# CO-EXPRESSION OF ALDO-KETO REDUCTASE (AKR1) AND PROTEIN L-ISOASPARTYL METHYLTRANSFERASE (PIMT2) TO ENHANCE SEED VIABILITY, SEEDLING VIGOUR AND ABIOTIC STRESS TOLERANCE IN GROUNDNUT TRANSGENICS (*Arachis hypogaea* L.)

**NAMRATHA, M. R.**

**PALB 3185**

**DEPARTMENT OF CROP PHYSIOLOGY**

**UNIVERSITY OF AGRICULTURAL SCIENCES**

**BENGALURU**

**2015**

# CO-EXPRESSION OF ALDO-KETO REDUCTASE (AKR1) AND PROTEIN L-ISOASPARTYL METHYLTRANSFERASE (PIMT2) TO ENHANCE SEED VIABILITY, SEEDLING VIGOUR AND ABIOTIC STRESS TOLERANCE IN GROUNDNUT TRANSGENICS (*Arachis hypogaea* L.)

**NAMRATHA, M. R.**

**PALB 3185**

*Thesis submitted to the*

**UNIVERSITY OF AGRICULTURE SCIENCES, BENGALURU**

*In partial fulfillment of the requirements*

*For the award of the degree*

**MASTER OF SCIENCE (Agriculture)**

**in**

**CROP PHYSIOLOGY**

**BENGALURU** **JULY, 2015**

# DEPARTMENT OF CROP PHYSIOLOGY
# UNIVERSITY OF AGRICULTURAL SCIENCES
# BENGALURU- 560 065

## CERTIFICATE

This is to certify that the thesis entitled **"CO-EXPRESSION OF ALDO-KETO REDUCTASE (AKR1) AND PROTEIN L-ISOASPARTYL METHYLTRANSFERASE (PIMT2) TO ENHANCE SEED VIABILITY, SEEDLING VIGOR AND ABIOTIC STRESS TOLERANCE IN GROUNDNUT TRANSGENICS (*Arachis hypogaea* L.)"** submitted in partial fulfillment of the requirement for the degree of **MASTER OF SCIENCE (Agriculture)** in **CROP PHYSIOLOGY** to the University of Agricultural Sciences, Bengaluru, is a record of research work carried out by **Ms. NAMRATHA, M. R., ID No. PALB 3185,** during the period of her study in this University under my guidance and supervision and no part of the thesis has been submitted for the award of any other degree, diploma, associateship, fellowship or any other similar titles.

