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Original Article

# Isolation, Identification Similarity And Qualitative Expression Of Metallothionein Gene in IR-Bagendit Rice (*Oryza sativa*)

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**ABSTRACT**

Metallothionein (MTs) is an enzyme that plays a role in the binding of metals in plants. Various types of rice have been known to contain MTs and IR-Bagendit rice leaves have the highest MTs protein content compared to other rice varieties. However, MTs coding gene in IR-Bagendit rice variety is still unknown. OsRAC1 gene is reported as the down-regulator of MTs and there is an analogous gene for MTs-like gene using RAP1 and RAP2 primers in various plants. This study aimed to isolate, identification of similarity, and analysis of qualitative expression of MTs gene in IR-Bagendit rice as compared to Inpari, IR-36, and IR-34. The steps of this research were DNA isolation, PCR in OsRAC1 gene, RNA isolation and cDNA reverse transcription using primer RP1 and RP2, and agarose gel electrophoresis. Amplification quality of OsRAC1 gene in IR-Bagendit, Inpari, IR-36, and IR-34 showed the same result. Qualitative expression of MTs by reverse transcription showed that IR-Bagendit has the highest MTs-like gene compared to other samples.

**Key words:** Metallothionein, MTs-like gene, Rice, IR-Bagendit, Metal Exposure.

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## INTRODUCTION

The role of metallothioneins (MTs) in metal detoxification is related to the ability to bind toxic metals so it can be used as an indicator of metal pollution. One of the toxic metals is plumbum (Pb) which may accumulate in large amounts on various organs in the body, such as the liver and kidneys. Pb exposure can cause some health problems, like increasing blood pressure; damaging of kidney, nervos system, and brain; destruction of sperm; and disturbance of hemoglobin biosynthesis. Furthermore, it may cause anemia, IQ reduction, and lowers male fertility.[1]

Single metal ion for 2 or 3 sulfhydryls (SH) residues form a tetrahedral tetrathiolate structure. Cysteine residues are needed to detox heavy metals by binding to cations of transition metals.[2] Metallothioneins, which help bind heavy metals, can be found in some plants and animals. Rice, soybeans, beans, and corn contain a lot of MTs in its root, stems, leaves, and fruit.[3] IR-Bagendit rice leaves have the highest MTs content compared to other rice varieties.[4] The intervention of IR Bagendit leaf infusion-contains MTs to the Plumbum (Pb)-exposure-rats has been proven to prevent kidney damage and prevent hematopoiesis.[3,5] Based on this study, we predict that each plant has a different genetic combination of MTs gene.

A study to identify MTs-like protein using bioinformatics to determine the diversity of genetic codes for amino acid sequences has produced 97% identical to MTs type 2 encoded by *RicMT* gene rice (*OsMt2c*, accession no. AB002820).[6] Research conducted by Zhang *et.al* 2009 proves that MTs-like protein expression has increased in rice leaves induced by Cu.

Various types of rice have been known to contain MTs, however, the protein-coding gene in IR-Bagendit rice varieties shows unknown. The MTs genetic code on IR-Bagendit rice leaves is important to know so that further research can be done with plasmid cloning as prevention of kidney damage in *Rattus norvegicus* exposed by Pb.

This study aimed to isolate, identification of similarity, and analysis of qualitative expression of MTs gene in IR-Bagendit rice as compared to Inpari, IR-36, and IR-34.

## METHOD

### Sample

Rice leaf samples came from 4 cultivars, they are IR Bagendit, Inpari, IR-36, and IR-34. Samples were taken from the Boja, Kendal, Central Java. As much as 20 mg leaves used in this study. The thin-boned leaves chosen to ease the cell destruction process by liquid nitrogen. DNA isolation process was done using a plant DNA extraction kit from *Geneaid*. Isolated DNA purity and concentration were measured using nanodrop spectrometry.

### Database search and sequence analysis

Data sequences were analyzed using the keyword "metallothioneins" and search locations in DDBJ/EMBL/GenBank databse using MTs gene sequences from rice (U77294) and wheat (p30570). The *ricMT* homologous DNA sequence is then used to database screen the Rice Genome Research Program (RGP) (http://rgp.dna.affrc.go.jp) then the target is reconfirmed.

