 seeds) with 100 ml autoclaved water for 30 seconds. Also, one set of seeds needs 100 ml of 70% ethanol, 100% bleach and 0.1% $HgCl_2$ each.

Water - Autoclaved water

Ethanol - 70% absolute ethanol (70 ml absolute ethanol + 30 ml ddH2O)

The cell wall is more thoroughly penetrated by 70% denatured alcohol, which permeates the entire cell, coagulates all proteins and kills the bacterium.

Bleach - 100% Bleach (100 ml Sodium hypochlorite + two drops of tween80)
Viruses and some bacterial infections can be successfully eliminated by bleach. Tween80 helps wet the seed's surface or drastically minimises the contact angle as it's a hydrophilic non-ionic surfactant.

$HgCl_2$ - 0.1% of $HgCl_2$ (100 mg $HgCl_2$ dissolved in 100 ml ddH2O)
It has strong antibacterial properties and works to combat fungi and bacteria. It also can kill seeds hence it should be exactly 2 min wash, more than that can be lethal to seeds.

## 1. Hormones preparation

**Kinetin stock 1 mM**

Molecular weight - 215. Dissolve 21.5 mg in 1 ml 1N HCl, add ddH2O gradually and make the volume up to 100 ml. Store in 4℃.

**NAA stock 1 mM**

18.62 mg in 0.5 ml DMSO. Add ddH2O gradually to make up to 100 ml. Store in 4℃.

**2-4-D**

22.1 mg in 0.5 ml 1N NaOH. Add ddH2O gradually to make up to 100 ml. Store in 4℃.

2. **½ MS**

| Components | For 1 litre |
|---|---|
| Murashige and Skoog Modified Medium | 17.21 g |
| Phytagel | 3 g |

Before adding phytagel, set the pH to 5.8 with the help of 1N NaOH. Autoclave and pour into containers.

3. **Callus induction media**

| Components | For 1 litre |
|---|---|
| Murashige and Skoog Modified Medium | 34.42 g |
| Casein Hydrolysate | 300 mg |
| L - Proline | 500 mg |
| 2-4-D | 11.3 ml |

| | |
|---|---|
| Phytagel | 2.250 g |

Before adding phytagel, set the pH to 5.8 with the help of 1N NaOH. Give for autoclaving. Photosensitive, stored in a dark place.

**4. Selection media**

| **Components** | **For 1 litre** |
|---|---|
| Murashige and Skoog Modified Medium | 34.42 g |
| Casein Hydrolysate | 300 mg |
| L - Proline | 500 mg |
| 2-4-D | 11.3 ml |
| Phytagel | 4 g |

Before adding phytagel, set the pH to 5.8 with the help of 1N NaOH. Give for autoclaving.
Photosensitive, stored in a dark place. While pouring add antibiotics (Hygromycin - 100 µl/100 ml, Cefotaxime - 100 µl/100 ml) when the media is cold enough and about to solidify.

5. **Regeneration media**

| Components | For 1 litre |
|---|---|
| Murashige and Skoog Modified Medium | 34.42 g |
| Casein Hydrolysate | 300 mg |
| 1 mM NAA | 8.1 ml |
| 1 mM Kinetin | 13.9 ml |
| Phytagel | 6 g |

Before adding phytagel, set the pH to 5.8 with the help of 1N NaOH. Give for autoclaving. Photosensitive, stored in a dark place. While pouring add antibiotics (Hygromycin - 80 µl/100 ml, Cefotaxime - 60 µl/100 ml) when the media is cold enough and about to solidify.

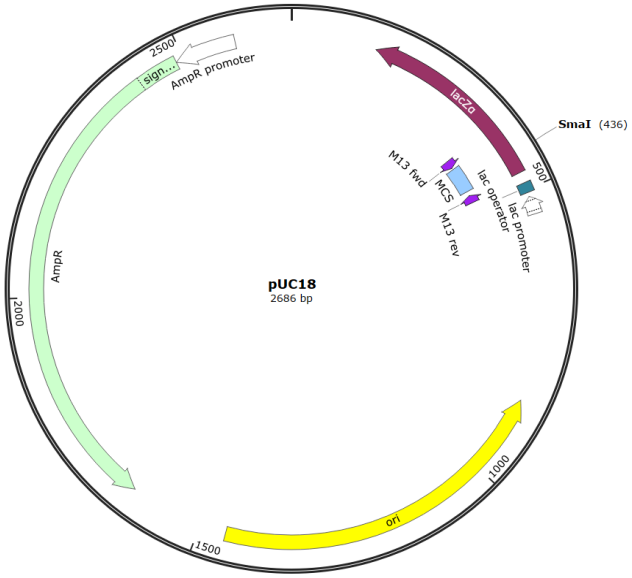
Transgenic rice plants were initially maintained in a tissue culture facility at 24°C, under 16 h/8 h light/dark cycle and 70% RH. Embryogenic rice calli were induced and maintained in darkness.

Rice plants were grown in the greenhouse under natural light conditions and 70% relative humidity (RH).

# Chapter 3 Materials

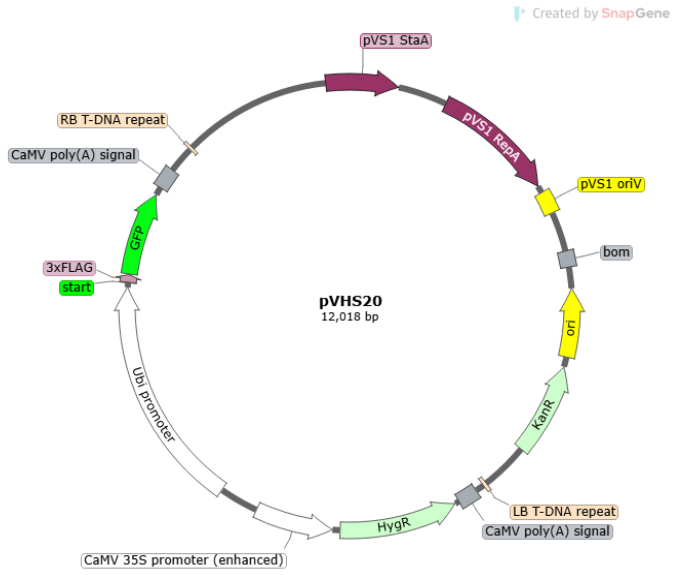
## 3.1 Plasmids used

1. **pUC18 (pE586)**



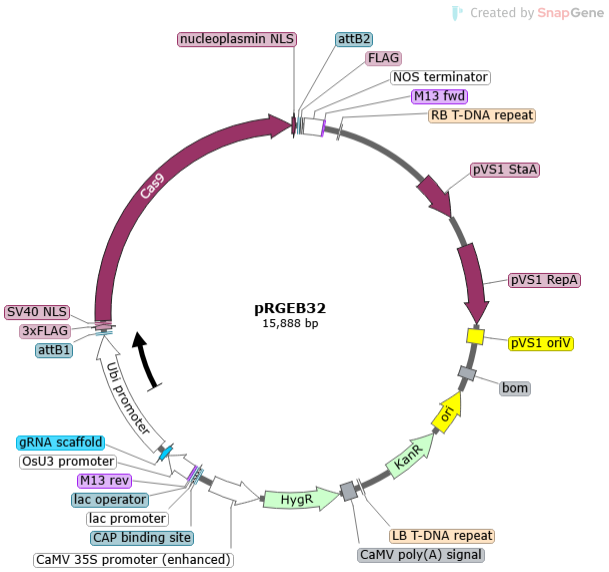

- High copy cloning vector
- Multiple cloning site
- Carbenicillin resistant
- Sequencing primers available

## 2. pVHS20 (pE489)




- Rice plant binary vector used for *Agrobacterium-mediated* transformation
- Derivative of pCambia1300, contains 3xFLAG tagged eGFP
- Driven by the strong *Zea mays* ubiquitin promoter
- kanamycin resistance (bacterial selection marker) and a hygromycin resistance (plant selection marker)
- High copy plasmid