Bengaluru
July, 2015

**(B. MOHAN RAJU)**
Major Advisor

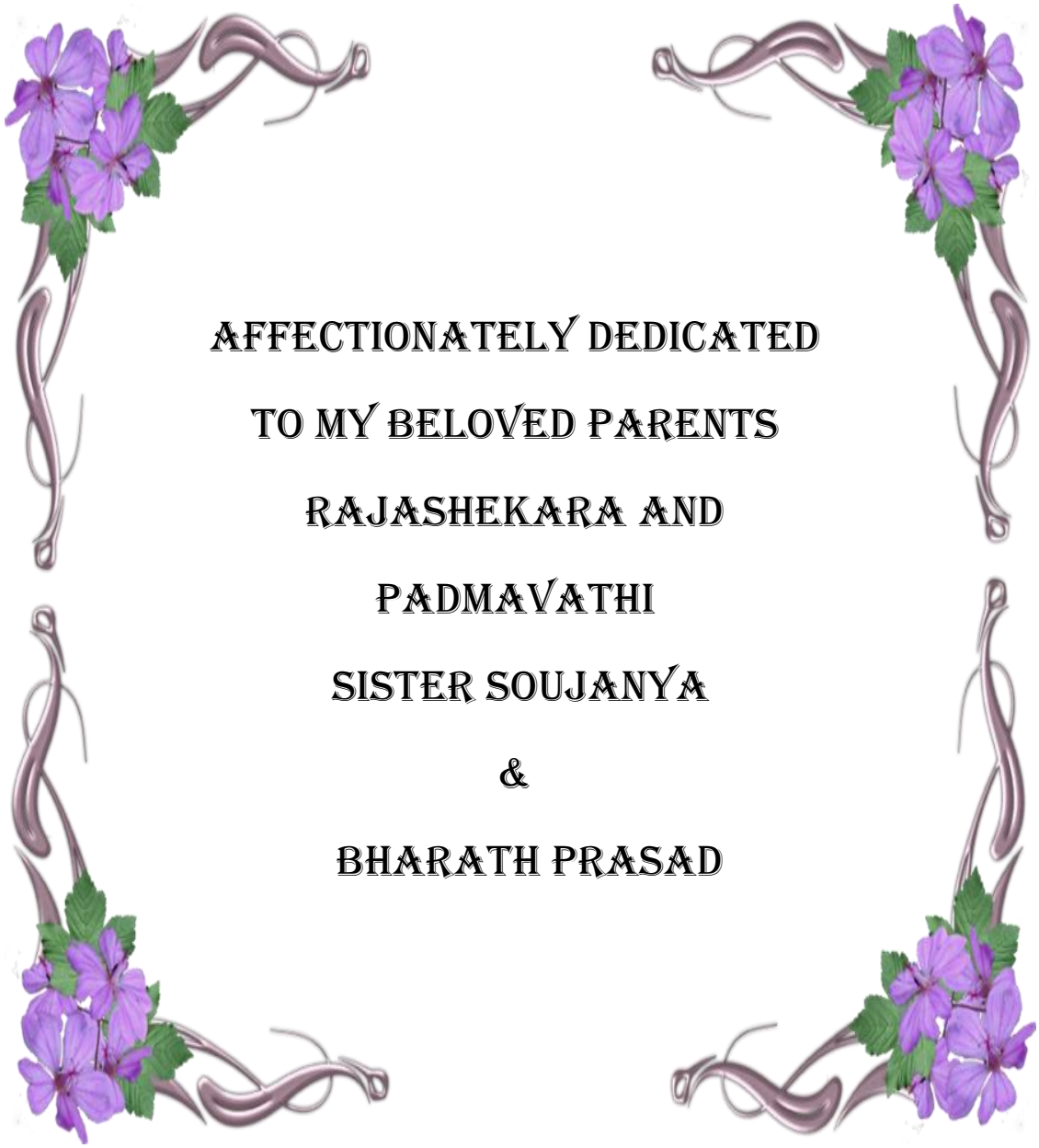
**APPROVED BY:**

**Chairman: _____**
**(B. MOHAN RAJU)**

**Members: 1. _____**
**(M. UDAYAKUMAR)**

**2. _____**
**(M. S. SHESHSHAYEE)**

**3. _____**
**(K. V. RAVISHANKAR)**



AFFECTIONATELY DEDICATED

TO MY BELOVED PARENTS

RAJASHEKARA AND

PADMAVATHI

SISTER SOUJANYA

&

BHARATH PRASAD

# *Acknowledgement*

*With regardful memories . . . . . .*

*There are many people who made this journey easier with words of encouragement and made my life more intellectually satisfying by offering different options to look to expand my dreams, theories and ideas.*

*It is my privilege to express my sense of reverence, gratitude and thanks to Dr.* ***B. MOHAN RAJU****, Professor, Department of Crop Physiology, UAS, Bengaluru and Chairman of the Advisory Committee for his able guidance, valuable suggestions, and ceaseless help. I just find it difficult to forget the ease with which he could induce the confidence and optimism in me without which this work would have been far from reality.*

*A special word of thanks to Professor* ***M. Udayakumar****. He challenged me to set my benchmark even higher and look for the solution to problems rather than focus on the problem. I appreciate all his contribution of valuable time, ideas, and finding my MSc experience productive and stimulating. I also owe my heartful thanks to the members of my Advisory Committee,* ***Dr. M. S. Sheshshayee,*** *Professor, Department of Crop Physiology, UAS, GVKV, Bengaluru and* ***Dr. K.V. Ravishankar****, Prinicipal Scientist, IIHR, Bengaluru for their valuable and constructive suggestions, helpful comments, encouragements and evaluation of the manuscript.*

*I place on record my deep sense of gratitude and heartfelt respect and thank to* ***Dr. T. G. Prasad****, for this subtle guidance, support sand continued calm endures. On this occasion I also whole heartedly thank and express my gratitude towards* ***Dr. P. Venkataravana****, Professor and groundnut breeder, ARS, Chintamani and* ***Dr.Manoj Mazee****, NIPGR, New Delhi for making the seed material and the gene available for my research.*

*I avail this opportunity to express my sincere thanks to my beloved seniors Rajashekar Reddy, Ramu, Babitha, Amaranath reddy, Swetha, Pavitra, Sheela, Mahesh salimat, Parvathi, Jalendra, Mahesh patil, Salishibhushan, Sindhu and all my friends for their constant help and encouragement during my research program. I wish to convey my heartfelt thanks to all the teaching and non-teaching staff of Department of Crop Physiology for their timely help and cooperation during my study.*

*On a personal note, I would like to express my gratitude and respect to my father Late.Rajashekar, mother Padmavathi, sister Soujanya, uncle Krishna swamy, aunt Radhika and beloved bharath for their abundant love, affection and care. I record my respectful indebtedness and gratitude to my best friends Shama, Priyanka, Jayashree, Shruthi and pruthvi for their wishes, encouragement, unquantified love and affection. It was been a worthy privilege for me to have such wonderful friends, thanks for being there for me whenever I was need. Thanks buddies.*

*Finally, I frankly admit that it is not possible to remember all the faces that stood behind the façade at this juncture and omission of any name shall not be taken as lack of gratitude.*

*Bengaluru* *Namratha. M. R.*

*July, 2015*

# CO-EXPRESSION OF ALDO-KETO REDUCTASE (AKR1) AND PROTEIN L-ISOASPARTYL METHYLTRANSFERASE (PIMT2) TO ENHANCE SEED VIABILITY, SEEDLING VIGOR AND ABIOTIC STRESS TOLERANCE IN GROUNDNUT TRANSGENICS (*Arachis hypogaea* L.)

NAMRATHA, M. R.