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## DNA isolation

Total DNA isolation was carried out using the FavorPrep Plant Genomic DNA Extraction Mini Kit (Favorgen). The principle of DNA isolation was solid base using silica filter column and DNA elution buffer. Samples of rice leaves were added liquid nitrogen then grinded and put into a tube then added buffer FAPG1 (preheat 65° C) and RNase A, incubated for 20 minutes at 65° C. Then the FAPG2 buffer was added and incubated for 5 minutes in the ice box. After that, the mixture was transferred to the tube filter column and centrifuged at 12,000 rpm for 3 minutes. The filtrate from the collection tube was transferred to the new tube and a FAPG 3 buffer is added which has been added with 96% ethanol. The mixture transferred to the FAPG column and centrifugate 12,000 rpm for 1 minute. Next wash buffer 1 added with ethanol before added to FAPG column then centrifuged 12,000 rpm for 30 minutes. Next wash buffer 2 added with ethanol before and centrifuged 12,000 rpm for 30 minutes, this was repeated twice. Elution buffer was added which was preheated 65° C and centrifuged 12,000 rpm for 3 minutes. DNA is in the collection tube.

## RNA isolation

RNA isolation process was done using the FavorPrep Plant Total RNA Purification Mini Kit (Favorgen). The rice leaf samples were added with liquid nitrogen and then grinded. Leaf extract was added to the tube then supplemented with FARB buffer which was added by β-mercaptoethanol before. Column filters and collection tubes are placed and samples added then centrifuged at 12,000 rpm for 1 minute. Ethanol 70% was added then the mixture transferred to the FARB mini column and centrifuged 12,000 rpm for 1 minute. Wash buffer 1 is added to the FARB Mini column then centrifuged 12,000 rpm for 1 minute. Wash buffer 2 that has been added with ethanol was added to the FARB Mini column then centrifuged at 12,000 rpm for 1 minute, this was repeated again with centrifugation for 3 minutes. FARB Mini columns are attached to the elution tube. RNase free water is added and allowed to stand for 1 minute then centrifuged 12,000 rpm for 1 minute. RNA was in the collection tube.

## PCR

PCR was used to amplificate the target genes which was involved in MTs-like rice gene. Kit PCR Promega GoTaq PCR Master Mix was used. The complete coding region of M-like protein in the rice was being amplified using PCR primer 5'- GGATCCAAGATGTCTTGCAGC-3' and 5'-CTCGAGATCTTAGCAGTTGCA-3'.[7]

## Electrophoresis agarose

The result of PCR was then confirmed using electrophoresis agarose 2%. 100bp ladder to identify PCR products. Meanwhile, the loading dye used was 2 µl with a sample as much as 5 µl.

# RESULTS

## Measurement of DNA concentration and purity

DNA concentration used to determine how much DNA template amplified in the PCR process. The same template concentration will produce the same thickness of the amplified band DNA when MTs gene found in the samples hence the results of the DNA band are optimum to see. The DNA template used in the PCR process was 150 ng/µl.

DNA is considered to be pure when optical density (OD) 1.8-2. DNA purity is determined by the presence or absence of contamination for example RNA, protein, and remaining reagents. DNA purity with OD below 1.8 indicates protein contamination, whereas DNA absorbance above 2 indicates RNA contamination. IR-36 and IR-34 DNA isolates showed low DNA purity because they had protein content as contaminants, while other DNA isolates were pure. The DNA sample concentration and purity showed in Table 1.

## RNA concentration of paddy leaves

RNA isolation from leaf samples was done using RNA isolation kit from Favorgen and cell lysis using liquid nitrogen. RNA from various cultivars was used to find out the expression level of the MTs gene. The gene expression began with mRNA and be translated into the MTs protein.[8]

The results of the concentration and RNA purity calculation using nanodrop showed that the sample used in this research have the almost same purity with OD above 2.0. This result indicated that the sample was pure RNA. The highest RNA samples were in Inpari. The amount of RNA isolates in the samples will affect the calculation of MTs gene expression results. The same concentration of RNA template must be given to measure MTs gene expression with the same initial RNA source. The Concentration and purity of RNA of rice leaves from various cultivars were shown in Table 2.