## 3. pRGEB32 (pE373)

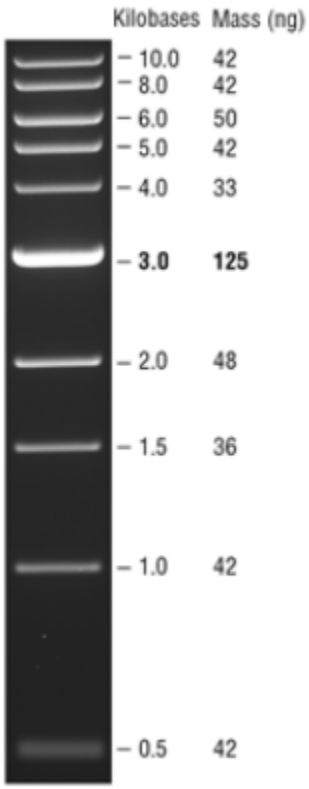


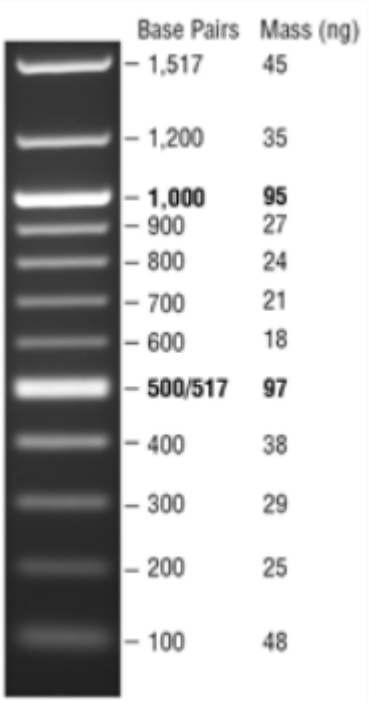

- Plant binary vector used for *Agrobacterium*-mediated transformation
- Contains Cas9 endonuclease under rice polyubiquitin high-copy promoter
- Contains target specific designed guide RNA(gRNA) under rice U3 high-copy promoter
- Kanamycin resistance (bacterial selection marker) and a Hygromycin resistance (plant selection marker)
- High copy plasmid

## DNA Ladder

1Kb and 100 bp DNA ladders were used for the project. The product was from New England Biolabs, USA. 6 µl of loading dye (Purple dye 6X, NEB, USA) added DNA ladder was loaded each time in each well. The mass and length of DNA observed by EtBr staining are shown in the following:

**1 Kb ladder** **100 bp ladder**




**(Size range: 500 bp to 10 Kb)** **(Size range: 100 bp to 1,517 bp)**

## 3.2 Strains used for transformation

1. *Escherichia coli* (DH5α) competent cells.
2. *Agrobacterium tumefaciens* (LBA 4404 with pSB) cells.

## 3.3 Table 3. List of materials used

| Material | Manufacturer | Material | Manufacturer |
|---|---|---|---|
| Glucose | Qualigen | Phytagel | Sigma Aldrich |
| EDTA | Fisher Scientific | Cas amino | Millipore |
| Tris Cl | Himedia | Isopropanol | Qualigen |
| Sodium Chloride | Himedia | Chloroform | Qualigen |
| Tris buffer | Qualigen | Yeast extract | Himedia |
| Boric acid | Qualigen | Meat extract | Himedia |
| EtBr | Himedia | Peptone | Himedia |
| LB | Himedia | MgCl2 | Fisher Scientific |
| Agar | Himedia | Sucrose | Qualigen |
| Potassium acetate | Fisher Scientific | Isoamyl alcohol | Himedia |
| Glacial acetic acid | Qualigen | Kinetin | Sigma-Aldrich |
| NAA | Sigma-Aldrich | 2-4-D | Sigma-Aldrich |

## 3.4 Machines used

1) Thermocycler - Life technologies, Proflex PCR system - Used to set up all types of PCR.
2) Laminar air flow cabinet - Thermo Scientific, 1300 series A2 - Used for bacterial and tissue culture work.
3) Gel Electrophoresis - BioRad - Powerpac HC High-Current power supply - Used for running DNA and RNA gel.
4) Gel Documentation - Lab India, Serial N 12 200706 - Used to visualise gel images.
5) Crosslinker - Artisan, CL-1000 Ultraviolet Crosslinker - Used to give UV treatment to rice seedlings.
6) Spectrophotometer - Thermo Scientific, Nanodrop 2000 spectrophotometer - Used to get nanodrop readings of extracted samples like DNA and RNA.
7) Centrifuge - Eppendorf centrifuge 5425R - Used for high-speed rotations.
8) Thermomixer - Eppendorf thermomixer comfort - Used for incubation.

## 3.5 Software used

1) Rap-DB - The Rice Annotation Project (RAP) was conceptualised upon the completion of the rice genome sequencing in 2004 with the aim of providing the scientific community with an accurate and timely annotation of the rice genome sequence. It is used to get the rice genome.
2) Ensemble Plant - Ensembl provides genes and other annotations such as regulatory regions, conserved base pairs across species, and mRNA protein mappings to the genome.
3) TAPIR - The TAPIR web server predicts targets for plant miRNAs. It is used to find the miR1880 targeting region in REX1S, and also to see the binding of miR1880 to REX1S when the targeted site is mutated.

4) Image J - ImageJ is a public domain Java image processing program inspired by NIH Image for the Macintosh. It is used to invert, adjust and quantify agarose gel images.
5) Mega11 - Mega11 is a software for analysing DNA and protein sequence data from species and populations. It is used to make the phylogenetic tree.
6) TAIR - The Arabidopsis Information Resource (TAIR) maintains a database of genetic and molecular biology data for the model higher plant *Arabidopsis thaliana*. We used it to refer to the Arabidopsis genome.
7) SnapGene - SnapGene offers a way to plan, visualise, and document DNA cloning and PCR and that's what it is used for.
8) NEB calculator - It is used to calculate appropriate annealing temperature based on the primer length and amount of DNA that has to be taken in the ligation reaction.
9) miRBase - The miRBase database is a searchable database of published miRNA sequences and annotations. It is used to find out the miR1880 sequence.
10) CRISPR-PLANT - Used for utilising the CRISPR-Cas9 system for plant genome editing.

## 3.6 Table 4. List of antibiotics used

| Component | Making in | Final stock | Manufacturer |
|---|---|---|---|
| Carbenicillin | Ultrapure water | 100 mg/ml | Himedia |
| Kanamycin | Ultrapure water | 100 mg/ml | Himedia |
| Cefotaxime | Ultrapure water | 250 mg/ml | Taxim |
| Hygromycin | Ultrapure water | 50 mg/ml | Sigma-Aldrich |
| Tetracycline | 70% ethanol | 12.5 mg/ml | Himedia |

| Component | Making in | Final stock | Manufacturer |
|---|---|---|---|
| Carbenicillin | Ultrapure water | 100 mg/ml | Himedia |
| Kanamycin | Ultrapure water | 100 mg/ml | Himedia |
| Rifampicin | Methanol | 10 mg/ml | Himedia |
| IPTG | Ultrapure water | 1 M | Himedia |
| X-gal | DMSO | 25 mg/ml | Himedia |

## 3.7 Table 5. List of recombinant clones generated

The clones are transformed into competent *Escherichia coli* (DH5α) cells.

| Plasmid | Description | Stock number |
|---|---|---|
| pGM1 | pGM1 is a derivative of pVHS20. The coding sequence of the OsREX1S (Os07g0573600) gene is inserted into pVHS20 in SmaI and BamHI sites. This construct contains just the CDS without any tag. | pE1068 |
| pGM2 | pGM2 is a derivative of pVHS20. The coding sequence of the OsREX1S (Os07g0573600) gene is inserted into pVHS20 in SmaI and BamHI sites with a C terminal 2X Myc tag. | pE1069 |
| pGM3 | pGM3 is a derivative of pUC18. The coding sequence of the OsREX1S (Os07g0573600) gene is inserted into pUC18 inside the SmaI site. | pE1070 |
| pGM4 | pGM4 is a derivative of pVHS20. The coding sequence of the OsREX1 (Os05g0198700) gene is inserted into pVHS20 in SmaI and BamHI sites. This construct contains just the CDS without any tag. | pE1102 |
| pGM5 | pGM5 is a derivative of pVHS20. The Coding sequence of the OsREX1 (Os05g0198700) gene is inserted into pVHS20 in the SmaI and BamHI sites with the C terminal 2X Myc tag. | pE1103 |
| pGM6 | pGM6 is a derivative of pRGEB32. gRNA targeting the coding sequence of miR1880 (miRBase accession | pE1127 |

| | | |
|---|---|---|
| | number - MI0008286) is inserted to pRGEB32 in BsaI and HindIII sites. | |
| pGM7 | pGM7 is a derivative of pRGEB32. gRNA targeting the coding sequence of XCT (Os09g0535300) is inserted into pRGEB32 in BsaI and HindIII sites. | pE1128 |

## 3.8 Table 6. List of *A. tumefaciens* strains generated

The clones are transformed into competent *agrobacterium tumefaciens* (LBA 4404 with pSB) cells.