## THESIS ABSTRACT

Groundnut seeds deteriorate rapidly during storage due to accumulation of cytotoxic compounds like Reactive Oxygen Species, Reactive Carbonyl Compounds and also due to formation of abnormal iso-aspartyl residues which cause age related protein damage. Therefore, detoxification of cytotoxic compounds and performing protein repair mechanisms has lot of relevance in improving seed viability and vigor. Plants have developed many defense mechanisms to detoxify cytotoxic compounds and one among them is Aldo-keto Reductases (AKR1) which scavenges cytotoxic compounds and prevents oxidative stress damage. Similarly, Protein L-isoaspartyl Methyltransferase (PIMT2) converts abnormal isoaspartyl residue into normal asparate residue thereby maintains overall protein integrity. In this study, genetic variability for seed viability, seedling vigor and accumulation of cytotoxic compounds was examined in selected groundnut genotypes upon aging treatment. Accordingly, some of the genotypes namely, KCG6 and ICGV9114 were found to be susceptible to aging treatment, showing reduced seed viability, poor germination with higher accumulation of cytotoxic compounds compared to tolerant genotypes like SB3 and SB15. Further, to study the relevance of AKR1 and PIMT2, modified *in planta* transformation technique was adopted to develop groundnut transgenics and promising transformants were identified based on physiological and molecular screens. These transgenic lines showed higher seed viability, enhanced germination and vigor with low level of cytotoxic compounds upon ageing and showed tolerance to salinity induced stress compared to wild type. Further, transgenics showed higher expression of AKR1 and PIMT2. Taken together, the experimental results signify the relevance of AKR1 and PIMT2 in regulating seed viability, seedling vigor and stress tolerance.

July, 2015 Signature of Major Advisor

Department of crop physiology
UAS, GKVK, Bengaluru-65

# ಅಲ್ಡೊ – ಕೀಟೊ ರಿಡಕ್ಟೇಸ್ (AKR1) ಮತ್ತು ಪ್ರೋಟೀನ್ ಎಲ್ ಐಸೊಆಸ್ಪರ್ಟೈಲ್ ಮೀಥೈಲ್ ಟ್ರಾನ್ಸ್ಫರೇಸ್ (PIMT2) ಅನುವಂಶಿಕಗಳ ಸಹ ಅಭಿವ್ಯಕ್ತಿತ ಶೇಂಗಾ ಸಸ್ಯಗಳಲ್ಲಿ ಬೀಜ ಮೊಳಕೆಯ ಪ್ರಮಾಣ ಮತ್ತು ಅಜೀವ ಒತ್ತಡಗಳ ಸಹನೆಯ ಸಾಮರ್ಥ್ಯದ ಅವಲೋಕನೆ

ನಮ್ರತಾ, ಎಂ. ಆರ್.