## Metallothionein gene amplification

Based on the PCR result with primer *OsRac1* (Rac1F::5′-AGATAGGGCCTATCTTGCTGATCATC-3′; Rac1R: 5′-CTAGAGT TTCCTCCTAGCTGCAAGC-3′)[9] and annealing temperature on 55°C, It showed that the was only one specific band which is linear with all rice sample from the varied cultivars. DNA band thickness of the *OsRac* gene for MTs regulation in varied rice cultivars was the same. It might be concluded that MTs genes in the rice had the same quality although it came from different cultivars.

Based on the PCR result using RP 1, two amplification regions were shown. Primer RP1 5'-GGATCAAGCTGCGGCTGCGGCTCA A-3' and RP2 5'-GCAGTTGCAAGGGTCGCACTTGCAG-3'[10] with annealing temperature was 63°C could cope two regions. All the rice sample from varied cultivars has two target regions, so it led to unspecific target. Primer RP1 and RP2 were designed based on the cysteine-rich consensus region of the MTs-like gene from varied plants.[10]

## Genetical annotation analysis of MTs gene in *Oryza sativa*

Here is the alignment of MTs gene sequences from the NCBI database. The result of the In Silico sequence analysis of the MTs gene in *Oryza sativa* is described below.

>AF048750.1 Oryza sativa MTs (MTe) gene, complete cds

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>AF048750.1 Oryza sativa MTs (MTe) gene, complete cds GAATTCTTT-TAAAACCATTTGTACTGAATTTAAGAGAAAAATGTATCAC-

**Table 1: Concentration and purity of DNA isolates.**

| No | Rice Cultivars | Concentrations | Unit | Purities (260/280 nm) |
|---|---|---|---|---|
| 1 | Bagendit | 145,4 | ng/µl | 1,87 |
| 2 | Inpari | 13,9 | ng/µl | 1,87 |
| 3 | IR 36 | 13,6 | ng/µl | 1,14 |
| 4 | IR 34 | 40,6 | ng/µl | 1,36 |

**Table 2: Concentration and purity of RNA isolates.**

| No | Rice cultivars | Concentrations | Units | Purities (260/280 nm) |
|---|---|---|---|---|
| 1 | IR-Bagendit | 55,6 | ng/µl | 2,34 |
| 2 | Inpari | 248,9 | ng/µl | 2,14 |
| 3 | IR 36 | | ng/µl | |
| 4 | IR 34 | | ng/µl | |


**Figure 1:** The result of the primary amplification of *OsRAC1* from varied rice cultivars.

Samples:

1. Bagendit RAC
2. Inpari RAC
3. IR 36 RAC
4. IR 34 RAC


**Figure 2:** The amplification result of primer RP1 on DNA template from 4 rice cultivars.

Samples:

1. Bagendit RP1
2. Inpari RP1
3. IR 36 RP1
4. IR 34 RP1


**Figure 3:** The position of gen was spotted at chromosome 3, mainly on 130432 bp /locus_tag="OSJNBa0003G23.9"/note="putative stress-inducible protein".