| Plasmid | Description | Stock number |
|---|---|---|
| pGM1 | pGM1 is a derivative of pVHS20. The coding sequence of the OsREX1S (Os07g0573600) gene is inserted into pVHS20 in SmaI and BamHI sites. This construct contains just the CDS without any tag. | pA393 |
| pGM2 | pGM2 is a derivative of pVHS20. The coding sequence of the OsREX1S (Os07g0573600) gene is inserted into pVHS20 in SmaI and BamHI sites with a C terminal 2X Myc tag. | pA394 |
| pGM4 | pGM4 is a derivative of pVHS20. The coding sequence of the OsREX1 (Os05g0198700 ) gene is inserted into pVHS20 in SmaI and BamHI sites. This construct contains just the CDS without any tag. | pA395 |
| pGM5 | pGM5 is a derivative of pVHS20. The Coding sequence of the OsREX1 (Os05g0198700) gene is inserted into pVHS20 in the SmaI and BamHI sites with the C terminal 2X Myc tag. | pA396 |
| pGM6 | pGM6 is a derivative of pRGEB32. gRNA targeting the coding sequence of miR1880 (miRBase accession number - MI0008286) is inserted to pRGEB32 in BsaI and HindIII sites. | pA404 |

| | | |
|---|---|---|
| pGM7 | pGM7 is a derivative of pRGEB32. gRNA targeting the coding sequence of XCT (Os09g0535300) is inserted into pRGEB32 in BsaI and HindIII sites. | pA405 |

# Chapter 4 Results

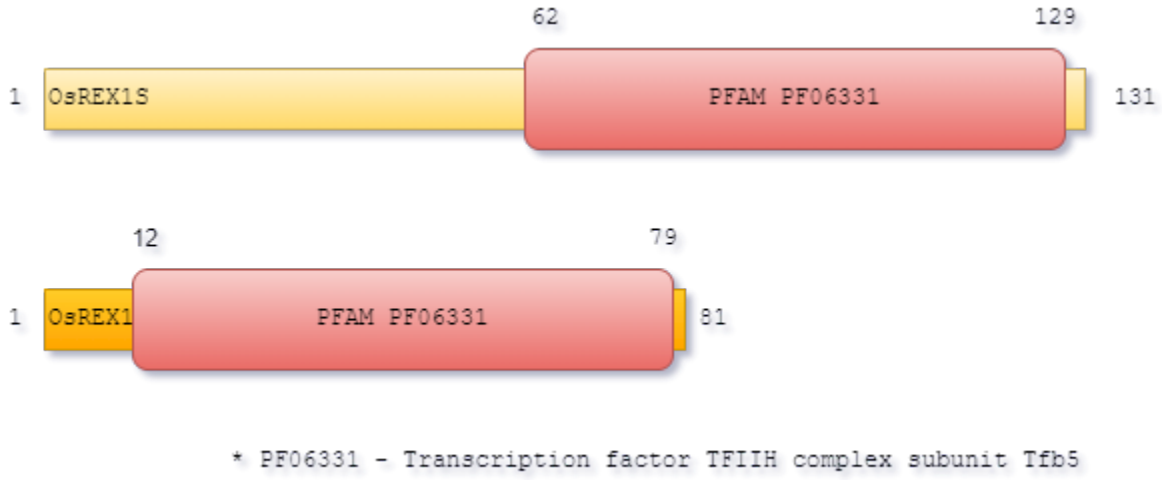



**Figure 4. Protein domain map of OsREX1S and OsREX1**

Two homologs of REX with gradated protein map. OsREX1S is 131 amino acids long and OsREX1 is 82 amino acids long. Both homologs are composed of the Tbf5 protein domain. This family is a component of the general transcription and DNA repair factor IIH. TFB5 has been shown to be required for the efficient recruitment of TFIIH to a promoter. Software Interpro is used for finding domains.

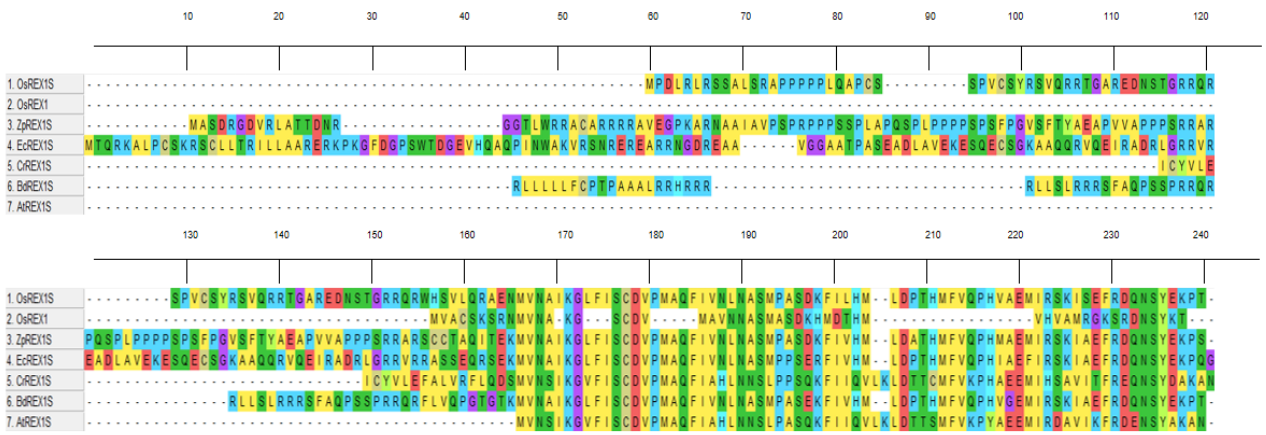



**Figure 5. Amino acid sequence alignment of OsREX1S and OsREX1 with related protein sequences.**

*Arabidopsis thaliana* - NP_001321467.1, *Brachypodium distachyon* - XP_010231853.1, *Capsella rubella* - EOA35953.1, *Eragrostis curvula* - TVU20482.1, *Oryza sativa* - XP_015645359.1, *Oryza sativa* - XP_015638314.1 *Zizania palustris* - KAG8084598.1

Schematic shows the amino acid alignment of OsREX1S and OsREX1 with 5 other monocot and dicot plants. The alignment was performed in the software Mega11.

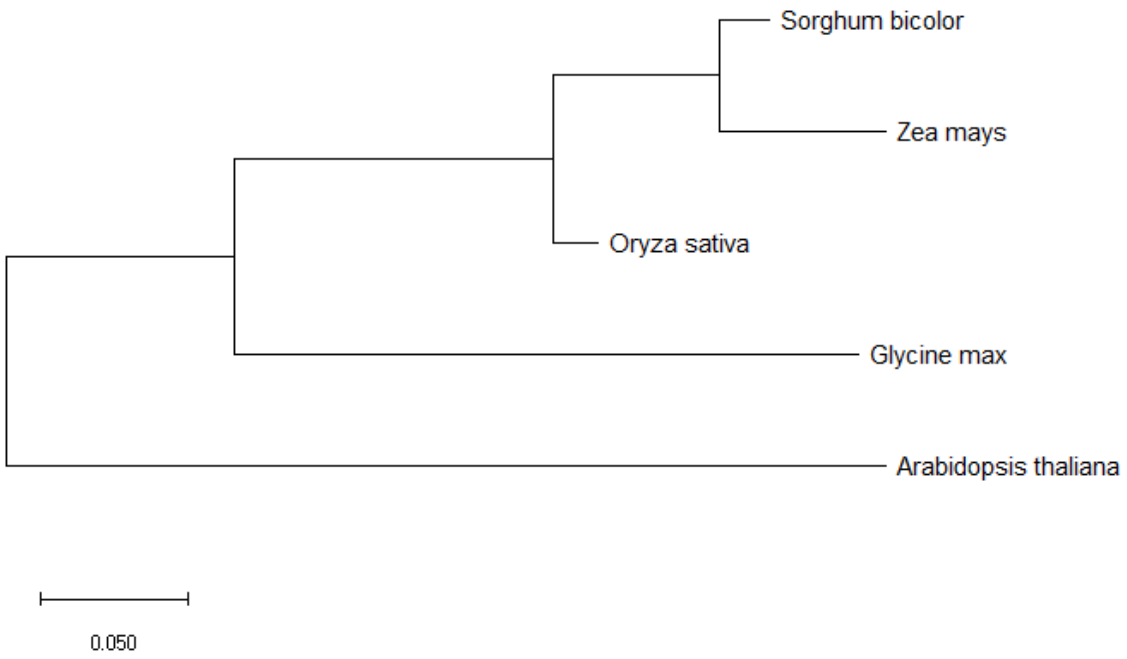



**Figure 5. Phylogenetic tree of OsREX1S**

Accession numbers are as following: *Arabidopsis thaliana* - NP_001321467.1, *Glycine Max* - XP_006584882.1, *Oryza sativa* - XP_015645359.1, *Sorghum bicolor* - XP_002440981.1, *Zea Mays* - NP_001281189.1

The Schematic shows the amino acid alignment of OsREX1S and OsREX1 with monocot and dicot plants performed in Mega11 with the maximum likelihood method.