## ಪ್ರಬಂಧದ ಸಾರಂಶ

ಶೇಂಗಾ ಬೀಜಗಳು ದಾಸ್ತಾನುವಿನ ಸಮಯದಲ್ಲಿ ತಮ್ಮ ಚೈತನ್ಯವನ್ನು ಕ್ರಮೇಣವಾಗಿ ಕಳೆದುಕೊಳ್ಳುತ್ತವೆ. ಇದಕ್ಕೆ ಮುಖ್ಯ ಕಾರಣ, ಪ್ರತಿಕ್ರಿಯಾತ್ಮಕ ಆಮ್ಲಜನಕ ಸಂಯುಕ್ತಗಳು ಮತ್ತು ಪ್ರತಿಕ್ರಿಯಾತ್ಮಕ ಇಂಗಾಲದ ಸಂಯುಕ್ತಗಲಾಗಿರುತ್ತವೆ ಹಾಗು ಸ್ವಾಭಾವಿಕ ವಯಸ್ಸಿಗೆ ಸಂಬಂಧಿಸಿದ ಸಸಾರಜನಕ ಹಾನಿಯಿಂದಾಗಿ ರಚಿಸಿದ ಅಸಹಜ ಐಸೊಆಸ್ಪರ್ಟೈಲ್ ಗಳು ಕಾರಣವಾಗಿರುತ್ತದೆ. ಆದ್ದರಿಂದ, ಹಾನಿಕಾರಕ ರಾಸಾಯನಿಕ ವಸ್ತುಗಳ ನಿರ್ವಹಣೆ ಮತ್ತು ಸಸಾರಜನಿಕ ದುರಸ್ತಿ ಯಾಂತ್ರಿಕ ವ್ಯವಸ್ಥೆಯಿಂದ ಬೀಜ ಚಟುವಟಿಕೆ ಮತ್ತು ಕಾರ್ಯಸಾಧ್ಯತೆಯನ್ನು ಹೆಚ್ಚಿಸಬಹುದಾಗಿದೆ. ಸಸ್ಯಗಳು ಈ ಹಾನಿಕಾರಕ ರಾಸಾಯನಿಕ ವಸ್ತುಗಳನ್ನು ಕ್ರಿಯಾಶೂನ್ಯಗೊಳಿಸಲು ಅನೇಕ ರಕ್ಷಣಾ ಕಾರ್ಯವಿಧಾನಗಳನ್ನು ಅಳವಡಿಸಿಕೊಂಡಿವೆ. ಅವುಗಳಲ್ಲಿ ಒಂದು ಅಲ್ಡೊ-ಕೀಟೊ-ರಿಡಕ್ಟೇಸ್ (AKR1) ಆಗಿದೆ, ಇದು ಉತ್ಕರ್ಷಕಾಶೀಲಹಾನಿಗಳನ್ನು ತಡೆಯುತ್ತದೆ. ಹಾಗೆಯೇ, ಪ್ರೋಟೀನ್ ಎಲ್ ಐಸೊಆಸ್ಪರ್ಟೈಲ್ ಮೀಥೈಲ್ ಟ್ರಾನ್ಸ್ಫರೇಸ್ (PIMT2) ಅಸಹಜ ಐಸೊಆಸ್ಪರ್ಟೈಲ್ ಸಂಯುಕ್ತವನ್ನು ಸಾಮಾನ್ಯ ಆಸ್ಪರ್ಟೇಟ್ ಗಳಾಗಿ ಪರಿವರ್ತಿಸುತ್ತದೆ, ತನ್ಮೂಲಕ ಒಟ್ಟಾರೆ ಸಸಾರಜನಕ ಸಮಗ್ರತೆಯನ್ನು ಸಹ ನಿಭಾಯಿಸುತ್ತದೆ. ಪ್ರಸ್ತುತ ಅಧ್ಯಯನದಲ್ಲಿ, ಆನುವಂಶಿಕ ಏರಿಳಿತ ಶೇಂಗಾ ಬೀಜಗಳ ಮೊಳಕೆ, ಕ್ರಿಯಾಶೀಲತೆ ಮತ್ತು ಹಾನಿಕಾರಕ ರಾಸಾಯನಿಕ ಸಂಯುಕ್ತಗಳು ಕ್ಕೋಢೀಕರಣವನ್ನು ಆಯ್ದ ಶೇಂಗಾ ತಳಿಗಳಲ್ಲಿ ಪರಿಶೀಲಿಸಲಾಗಿದೆ. ಅಂತೆಯೇ, ಅವುಗಳಲ್ಲಿ KCG6 ಮತ್ತು ICGV9114 ಎಂಬ ತಳಿಗಳ ಪ್ರಬೇಧಗಳು ವಯಸ್ಸಿನ ಉಪಚಾರ ಹಾಗು ಬೀಜದ ಕಾರ್ಯಸಾಧ್ಯತೆಗಳಿಗೆ ಒಳಪಡಿಸಿದಾಗ ನಿರೋಧಕ ತಳಿಗಳಾದ SB3 ಮತ್ತು SB15 ತಳಿಗಳಿಗಿಂತ ಹೆಚ್ಚಾಗಿ ಹಾನಿಗೊಳಗಾಗತ್ತವೆ. ಈ ಅಧ್ಯಯನವನ್ನು AKR ಮತ್ತು PIMT ವಂಶವಾಹಿನಿಯ ಪ್ರಾಭಲ್ಯತೆಯನ್ನು ಅರಿಯಲು, ಸಸ್ಯ ರೂಪಾಂತರ ತಂತ್ರಗಳನ್ನು ಬಳಸಲಾಗಿತ್ತು. ಶಾರೀರಿಕ ಮತ್ತು ಆಣ್ವಿಕ ಸಂಶೋಧನೆಗಳ ಆಧಾರದ ಮೇಲೆ ಇವುಗಳನ್ನು ಕುಲಾಂತರಿಸಿ ಭರವಸೆಯ ರೂಪಾಂತರಗಳೆಂದು ಪರಿಗಣಿಸಲಾಗಿದೆ. ಈ ರೂಪಾಂತರಗಳಲ್ಲಿ ಹಾನಿಕಾರಕ ರಾಸಾಯನಿಕ ವಸ್ತುಗಳು ಕಡಿಮೆ ಮಟ್ಟದಲ್ಲಿದ್ದು, ಅತೀ ಹೆಚ್ಚಿನ ಬೀಜ ಕಾರ್ಯಸಾಧ್ಯತೆ, ಮೊಳಕೆಯೊಡೆವಿಕೆ ಪ್ರಮಾಣ ಹೆಚ್ಚಾಗಿರುವುದು ಕಂಡುಬಂದಿದೆ ಹಾಗೂ ಈ ರೂಪಾಂತರಿ ಸಸ್ಯಗಳು ಲವಣಾಂಶ ಪ್ರೇರಿತ ಒತ್ತಡವನ್ನು ತಡೆದಿವೆ. ಈ ಎಲ್ಲ ಪ್ರಾಯೋಗಿಕ ಫಲಿತಾಂಶಗಳು, ಶೇಂಗಾ ಬೀಜ ಕಾರ್ಯಸಾಧ್ಯತೆ ಮತ್ತು ಒತ್ತಡ ಸೈರಣೆ ನಿಯಂತ್ರಿಸುವ ಶಕ್ತಿಯಲ್ಲಿ AKR1 ಮತ್ತು PIMT2 ಅನುವಂಶಿಗಳು ಹಾನಿಕಾರಕ ರಾಸಾಯನಿಕ ವಸ್ತುಗಳ ನಿರ್ವಹಣೆಯಲ್ಲಿ ಮುಖ್ಯ ಪಾತ್ರವಹಿಸಿರುವುದು ಕಂಡುಬಂದಿದೆ.

ಜುಲೈ, ೨೦೧೫ ಪ್ರಮುಖ ಸಲಹೆಗಾರರ ಸಹಿ

ಬೆಳೆ ಶರೀರ ಕ್ರಿಯಾಶಾಸ್ತ್ರ
ಯು ಎ ಎಸ್, ಜಿ ಕೆ ವಿ ಕೆ, ಬೆಂಗಳೂರು- ೬೫

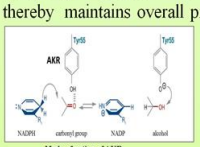
# Co-expression of AKR1 and PIMT2 to enhance seed viability in groundnut (*Arachis hypogaea L.*) transgenics.