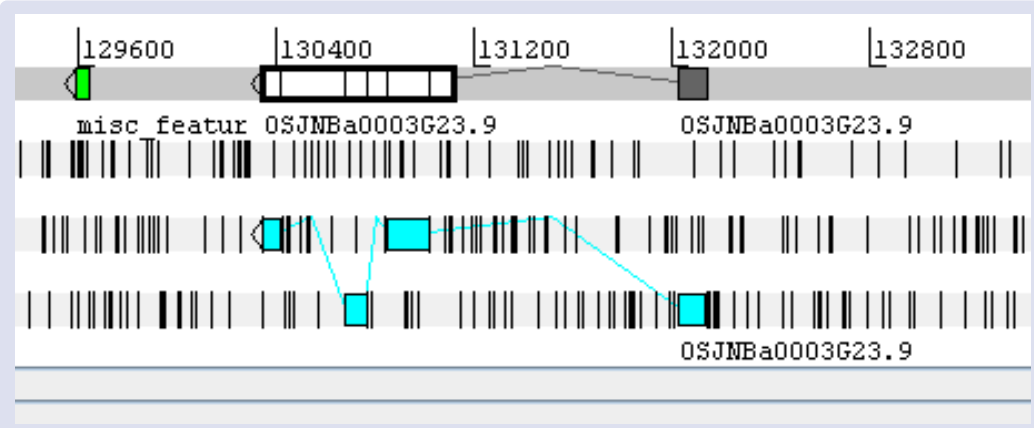


**Figure 4:** Position of gene in insert.

GAGGGAATATCCTGGAGGGATTATAATTCATCTCCTTAT-TATTTAGCACTTTTTTTTATCTACAGAGTTGAAAGGACATG-GCATCACAATTACTTTCATCTTTCATGCTCAGCCTCAGAC-GTCTAATTGGCTAGTATACTAGCTTGTTAGGTGCAAAGT-CAAGCAAGAGAATTTCAACTGTGGCGGTGTTTAATTCTCTT-TAAACTTCTAAAAATTCCGTCACATCAAATGTTTAAATGTG-GACAAAAAAAAAATAATTGCACAGTTTGTATGTAAATT-GCGAAACGAATCTTGTGAGTCTAATTACGCCATGATTT-GACAATGTTGTGCTACAGTAAACATGTGCTAATGATGTATTA-ATTAGATTTAATAGATTCATCTCGCAGTTTATATGTGGAATC-TATATATTTTGTTATTAATCTATATTTAATACTTAATACTTTT-GTCCGTGTATGTAAAAAAATTTTGACCAAACAACTGAACAC-GGCCTGTATATGTAACCAAAGAAAGATCAAAGGAGAGGAGG-TAGCTACTCCTACAAAGAAAGAAGAAACGATGAGATTTGATT-GCCTACTACTCCAGCTAGCTAGTATACAGTACTCATACGT-GTCTATCCATATTCCTGCTCCCAATGCAAAAATGCAATGGC-GAGATTGCAAGGTTGTGTGGGTGGTGGACCCTGGATCGAT-CACCTCCATTCTTTCTCCGCATCTCGCCACAGTACGCTTC-GCTGCTCTCCCGCCTATATATACCACCTCCTCCTCGATCAT-CAGTTCATCAGCAACCAAAGCAAGAAGCAATTCTTGAGCT-CAATCAGCAACAACATCTATCTCCTTCTTGCTTAGTTA-AGTCCTTCGCCCTCCCAAGAAGAAGAAGATGTCTTGCTGC-GGCGGCAACTGCGGCTGCGGCAGCGGCTGCCAGTGCGGC-GGCGGCTGCGGCGGGTAATTAACTAACTAACTAACCAACT-GCTAATTAATTCACTCAAGAAACCATTGTGACACGCACA-GATCGATAACTGATCAATATTAACATTAATATGCATGGATG-CAGATGCAAGATGTTCCCTGATGTGGAGGCCACAGCCAC-CACCAAGACCTTCGTCCTCGCTGCTCCATCCAACAAGGCG-TAAGTTTCATCATTAATGCTAATTAATTGATCTGTCATTCCT-GTGTGTGTTCATCATTAGTGTTAATTAATTGATTGATCTGC-CATTCCGAAACAAAATTGTGCTGGATTACAGGAGCTCTG-GAGGAATGGAGATGGCGGTGGAGAGCGGCGAGAACGGCG-GCTGCGGCTGCAACACCTGCAAGTGTGGCACCAGCTGCAGC-GGCTGCTCCTGCTGCTCCTGCAACTGAATCTATCGTCGTC-GTCGCCCGGCTGCATGAGGATTTATCGTATGGATGCTGC-TACTGTCGATCAGAGCTTTGATCGAGGCCTTAATTTGCTTG-CATTAGTACCCAGCTTATATGTAGGCAGGCCTTGCCTTTT-GCTCTGACGCCTAAATAAAACCGTCGTCGTCGTTGTCAGT-GTGTGCGTGTGTCGATCAATGTTGGATGGATCCCTAGC-TAGCTTGGATGGATCATCTATCATCATGGTGATCTCATCAT-GTACTCCTGCTCCATCTCCTCATGCGTGCCAGGCTTCTTA-ATATAATCTACCTCTGCTTTCATCCCATTTCTCTTACTGCT-TACTGCCCTTGTTTAATTTCCCTTTGGTTCTAATTACCAC-GAGGAAATTGTTGTTTTTTCACTAATGAAAACTCTGCAAAGT-TAAACTGACCACAAACTAGTTAACACAGAGACTGCATCAGCT-GBased on *in silico* analysis using the NCBI on MTs sequence in *Oryza sativa* was 1862 bp.[11] The sequence was then confirmed with the MTs database from UniProt. Here is the arrangement of amino acids in the FASTA format of MTs protein.