## 4.1 Cloning and expression of OsREX1S

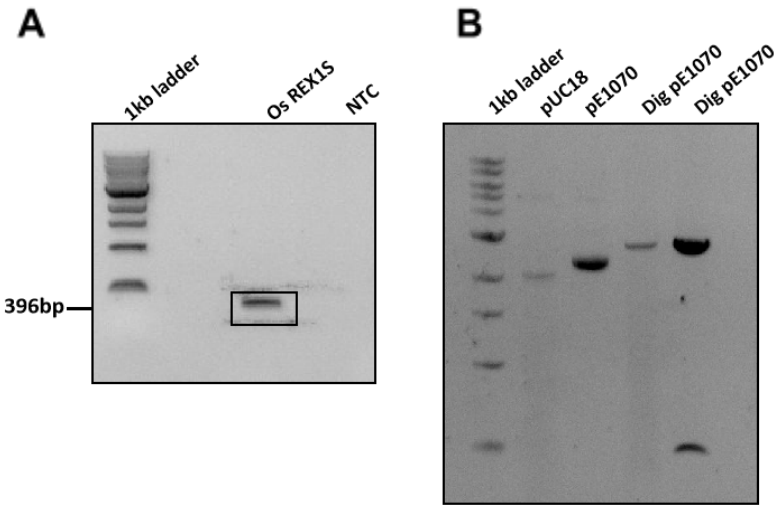
Amplification of OsREX1S is done from cDNA extracted from flag leaf using Phusion DNA polymerase. Following primers are being used to amplify the gene:-

Forward primer - ATGCCTGACCTCCGCCT
Reverse primer- TCATGTTGGCTTCTCGTAGCTG

Programme set up in thermocycler.

| Step | Temperature | Time |
|---|---|---|
| Initial denaturation | 98℃ | 2 min |
| Denaturation | 98℃ | 20 sec |
| Annealing | 60℃ | 20 sec |
| Extension | 72℃ | 40 sec |
| Final extension | 72℃ | 5 min |
| Hold | 14℃ | 15 min |

The clone is confirmed by the restriction digestion with the enzymes HindIII and EcoRI followed by Sanger sequencing.




C

| Construct | HindIII + EcoRI |
|---|---|
| pUC18 (2686 bp) | 2.6 kb + 51 bp |
| pE1070 (3082 bp) | 2.6 kb + 447 bp |

**Figure 7. Cloning of OsREX1S into base plasmid**

A) PCR product of OsREX1S

B) Restriction digestion of clones using HindIII and EcoRI

C) Table of fragments obtained in restriction digestion

## 4.2 Studies to overexpress OsREX1S

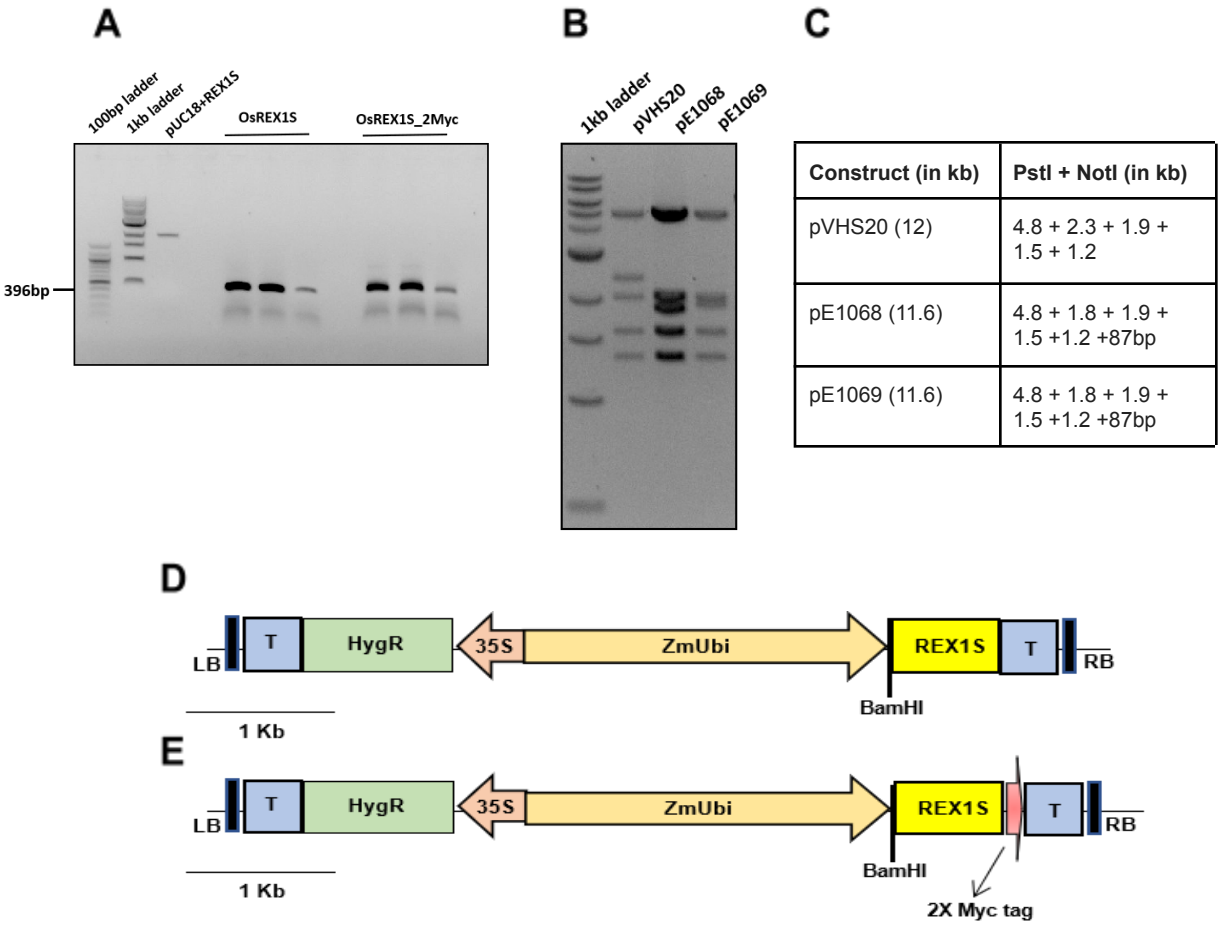
Amplification of OsREX1S, with and without myc tag is done from pE1070 using Phusion DNA polymerase. The following primers are being used to amplify the gene:-

No tag:
Forward primer - GACGGATCCGACATGCCTGACCTCCGC
Reverse primer- GCCaagcttctaTCATGTTGGCTTCTCGTAG
2x Myc tag:
Forward primer - GACGGATCCGACATGCCTGACCTCCGC
Reverse primer -
GCCaagcttctacaaatcttcttcagaaatcaacttttgttccaaatcttcttcagaaatcaacttttgttcTGTTGGCTT
CTCGTAGC

Programme set up in thermocycler.

| | **Without Tag** | | **With 2x Myc Tag** | |
|---|---|---|---|---|
| **Step** | **Temperature** | **Time** | **Temperature** | **Time** |
| Initial denaturation | 98℃ | 2 min | 98℃ | 2 min |
| Denaturation | 98℃ | 20 sec | 98℃ | 20 sec |
| Annealing | 66℃ | 20 sec | 72℃ | 20 sec |
| Extension | 72℃ | 40 sec | 72℃ | 40 sec |
| Final extension | 72℃ | 5 min | 72℃ | 5 min |
| Hold | 14℃ | 15 min | 14℃ | 15 min |

- The amplified product is extracted from the gel and purified (3.5).
- The purified product is digested with BamHI to make a sticky end.
- Simultaneously pVHS20 is digested with SmaI and BamHI, and the 11kb backbone is purified from the gel.
- Both the products are then ligated.
- Clones are confirmed with multiple digestion using PstI and NotI, followed by Sanger Sequencing.




| Construct (in kb) | PstI + NotI (in kb) |
|---|---|
| pVHS20 (12) | 4.8 + 2.3 + 1.9 + 1.5 + 1.2 |
| pE1068 (11.6) | 4.8 + 1.8 + 1.9 + 1.5 +1.2 +87bp |
| pE1069 (11.6) | 4.8 + 1.8 + 1.9 + 1.5 +1.2 +87bp |

**Figure 8. Cloning of OsREX1S into binary vector**

A) PCR product of OsREX1S with and without 2xMyc tag

B) Restriction digestion of clones using PstI and NotI

C) Table of fragments obtained in restriction digestion

D) Linear map of OsREX1S inserted into binary vector

E) Linear map of OsREX1S with 2xMyc tag inserted into binary vector

## 4.3 Studies to overexpress OsREX1

Amplification of REX1, with and without myc tag is done from pE1045 using Phusion DNA polymerase. The following primers are being used to amplify the gene:-

No tag:
Forward primer - gaGACGGATCCGACatgGTGGCATTCTGT
Reverse primer- GCCaagcttctaTCATGTTGGCTTCTCGTAG
2x Myc tag:
Forward primer - gaGACGGATCCGACatgGTGGCATTCTGT
Reverse primer -
GCCaagcttctacaaatcttcttcagaaatcaacttttgttccaaatcttcttcagaaatcaacttttgttcTGTTGGCTT
CTCGTAGC

Programme set up in thermocycler.

| | **Without Tag** | | **With 2x Myc Tag** | |
|---|---|---|---|---|
| **Step** | **Temperature** | **Time** | **Temperature** | **Time** |
| Initial denaturation | 98℃ | 2 min | 98℃ | 2 min |
| Denaturation | 98℃ | 20 sec | 98℃ | 30 sec |
| Annealing | 66℃ | 20 sec | 66℃ | 30 sec |
| Extension | 72℃ | 40 sec | 72℃ | 40 sec |
| Final extension | 72℃ | 5 min | 72℃ | 5 min |
| Hold | 14℃ | 15 min | 14℃ | 15 min |

- The amplified product is extracted from the gel and purified (3.5).
- The purified product is digested with BamHI to make a sticky end.
- Simultaneously pVHS20 is digested with SmaI and BamHI, and the 11kb backbone is purified from the gel.
- Both the products are then ligated.
- Clones are confirmed with multiple digestion using PstI and NotI, followed by Sanger Sequencing.