**NAMRATHA., M.R.**

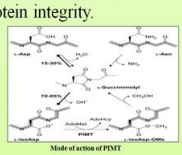
**Department of Crop Physiology, University of Agricultural Sciences, GKVK, Bangalore-560065**

## INTRODUCTION:

- Groundnut (*Arachis hypogea L.*) is one of the important oilseed crops which provides high quality edible oil. During storage, groundnut seeds deteriorate rapidly mainly due to accumulation of cytotoxic compounds like Reactive Oxygen species and Reactive Carbonyl compounds leading to generation of Advanced Glycation End products (AGE´s) and Advance Lipoxidation End products (ALE´s). Seed deterioration is also due to protein inactivation by spontaneous formation of abnormal iso-aspartyl residue, which also results in loss of seed viability and vigor. Plants have developed many mechanisms to detoxify these cytotoxic compounds. Aldo Keto Reductase (AKR) scavenge RCCs and prevent oxidative stress damage, while Protein L-isoapartyl methyltransferase (PIMT) converts abnormal isoaspartyl residues into normal asparate residues, thereby maintains overall protein integrity.




Mode of action of AKR




Mode of action of PIMT

## OBJECTIVES:

- **Assessing the role of cytotoxic compounds for seed viability in groundnut genotypes.**
- **Development of AKR1 and PIMT2 double gene construct.**
- **Development and characterization of Groundnut transgenics overexpressing double gene cassette.**

## MATERIAL AND METHODS:

Seeds samples of 20 groundnut genotypes of same age were subjected to accelerated aging ( 6 days)

↓ (45°C,100%RH)

Seeds were tested for seed viability, germination, level of cytotoxic compounds (MDA, MG etc.) following standard protocol and identified short and long viable genotypes

↓

Relevance of AKR1 and PIMT2 for improving seed viability in short seed viable genotype through transgenic approach.

↓

Transgenic development through modified *inplanta* technique using marker free binary vector harboring AKR1( under RBCs) and PIMT2 ( napin promoter).

↓

$T_o$ as well as $T_1$ transformants were screened at 30 ppm glyphosate.

↓ tolerant plants were transplanted to pots and maintained in green house.

**Molecular characterization**

Genomic DNA extraction from putative T1 transformants and PCR confirmation was performed using respective primer combination for both the genes.

## RESULTS:

### Assessing role of cytotoxic compounds for seed viability in Groundnut genotypes.

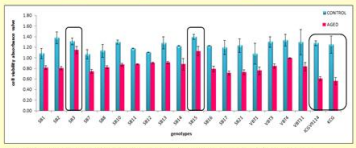



Fig1:Assessment of genotypic variability for seed viability in 20 Groundnut genotypes upon ageing treatment

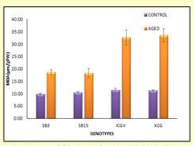



Fig 2: Quantification of Melondialdehyde upon aging.

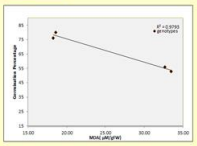


Fig 3: Relationship between germination and melondialdehyde content.

Genetic variability for seed viability was observed based on germination, TTC test ( Fig 1), MDA levels ( Fig 2) and other cytotoxic compounds and contrasting genotypes differing in seed viability identified (SB3 and SB15; longer seed viability) and ( ICGV9114 and KCG 6: short seed viability).

### Relevance of AKR1 and PIMT2 in improving seed viability


Vector map harboring AKR1 and PIMT2

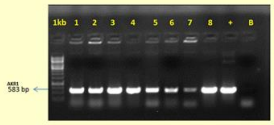



Plate1:PCR confirmation of AKR1 in binary vector cassette

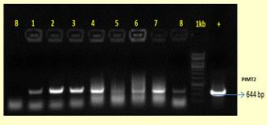



Plate2: PCR confirmation of PIMT2 in binary vector.

A double gene construct harboring AKR1 and PIMT2 genes was developed through Agrobacterium mediated *inplanta* transformation technique. KCG6 a low seed viable groundnut genotype was used for transgenic development

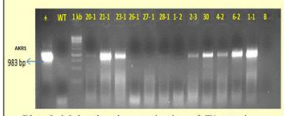
### Screening $T_1$ Putative AKR1-PIMT2 Transformants in 30ppm of glyphosate.

The $T_0$ seggregants were selected based on 30ppm of glyphosate screening.


Putative T1 Transformants identified by screening on 30 ppm of glyphosate.

Transplantation of glyphosate tolerant seedling and grown under control environment condition.

### PCR Confirmation AKR1 and PIMT2 in T1 Putative Transformants and Transformation efficiency



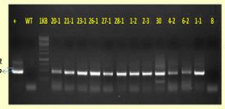




Plate 3: Molecular characterization of T1 putative groundnut transformants for AKR1 and PIMT2 genes.

| *inplanta* transformation | 700 seeds |
|---|---|
| No. of $T_0$ seeds screened | 500 seeds |
| No of $T_0$ plants survived | 31 plants |
| No. of $T_1$ seeds screened | 950 seeds |
| No. of $T_1$ plants survived | 110 Plants |
| No of PCR positive T1 plants | 35plants |
| Transformation efficiency | 4 % |

Table1:Transformation efficiency of putative transformants.

Further characterization of promising transformants for seed viability is in progress

## DISCUSSION:

- Aging leads to macromolecular damage arising from lipid peroxidation, accumulation of cytotoxic compounds and also from non-enzymatic modification of protein. In the present study, genotypic variability for seed viability was observed across 20 groundnut genotypes through accelerated aging technique. Accordingly, some of the groundnut genotypes namely, ICGV9114 and KCG6 showed early loss of seed viability with high level of cytotoxic compounds. In this scenario, it is crucial to develop crop improvement strategies. We adopted modified *inplanta* transformation to develop transgenics. Molecular characterization of glyphosate tolerant putative T1 transformants suggest that, stable transformation can be obtained by this method. Functional validation of T2 seeds will provide scientific evidences about the relevance of AKR and PIMT in improving seed viability.