>tr|A0A0D3FH51|A0A0D3FH51_9ORYZ Uncharacterized protein OS=Oryzabarthii

MSCSCGSSCGCGSNCTCGKMYPDLEEKSSSAQATVVLGVAPE-KAHFEAAAESGETAHGCGCGSSCKCNPCNC

The result of MTs gene sequencing in a paddy plant based on the expressed protein was 216 bp in length.

>reverse translation of sample sequence to a 216 base sequence of most likely codons.

ATGAGCTGCAGCTGCGGCAGCAGCTGCGGCTGCG-GCAGCAACTGCACCTGCGGCAAAATGTATCCGGATCTG-GAAGAAAAAAGCAGCAGCGCGCAGGCGACCGTGGT-GCTGGGCGTGGCGCCGGAAAAAGCGCATTTTGAAGCGGC-GGCGGAAAGCGGCGAAACCGCGCATGGCTGCGGCTGCG-GCAGCAGCTGCAAATGCAACCCGTGCAACTGC

The notation of the MTs gene in the paddy plant from the sequencing process was locus_tag="OSJNBa0003G23.9" similar to stress-inducible protein STI GB: CAA56165 GI:872116 [Glycine max]. codon_start=1. Product="putative stress-inducible protein. protein_id="AAK00971.1. translation="MKKCLEVLIPVTFRKELTYH-IWNSAIVTYTPVVFTTAIQLDPTDATLHSNRSFCYLKSGEAREA LVDAKTCIGLKPDWPKGYYRKGAALMSLKEYKEACDAFMDGV KLDPASGEMHEAFWEAAAALKKKHLAGKTVSSFD"

## DISCUSSION

*OsRac1* gene from rice was used to regulate MTs expression in rice plant. The gene may be a contributing factor to MTs gene expression down-regulation through elicitor, pathogen, and NADPH oxidation.[9]

The amplification process of *OsRac1* gene was done to show that the higher gene amplified, the lower MTs gene expressed. This study used IR Bagendit, Inpari, IR 36, and IR 34. However, the low expression showed in this procedure indicated that MTs gene was well-expressed in those cultivars.

Based on the amplification result using primer RP1 showed two MTs gene targets in all samples. IR-Bagendit sample had more MTs gene compared to the other sample. Meanwhile, the lowest amplification primer RP1 of the MTs gene was in Umbul sample. The level of MTs gene in IR-Bagendit rice showed the high MTs protein expression. A research study found that among some varieties, like black glutinous rice, red glutinous rice, red ride, chiherang, serang, umbuk, ciliwung, IR Bagendit, IR 64, and umbul rice, the highest MTs was shown in IR Bagendit rice.[3,4]

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MTs gene was in third chromosome of *Oryza sativa.* The MTs gene in *Oryza sativa* of which functioned as stress-inducible protein during drought and during cadmium, copper, zinc, and nickel contamination. The MTs content in rice plants can be used to reduce metal toxic on rats.[3,5] A research conducted by de Francisco *et al.,* (2016) reported that MTs family from *Tetrahymena* represents a group of protein structure which was uniquely conserved. There were varied level differences on Cd and CuMT subfamily characters, such as cysteine pattern, modular structure, codon usage for glutamine, and gene expression under metal exposure. Gene analogous through evolution was the same with a general genetical mechanism to form a new MTs gene that obtains isoform and able to improve its varied function.[12] Research by Liu *et al*., 2014 characterized the MTs gene in *Oxya chinensis* by looking at expression and role during metal pressure using a specific primer.[13] MTs gene was expressed as a primary controller on transcription level. The expression of Cd resistant cell was enhanced by MTs protein acceptance through MTs gene amplification 5' end on MTs-I and MTs-II gene contained TATA box (main element promoter) and some cis-got a role in responding the element (promoter-proximal elements). Cis- had a role to respond to the elements including a heavy metal-responsive element (MRE).