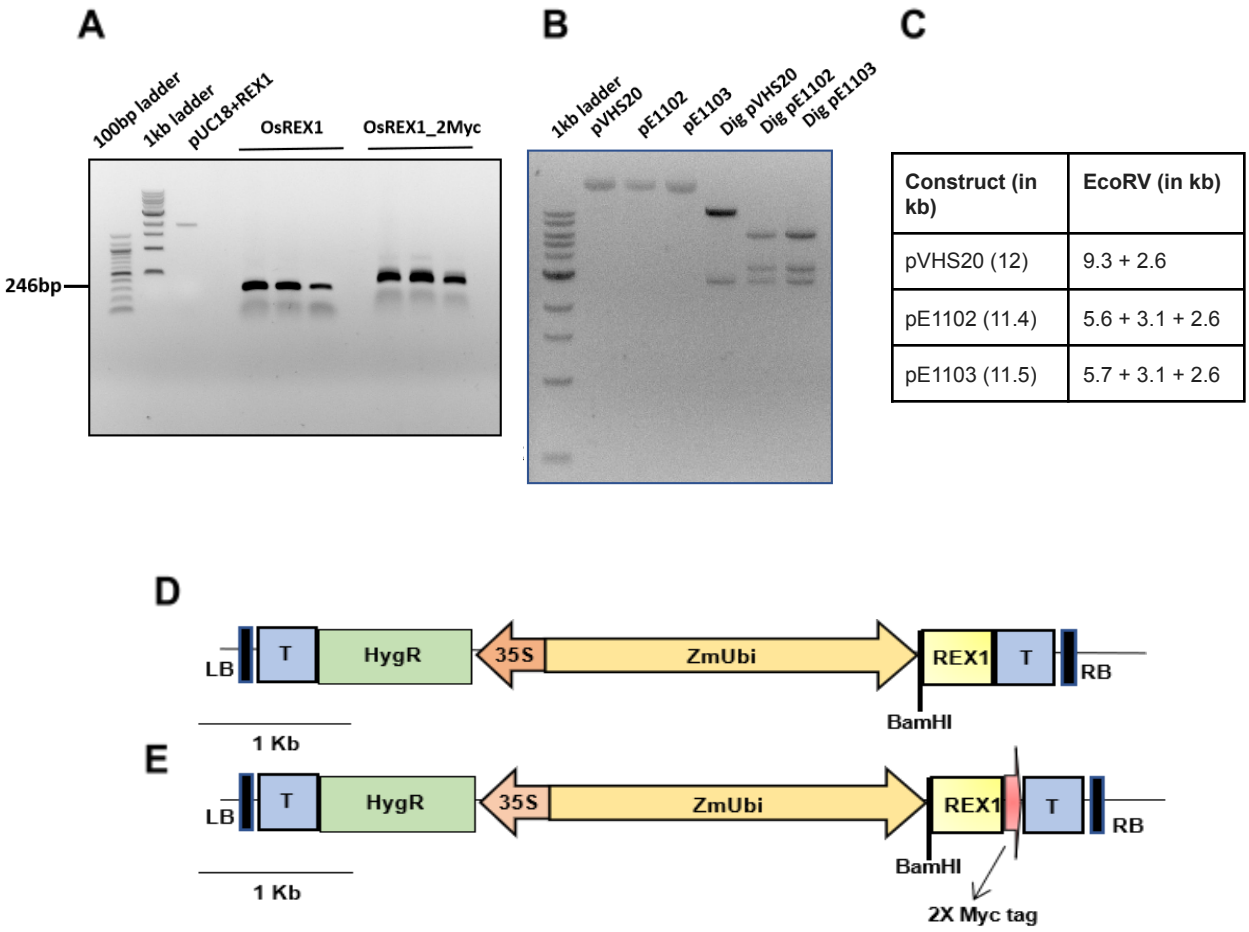



**Figure 9. Cloning of OsREX1 into binary vector**

A) PCR product of OsREX1 with and without 2xMyc tag

B) Restriction digestion of clones using EcoRV

C) Table of fragments obtained in restriction digestion

D) Linear map of OsREX1 inserted into binary vector

E) Linear map of OsREX1 with 2xMyc tag inserted into binary vector

## 4.4 Strategy to knockout OsREX1S, OsREX1 and OsXCT using gRNA

Amplification of both the gRNAs are done with the primers given below using Phusion DNA polymerase.

gRNA 1880:
Forward primer - CCGGCTCGTATGTTGTGTGG
Reverse primer-
attaggtctccaaacAGCGGGCCACTTAAGCATTCtgccacggatcatctgcacaac

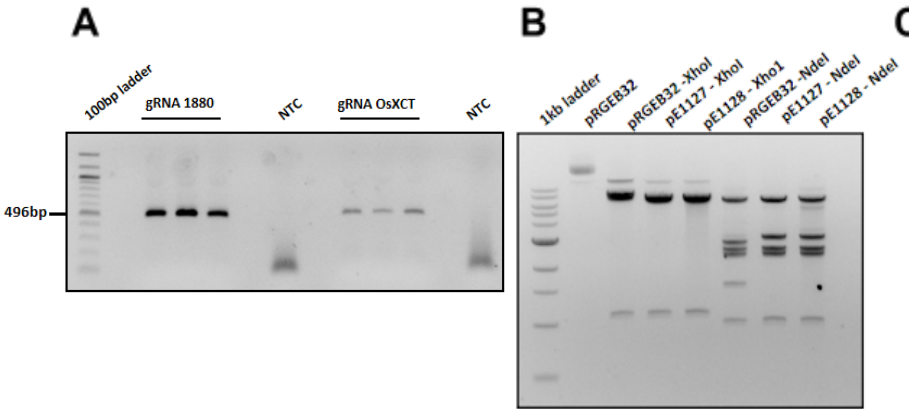
gRNA OsXCT:
Forward primer - CCGGCTCGTATGTTGTGTGG
Reverse primer -
attaggtctccaaacGGCCTGCTCCAGTTCGGCTCtgccacggatcatctgcacaac

Programme set up in thermocycler.

| Step | Temperature | Time |
|---|---|---|
| Initial denaturation | 98℃ | 3 min |
| Denaturation | 98℃ | 25 sec |
| Annealing | 66℃ | 20 sec |
| Extension | 72℃ | 30 sec |
| Final extension | 72℃ | 10 min |
| Hold | 14℃ | 15 min |

- The amplified product is extracted from the gel and purified (3.5).

- The purified product is digested with BsaI and HindIII to make sticky ends.
- Simultaneously pRGEB32 is digested with BsaI and HindIII, and the 15kb backbone is purified from the gel.
- Both the products are then ligated.
- Clones are confirmed with multiple digestion using XhoI and NdeI, followed by Sanger Sequencing.




| Construct (in kb) | XhoI (in kb) | NdeI (in kb) |
|---|---|---|
| pRGEB32 (15.4) | 7663bp + 7.1 + 1 | 7 + 3 + 2.4 + 2.3 + 1 |
| pE1127 (15.8) | 7667bp + 7.1 + 1 | 7 +3 + 2.4+ 2.3 + 1 |
| pE1128 (15.8) | 7667bp + 7.1 + 1 | 7 +3 + 2.4+ 2.3 + 1 |