## SUMMARY:

- We standardized the accelerated aging technique to study seed viability in groundnut genotypes. ICGV9114 and KCG6 genotypes showed low seed viability and accumulate high cytotoxic compounds.
- To improve seed viability, transgenic co-expressing AKR1 and PIMT2 were developed.
- By physiological screening and molecular characterization, putative transformants identified.
- Functional validation of these transformants for seed viability improvement is under progress.

## ADVISORY COMMITTEE:

**Dr. B. MOHAN RAJU ( Chair person)**
**Dr. M. UDAYAKUMAR ( Member)**
**Dr. M. S. SHESHSHAYEE ( Member )**
**Dr. K.V RAVISHANKAR ( Member)**

**Acknowledgement: I would like to thank Ramu., S.V. and Rajashekar Reddy for their techinal guidances's and support.**

**CONTENTS**

## LIST OF TABLES

## LIST OF FIGURES

## LIST OF PLATES

| | | |
|---|---|---|
| 13 | *Agrobacterium* colony PCR confirmation of NAPIN:PIMT2:TRBCS gene cassette in pCAMBIA2301 binary vector using gene specific primers | 37 – 38 |
| 14 | *Agrobacterium* colony PCR confirmation of AKR1 and PIMT2 (PRBCS:AKR1:TRBCS::NAPIN::PIMT2:TRBCS) in pmf12GW binary vector | 37 - 38 |
| 15 | 3-ketoglycosidase test for *Agrobacterium* cells. | 37 - 38 |
| 16 | Standardization of glyphosate lethal concentration for groundnut screening | 39 – 40 |
| 17 | Standardization of kanamycin lethal concentration for groundnut screening | 39 - 40 |
| 18 | Screening of putative $T_0$ putative groundnut transformants against 30 ppm of glyphosate | 39 – 40 |
| 19 | Screening of putative $T_0$ Groundnut transformants overexpressing PIMT2 against 400 ppm of kanamycin | 39 – 40 |
| 20 | Photograph showing $T_0$ transformants grown and maintained under greenhouse conditions | 39 – 40 |
| 21 | Screening of putative $T_1$ putative groundnut transformants against 30 ppm glyphosate and 400 ppm of kanamycin | 39 – 40 |
| 22 | Identification of glyphosate tolerant putative $T_1$ groundnut transformants co-expressing AKR1 and PIMT2 | 39 – 40 |
| 23 | Molecular characterization of glyphosate tolerant putative $T_1$ transgenic lines co-expressing AKR1 and PIMT2 through PCR | 39 – 40 |
| 24 | Identification of glyphosate tolerant putative $T_1$ groundnut transformants overexpressing AKR1 | 39 – 40 |
| 25 | Molecular confirmation of glyphosate tolerant $T_1$ putative transgenic lines overexpressing AKR1 | 39 – 40 |

# I INTRODUCTION

Groundnut (*Arachis hypogaea* L.) is one of the world's most important leguminous crops and an economically important oilseed crop which provides high quality edible oil (48-50 %) and easily digestible protein (26-28 %). Groundnut ranks 3rd among the oilseed crops and 13th among the food crops of the world. It is grown nearly in area of 23.95 million ha worldwide with a total production of 36.45 million tons and an average yield of 1529 kg/ha (FAOSTAT, 2011). Two-third of world's peanut production come from India and China, where one third of global peanut production is used for the food and remaining is used for the oil production. Groundnut is mainly cultivated in tropical and sub-tropical region of developing countries (Feng *et al*., 2012).

There are several constraints to the productivity of peanut crop that results in greater economic losses annually. Although some of the wild relatives of *A. hypogaea* have been identified as a resistance source to several biotic and abiotic stresses, the success of transferring the desirable traits to cultivated varieties have been limited due to reproduction barrier and frequent failure in the interspecific crosses. Therefore, application of biotechnological methods for the improvement of important crop plant of semi-arid tropics has been shown to hold greater potential. Genetic transformation approach allows for introducing novel genes that are not accessible normally by conventional crossbreeding, i.e., limited by sexual incompatibility. Although several reports on efficient regeneration from diverse explants of peanut have been published, not much success with genetic transformation of *Arachis* species has been achieved. This is due to lack of efficient protocol to generate whole plants through *in vitro* regeneration of adventitious shoot buds from the transformed tissue. This has prompted to adopt non-tissue culture based approaches that do not depend on the regeneration of adventitious shoot buds for generating transgenic plants of groundnut.

Groundnut being one of the important oilseed crops rapidly deteriorates during storage which causes complex physiological changes leading to loss of viability/or reduction in seed vigour (Chakraborty *et al.,* 1991). Seed deterioration during storage accounts for 100 % loss in seed vigour (Bewley and Black, 1994) which is accompanied by loss in membrane integrity, chromosomal damage and loss in enzyme activity (Priestly *et al.,* 1986). During seed deterioration, there is a production of reactive oxygen species which are highly toxic and cause damage to proteins, lipids, carbohydrates and DNA resulting in cell death. Generally ROS production is counteracted by enzymatic antioxidant systems that include a variety of scavengers which are the first line of defense such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX). In one of the studies, it has been demonstrated that transgenic tobacco over expressing Cu/Zn-SOD showed tolerances to multiple stresses (Badawi *et al.,* 2004). Similarly, overexpression of APX in *Nicotiana tabacum* chloroplast enhanced plant tolerance to salt and water deficit and transgenic plants overexpressing OsAPXa /OsAPXb exhibited increased salt tolerance (Lu *et al.,* 2007).