MTs gene was spotted on the third chromosome as the induced-protein by considering environment stress such as metal pollution. It was in line with the research found that the expression of the MTs encoding gene was improved during heavy metal induction such as Cu, Zn, Ni, Cd. MTs are a good heavy metal isolating ligand in plants. An experiment using Scrobicularia plant with heavy metal exposure such as Zn, Cd, Pb, and Cu for 15 days showed the improvement of MTs concentration, especially from Zn exposure.[14] MTs synthesis was the result of physiology change from metal exposure,[15,16] one of the metals is lead (Pb).[17]

## CONCLUSION

1. RP 1 and RP2 as MTs-like gene varied plants strong amplification in Bagendit cultivars seen from the thickness of DNA band.
2. The osRAC 1 gene that functions as a regulator of MTs gene expression has the same amplification in all types of rice agendit, Inpari, IR 36, IR 34.
3. Based on the sequences from NCBI, the metallothionein gene is located on chromosome 3 Oryza sativa which functions to inducible stress protein exposure to metal cadmium, copper, zinc, and nickel.

## ACKNOWLEDGMENTS

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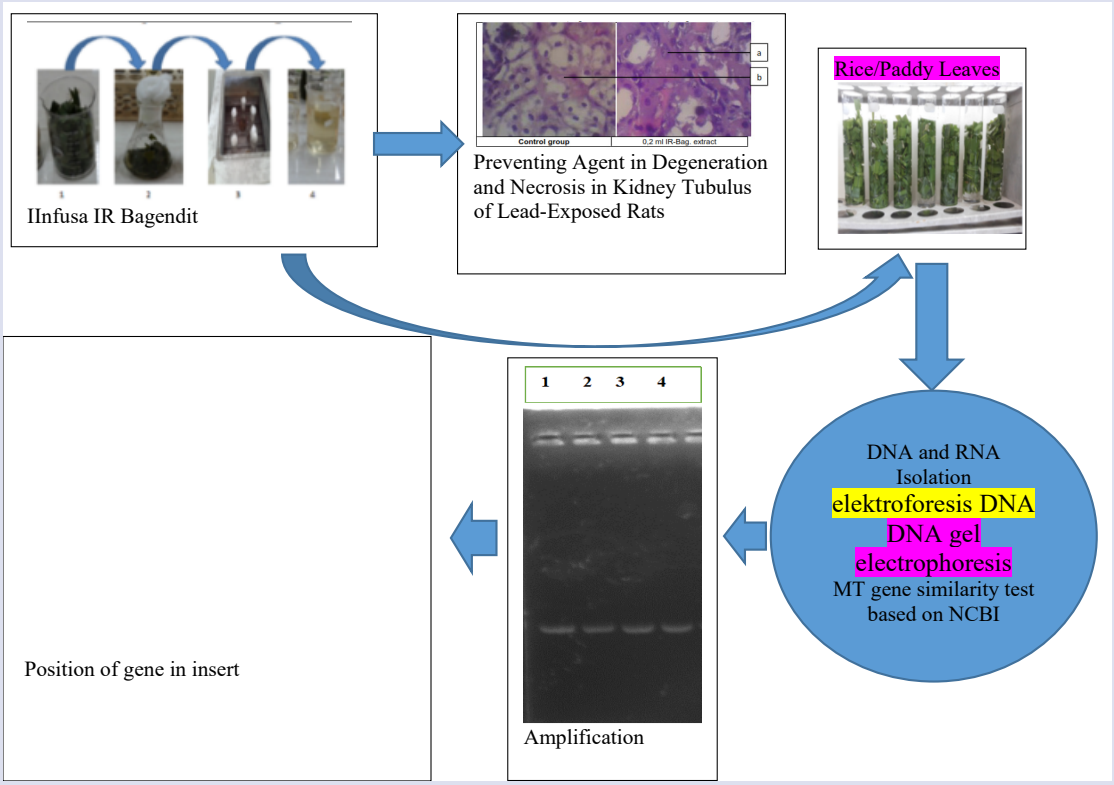
## GRAPHICAL ABSTRACT




## SUMMARY

1. RP 1 and RP2 as MTs-like gene varied plants strong amplification in Bagendit cultivars seen from the thickness of DNA band.
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