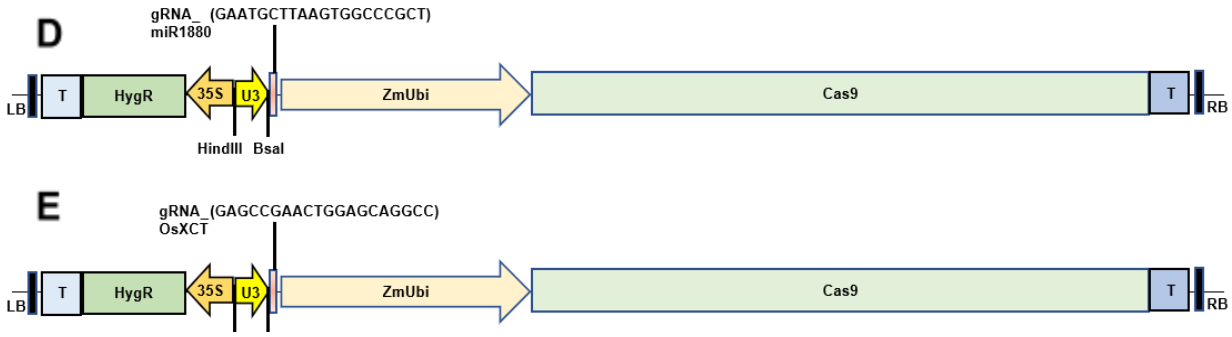



**Figure 10. Cloning of gRNA targeting miR1880 and OsXCT into binary vector**

A) PCR product of gRNA targeting miR1880 and OsXCT

B) Restriction digestion of clones using XhoI and NdeI

C) Table of fragments obtained in restriction digestion

D) Linear map of gRNA targeting miR1880 inserted into binary vector

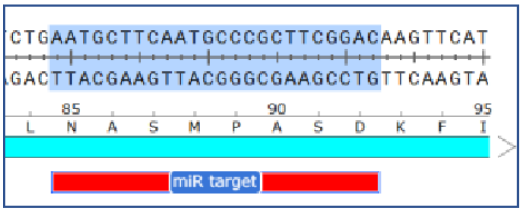
E) Linear map of gRNA targeting OsXCT inserted into binary vector

## 4.5 Mutating miR1880 binding site

Strategy:

1) We first identified the miR1880 sequence in miRBase.
2) Then checked miR1880 target prediction in TAPIR taking OsREX1S as the target region.
3) We then mutated the target region so the miR1880 can not bind to REX1S anymore in snapgene.
4) Here we ensured that the amino acids didn't change and remained stable even though we changed the targeting site.
5) Then we designed the primers having these mutations as overhangs.
6) Two primers will amplify the first region and the other two amplify the remaining region.
7) Now we have to combine both fragments with overlapping PCR.
8) Insert the amplicon into the base vector.

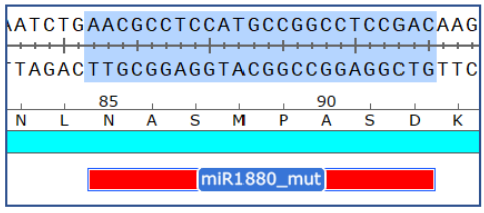
**miR1880_REX1S**




```
#------------------------
# Search parameters
# score <= 6
# mfe ratio >= 0.2
#------------------------

target      REX1S
miRNA       miR1880
score       6
mfe_ratio   0.65
start       253
seed_gap    0
seed_mismatch 1
seed_gu     1
gap         1
mismatch    2
gu          0
UUACGAAUUCACCGGGCGAACCUU miR
|||||||.|.|.|||||||.|o|
AAUGCUUCAAU-GCCCGCUUCGGA target
//
```

**miR1880_mut_REX1S**




```
#------------------------
# Search parameters
# score <= 20
# mfe ratio >= 0.3
#------------------------

target      REX1S_mut
miRNA       miR1880
score       20
mfe_ratio   0.31
start       319
seed_gap    0
seed_mismatch 7
seed_gu     0
gap         0
mismatch    6
gu          0
UUACGAAUUCACCGGGCGAACCUU miR
.|..||.|.|||||.....||...
CAGCCUCAUGUGGCGGAGAUGAUC target
//
```

## 4.6 The stress assay

In order to check the abundance of microRNA 1880 and its target REX1S across a wild and domesticated variety of rice when subjected to stress, we treated *O. nivara* and Pusa Basmati 1 (PB1) with ultraviolet light and bleomycin.

Ultraviolet stress - Two doses in the form of 1 kJ and 3 kJ UV light were given to *O.nivara* and PB1 4-week-old seedlings using a crosslinker. After 20 minutes of recovery, seedlings are snap-chilled in liquid nitrogen and stored for RNA extraction at -80℃.

The UV radiation causes dimerization of the pyrimidines in the form of cyclobutane pyrimidine dimers and 6-4 photoproducts. These lesions disrupt DNA structure in the form of kinks which leads to problems in transcription and replication. Repair of such lesions sometimes causes DNA damage (Rastogi et al., 2010).

Bleomycin stress - Two doses in the form of 4 mg/L and 10 mg/L are given to *O.nivara* and PB1 4-week-old seedlings. Bleomycin was dissolved in the autoclaved water and plants was placed in it for 8 hours. Samples were then snap-chilled in liquid nitrogen and stored for RNA extraction at - 80℃.
Bleomycin $Fe^{2+}$ binds to DNA, this complex undergoes oxidation hence liberating electrons. The electrons react with oxygen which in return produces hydroxyl radicals. It attacks the phosphodiester bond which leads to DNA damage (Chen et al., 2008).