However, in spite of efficient scavenging system, still some amount ROS escapes leading to the production of reactive carbonyl compounds via lipid peroxidation and also

through glycation. These RCC's such as methyl glyoxal, 3-deoxyglucosone, melondialdehyde are highly cytotoxic leading to production of Amadori/Maillard products (Mano *et al.,* 2012). Many of these reactions are found to be active in dry system and play an important role in deterioration of seeds. Loss in germination occurs at the time when Amodari and maillard products increase in the soybean axes. These results suggest the role of non-enzymatic glycation in seed deterioration (Scott *et al.,* 1991). Similarly, AGE product (Advanced Glycation End-products) and ALEs (Advanced Lipoxidation End-products) from glycation and lipid degradation respectively resulting in early loss of seed viability and vigour has been demonstrated in barley seeds upon ageing treatment (Jun *et al.,* 2009).

Plants possess many defense mechanisms to scavenge RCCs compounds. One such enzyme is Aldo-Keto Reductases (AKR) is known to play a pivotal role in detoxifying RCCs in plants. The AKR superfamily is a large enzyme group of NADP-dependent oxidoreductases with numerous roles in metabolism of steroids, sugars and other carbonyls and is widely distributed in animals, plants and bacteria and has various physiological functions (Simpson *et al.,* 2009). AKR gene expression also increases due to various other abiotic stresses, including high temperature, drought, heavy metals, and UV-B in digitalis and alfalfa (Hegedus *et al*., 2004; Hideg *et al.,* 2003; Oberschall *et al.,* 2000). They have also frequently been implicated in the metabolism of exogenous and endogenous toxicants including those stimulated by stress. The aldo-keto reductase (AKR) super family comprises a range of generally monomeric 34 to 37 kDa proteins that are NADPH-dependent and share a common (α/β) 8-barrel structural motif. AKRs typically catalyze the reduction of a number of carbonyl compounds to the corresponding alcohols or the reverse oxidation reactions. It has been argued that, in some cases, their ability to accept multiple substrates is linked to a function in alleviating stress, in that, some are capable of detoxifying toxic carbonyls including both endogenous stress-induced aldehydes such as 4-hydroxy-2-nonenal (HNE), malondialdehyde and methyl glyoxal derived from lipid or sugar oxidation and xenobiotic and this role has been suggested for an aldose reductase from *medicago* (Oberschall *et al.,* 2000). The role of AKR's in detoxifying the RCCs has also been reported by Nisarga (2013) in rice and tobacco where the level of cytotoxic compounds was found to be low in AKR transgenic compared to wild type rice and tobacco.

Deterioration of seeds during storage also involves change in seed components which includes loss in protein integrity. Deteriorated seeds showed a reduced capacity to synthesize a new protein which has been demonstrated in rye embryo protein (Sen *et al.,* 1977). Non–enzymatic deamidation of peptides and proteins represents an important degradation reaction occurring in vitro in the course of isolation or during storage and in vivo during development or ageing of cells. Proteins and enzymes are described as factors that may determine seed longevity. Heat stress transcription factor-over accumulating seeds of transgenic *Arabidopsis* displayed enhanced accumulation of Heat Stress Protein and improved tolerance to ageing (Priet-Dapena *et al.,* 2006).

The accumulation of spontaneously damaged proteins in seeds due to ageing/ stress/ storage often adversely affects the seed vigour and viability (Oge *et al.,* 2008). Over time and under stress conditions, proteins may undergo a variety of modifications and/ or damage. Among the known covalent damage that can occur spontaneously to

protein, the formation of iso-aspartyl linkage through deamination of asparagine and isomerization of aspartate may be one of the most rapid forms under physiological pH and temperature (Clarke *et al.,* 2004). The formation of abnormal non-functional iso-aspartyl residue can result in structural heterogeneity or loss of protein function resulting in loss of seed viability (Verma *et al.,* 2013). Therefore, in the context, Protein repair mechanisms appear to play a key role in long-term survival of seeds in the dry state.

One enzyme that repairs such damaged protein is Protein L-isoaspartyl Methyltransferase (PIMT) is a widely distributed protein-repair enzyme that catalyzes the conversion of abnormal L-isoaspartyl residues synthesized spontaneously in proteins to normal aspartyl residues (Verma *et al.,* 2013). In plants, PIMT activity is primarily localized in seed tissue during late stages of embryogenesis during and after maturation desiccation, suggesting the role in rescuing the functionally active conformation of the orthodox seeds (Thapar *et al.,* 2001).

Protein repair appears to play a key role in the long-term survival of seeds in the dry state. PIMT (Protein L-isoaspartyl Methyltransferase), which limits and repairs age-damaged aspartyl and asparaginyl residues in proteins, has been associated with greater seed longevity because, it is highly accumulated in sacred lotus seeds (*Nelumbo nucifera*), one of the world's longest living seeds (1,300 years; Shen-Miller, 2002). Overexpression of PIMT1 in Arabidopsis enhanced both seed longevity and germination vigour, whereas reduced PIMT1 expression led to increased sensitivity to ageing treatments and loss of seed vigour under stressful germination conditions (Ogé *et al*., 2008). PIMT exhibited a decreased activity in naturally aged barley (*Hordeum vulgare*) seeds (Mudgett *et al.,* 1997). In addition, PIMT enzyme activity and accumulation was found to high in seedling which was induced by water and salt stress (Mudgett and Clarke, 1994; Thapar *et al.,* 2001; Xu *et al.,* 2004).