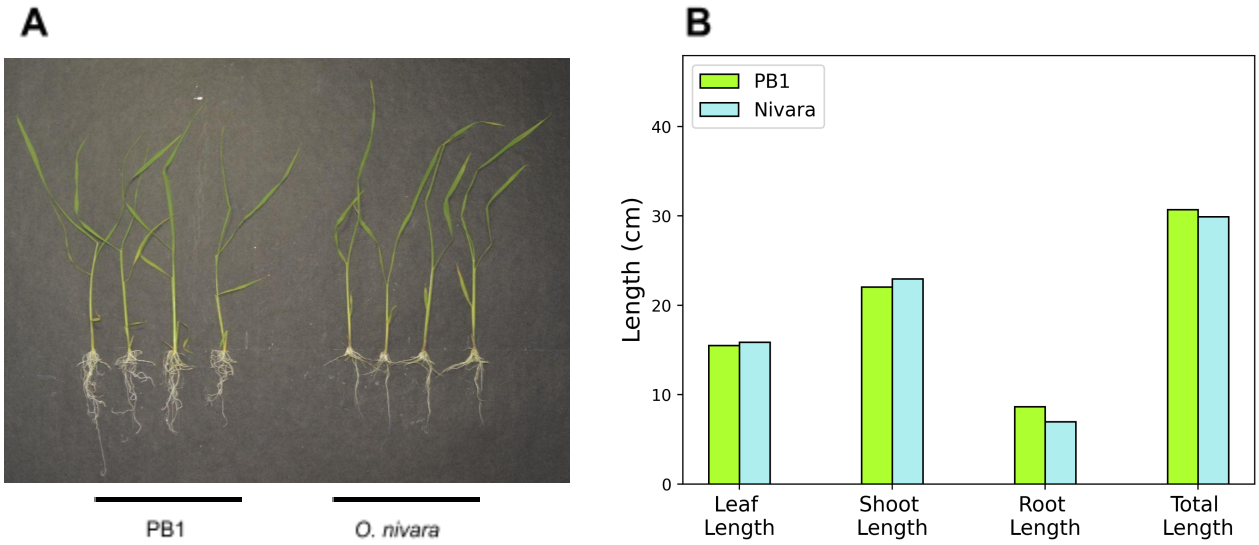



**Figure 11. Domesticated and wild variety of rice.**

A) Picture of domesticated (PB1) and wild (*O. nivara*) variety of rice

B) Dimensions of domesticated (PB1) and wild (*O. nivara*) variety of rice

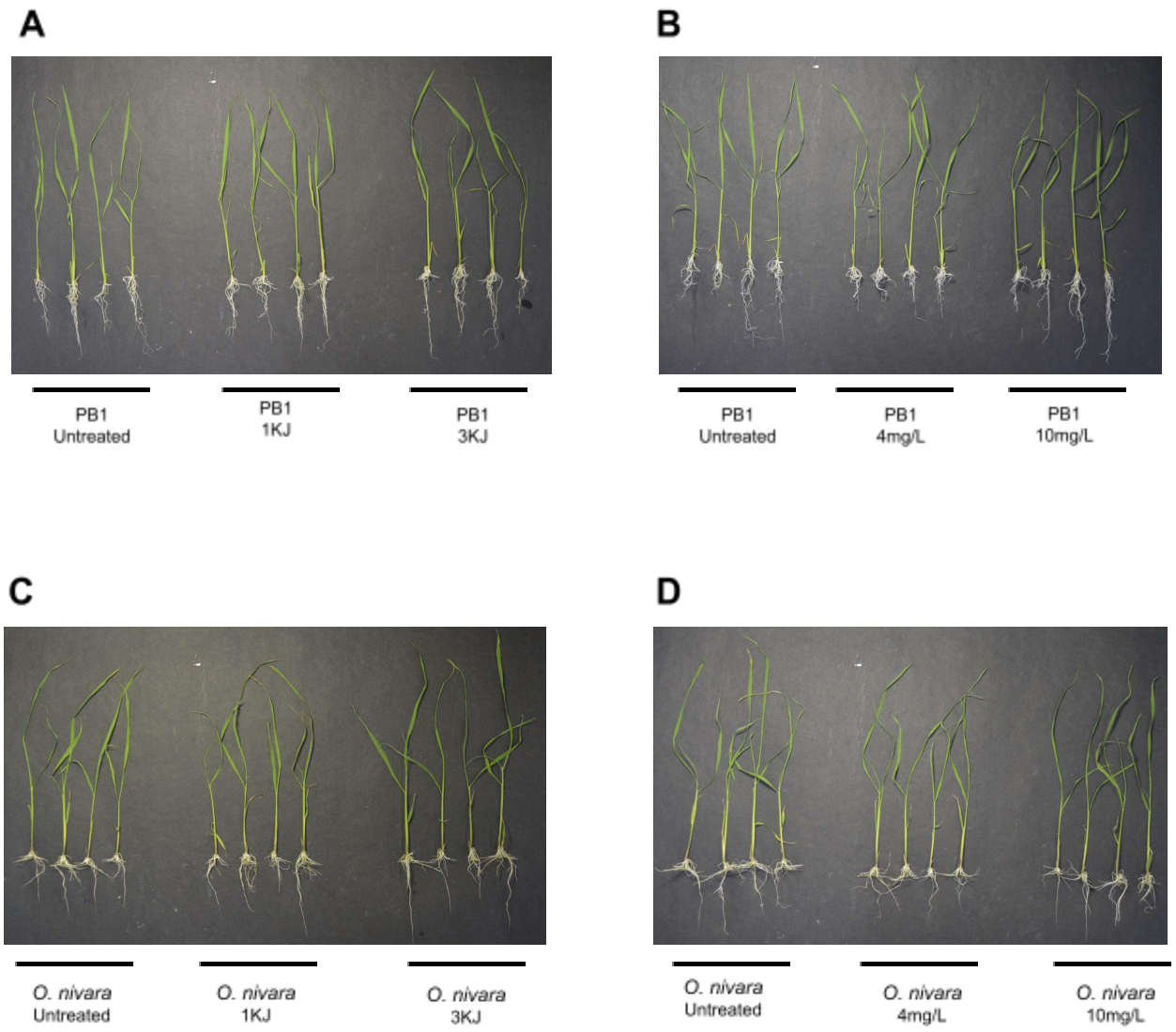



**Figure 12. Domesticated and wild variety of rice treated with stress**

A) 4 week old PB1 seedlings with (1KJ and 3KJ) and without UV stress.

B) 4 week old PB1 seedlings with (4mg/L and 10mg/L) and without Bleomycin stress.

C) 4 week old *O. nivara* seedlings with (1KJ and 3KJ) and without UV stress.

D) 4 week old *O. nivara* seedlings with (4mg/L and 10mg/L) and without Bleomycin stress.

## 4.7 Transgenic Plants

### 1. pA393 - OsREX1S Overexpression

| Subculture | Selection -1 | Selection -2 | Selection -3 | Regeneration-1 | Regeneration-2 | Regeneration-3 |
|---|---|---|---|---|---|---|
| Number of calli | ~520 | ~500 | ~240 | ~40 | ~20 | ~15 |

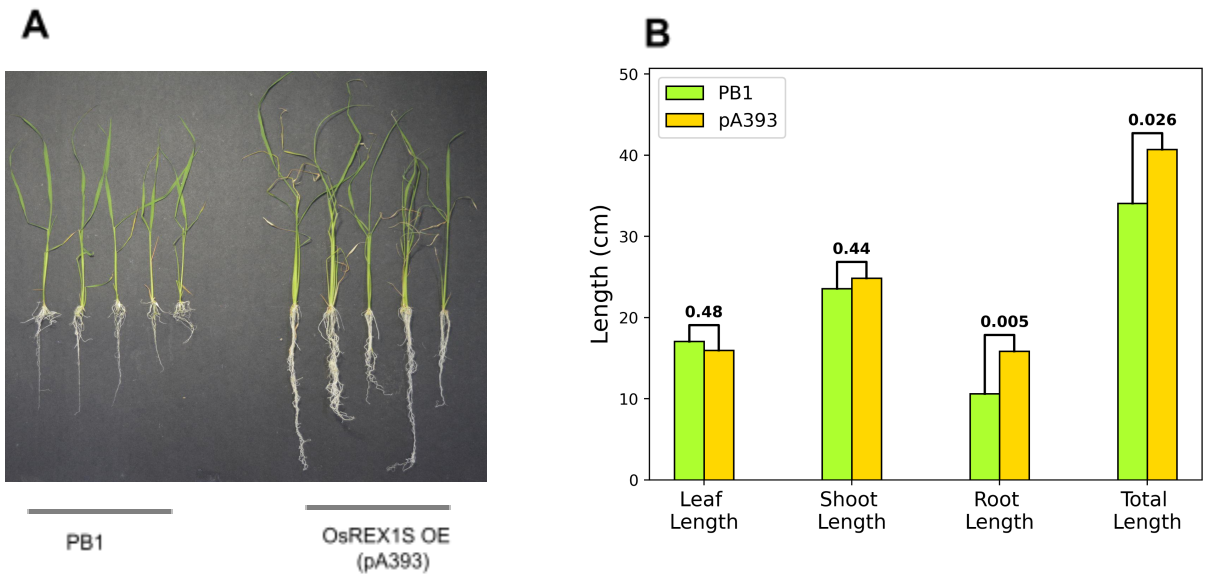



**Figure 13. Control Vs OsREX1S OE**

A) Picture of control (PB1) and OsREX1S (pA393) overexpression seedlings.

B) Comparison between control (PB1) and OsREX1S (pA393) overexpression seedlings.

## 2. pA396 - OsREX1 Overexpression with 2X Myc tag

| Subculture | Selection -1 | Selection -2 | Selection -3 | Regeneration-1 | Regeneration-2 | Regeneration-3 |
|---|---|---|---|---|---|---|
| Number of calli | ~250 | ~220 | ~80 | ~25 | ~20 | ~15 |

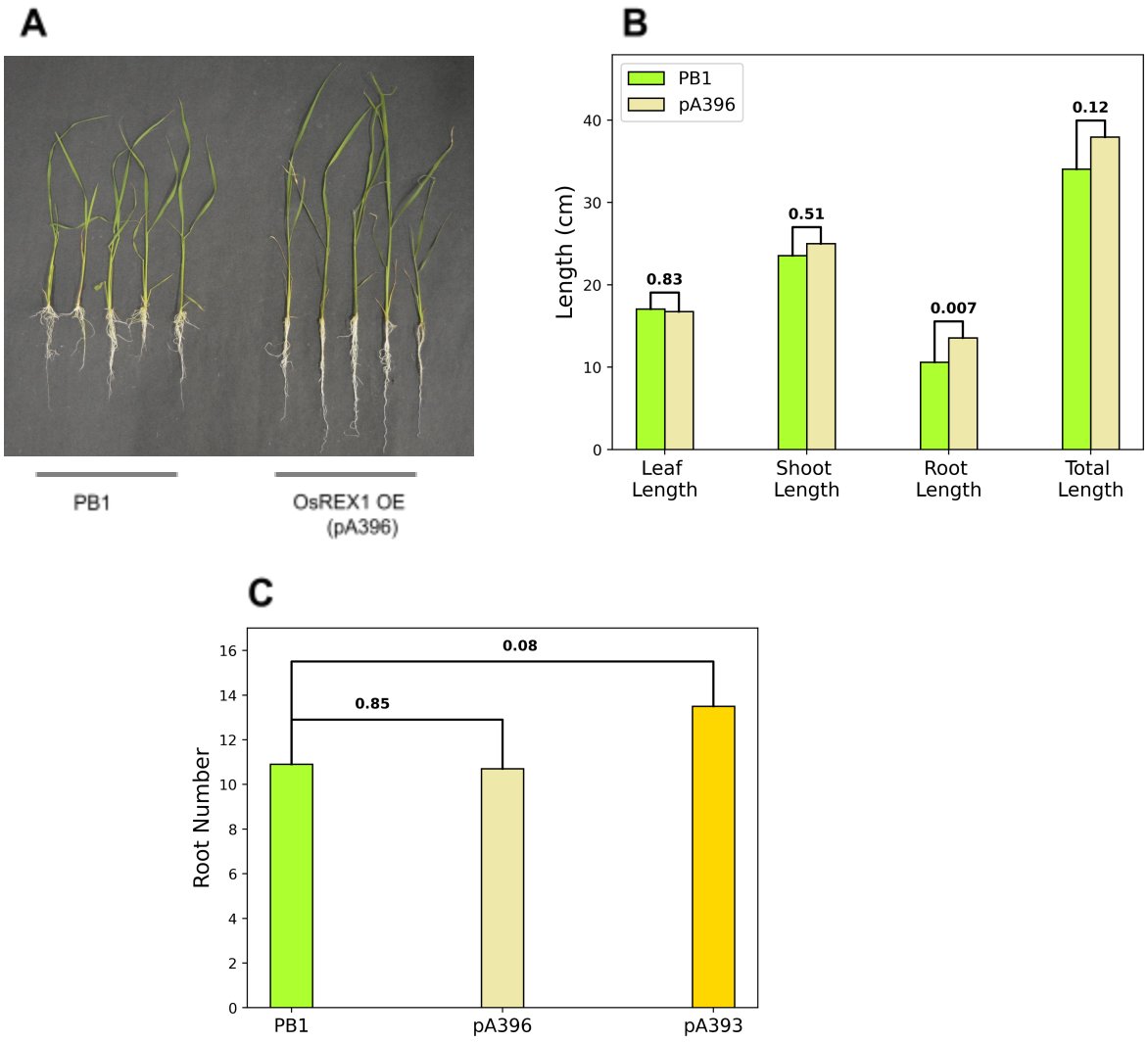



**Figure 14. Control Vs OsREX1 OE**

A) Picture of control (PB1) and OsREX1S (pA396) overexpression seedlings.

B) Comparison between control (PB1) and OsREX1S (pA396) overexpression seedlings.

C) Comparison between root number of PB1, OsREX1S OE and OsREX1 OE.

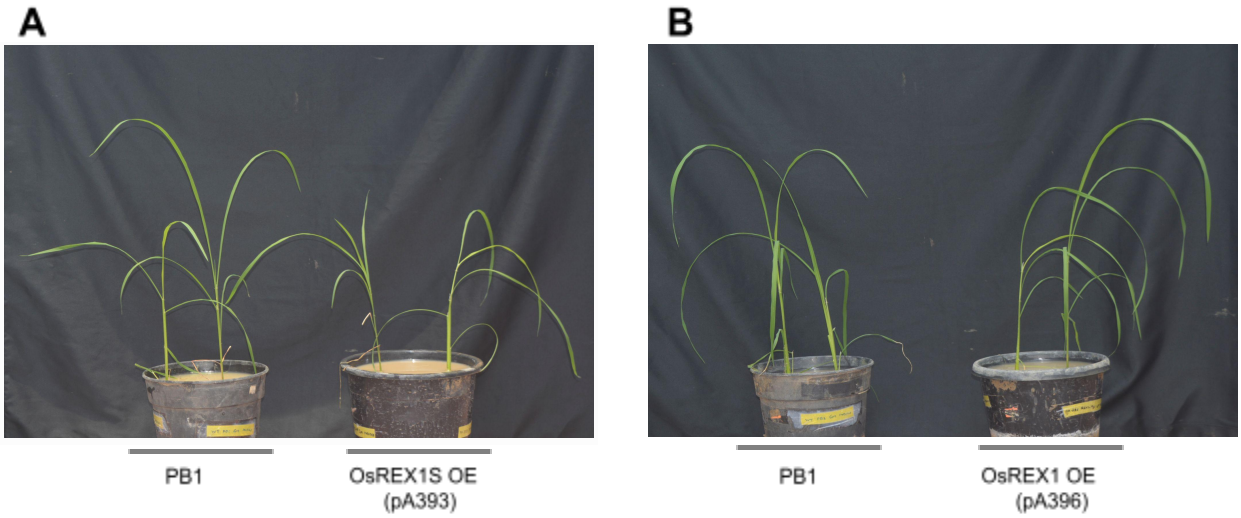



**Figure 15. Plants transferred in greenhouse**

A) Picture of control (PB1) and OsREX1S (pA393) overexpression plants.

B) Picture of control (PB1) and OsREX1 (pA396) overexpression plants.

**10 Plants of each transgenic(pA393 and pA396) has been transferred to green house on 14/3/23 along with 2-2 control PB1 plants.**

3. **pA404 - miR1880 CRISPR Knockout**

| Subculture | Selection-1 | Selection-2 | Selection-3 | Regeneration-1 |
|---|---|---|---|---|
| Number of calli | ~220 | 200~ | ~60 | ~30 |

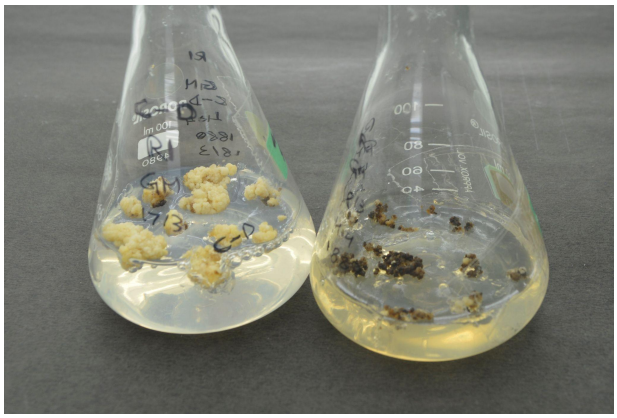


Figure 16. gRNA1880 CRISPR knockout (pA404) in Regeneration-1 media

**4-5 promising calli can be observed in the current subculture. The plants are expected in 45 days.**

## 4. pA405 - OsXCT CRISPR Knockout

| Subculture | Selection-1 | Selection-2 | Selection-3 |
|---|---|---|---|
| Number of calli | ~250 | ~220 | ~100 |

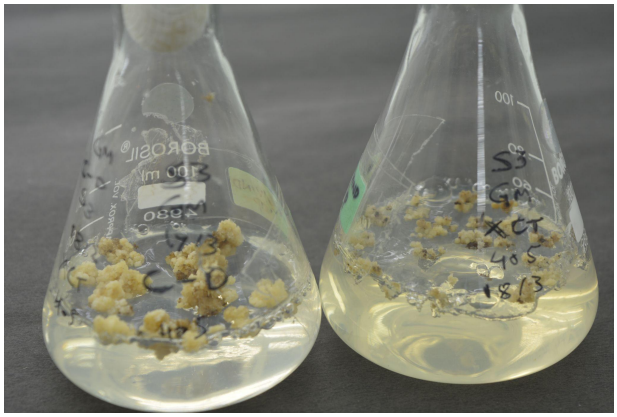


**Figure 17. gRNA OsXCT CRISPR knockout (pA405) in Selection-3 media**

5. **pA395 - OsREX1 Overexpression**

| Subculture | Selection-1 | Selection-2 |
|---|---|---|
| Number of calli | ~250 | ~230 |

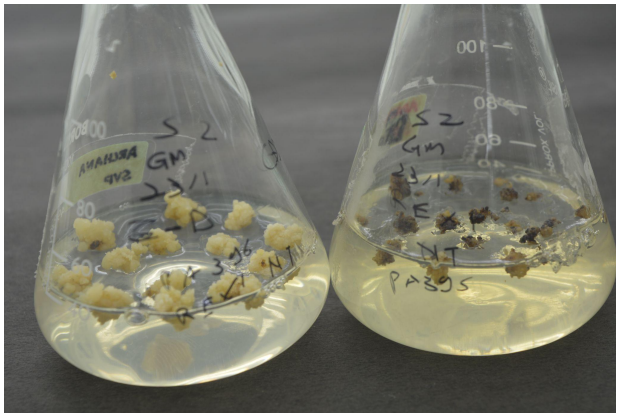


**Figure 18. OsREX1 overexpression (pA395) in Selection-2 media**

# Chapter 5 Discussion

Domestication of plants was a major expedite in the history of modern civilization. We still need a lot of understanding about domestication to exploit the process in its full potential. As gradually science progresses, no one knows what wonders can be achieved with the application of this knowledge. But for now we know that factors like amino acid changes in a gene, epigenetics and small RNA pathways plays important role in domestication. Chenna et al., already showed miRNA397's crucial role in the lignification of rice.

In this study we have identified a novel miRNA, miR1880 which is found to target a gene called OsREX1S. Both the target and the gene showed differential expression in wild and cultivated rice varieties which enabled us to think about the putative miRNA target gene module in domestication. In several model systems this gene is proven to be vital for DNA damage repair. To prove it explicitly need further understanding of the topic we generated transgenic lines to overexpress the target protein and to knockout the predicted miRNA. With that vision tools generated in this study have potential to appreciate its role in domestication. These overexpression lines can be tested with various stress assays to understand the importance of this genes in DNA damage repair. The CRISPR knockout lines will also be very useful to evaluate the differential expression of the gene in wild and cultivated genes. As per the literature if the hypothesis holds true, the overexpression lines will be able to do excision of pyrimidine dimers effectively than those of the control plants. The knockout lines should behave in the similar way because the regulation of gene is taken off. We also planned to mutate the miRNA binding site of the gene which will give the same effect as miRNA mutant.

The tools generated in the study will be handy for various future experiments including biochemical studies using tagged proteins. Proper expression analysis of the gene using qPCR and miRNA expression using northern blotting are important for further understanding of the topic. It is known that the DNA damage repair pathways in

eukaryotes are conserved, at this juncture the study have huge potential in understanding DNA damage repair pathways in different model organisms.

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