With this background, it is hypothesized that co-expression of Aldo-Keto Reductase (AKR1) and Protein L-isoaspartyl Methyltransferase (PIMT2) wound detoxify reactive carbonyl compounds and maintain over all protein integrity respectively, increases seed viability, seedling vigour and abiotic stress tolerances in transgenic groundnut. This hypothesis was tested by developing transgenic in groundnut overexpressing AKR1 and PIMT2 and co-expressing them together. Therefore, objectives set for this investigation are as follows:

OBJECTIVES:

1. Assessing the genetic variability for seed viability across the selected groundnut genotypes.
2. Construction of AKR1 and PIMT2 single gene cassette and AKR1+PIMT2 co-expressing gene cassettes.
3. Development of groundnut transgenics overexpressing these genes constructs.
4. Characterization of transgenic for-
    a) Seed viability and seedling vigour.
    b) Abiotic stress responses.

# II REVIEW OF LITERATURE

Seeds are uniquely equipped to survive as viable regenerative organisms until the time and place are right for the beginning of a new generation. They serve as vector of plant propagation. Seeds of many plant species are extremely tolerant to harsh environmental condition. However, like any other form of life, they cannot retain their viability indefinitely and eventually deteriorate and die (Rajjou *et al.,* 2008).

Seed viability and vigour are the two main important seed qualities for the success of plant propagation and food production and also a major challenge for the conservation of plant biodiversity. High vigour seed lots can maintain high germination rates and vigorous seedling development under adverse environmental condition. However, seeds gradually lose vigour and viability during storage and become more sensitive to stresses and lose germination ability (McDonald, 1999; Harington, 1972, Debeaujon *et al.,* 2007).

Germination is resumption growth of embryo which is a miniature plant contained within a seed. It is a complex process during which, the imbibed mature seed must quickly shift from a maturation to a germination driven program of development and prepare for seedling growth and hence, seed germination and establishment of seedling are considered as crucial phases in the life of a plant. Because of its high vulnerability to injury, disease and water/environmental stresses, seed germination is considered to be the most critical phase in the plant's life cycle. The seed germination process incorporates events starting with the uptake of water by mature dry seed and terminating with the protrusion of radical through the seed envelopes (Donohue *et al.,* 2005; Come *et al.,* 1989).

Seed performance can be measured by many parameters including emergence, plant growth, vegetative and reproductive development and yield. During photosynthesis, seeds accumulate reserves in multiple forms including carbohydrates, lipids and proteins. Mobilization of these reserves following germination is essential for the embryo to complete seedling establishment and also signals to start new cycle (Rao *et al.,* 1998).

## 2.1 Factors affecting seed deterioration process and lipid peroxidation

Seed deterioration is an irreversible, degenerative natural process that occurs during the ageing process or under adverse environmental conditions. The deterioration of seeds during dry storage is a complex phenomenon involving changes in many seed components. Several physiological and biochemical processes change as seed deteriorates. Along with a loss of protein integrity, oxidative damage to DNA, formation of sugar-protein adducts or iso-aspartyl residues, cell membrane degradation, fatty acid oxidation, can also occur. Further, there is encountered decline in the activity of numerous enzymes and decrease in the level of antioxidants (Scott *et al.,* 1991). In contrast, Heat Shock Proteins (HSPs) and other stress related proteins wound increase effect of ageing on seed vigour. Therefore, seed deterioration is expressed as loss of quality, viability and vigour which normally occurs during the process of ageing.

In fact, deterioration of seed during storage can be considered as ageing event (in contrast to a senescence event), which has been profound influence on agriculture and conservation (Osborne *et al.,* 1980). Species with shorter viability period requires at most care as they pose the threat of losing the material provided, if they are not stored

and conserved properly or sow them immediately to continue their generation. Like environmental condition, genetic factor also have substantial impact on seed viability and vigour. Genetic loci associated with seed vigour have been identified in rice, barley, wheat, oilseed rape, maize Arabidopsis thaliana using artificial ageing tests (Miura *et al.,* 2002; Contreras *et al.,* 2005). The storage conditions reported to alter the seed viability period and accordingly, at different storage conditions, rye seeds deteriorate faster than wheat (Robertson *et al.,* 1943). In addition, most temperate crops deteriorate less rapidly in cool dry conditions than hot and humid environment to indicate the storage conditions playing an important role in determining seed viability. In general, the cultivated species have shorter viability period than the perennials or wild species. The rate of deterioration is influenced by seed moisture content and the temperature during storage and alteration in any of the above conditions lead to rapid deterioration of seeds (Ellis *et al.,* 1992).

Most often, seed longevity is linked to seed water content which is regulated by relative humidity of the storage conditions (Walters, 1998). An exponential increase in longevity with decreasing water content for all grains types until seeds are dried to storable level noticed (Ellis *et al.,* 1992). The mode by which the seed moisture regulates ageing process is highly dependent on cell composition and structure. Even a small difference in water content larger has larger effects on seed deterioration rate. However with molecular constituents such as sugar and LEA proteins, the moisture content effects on seed viability can be minimized (Walters and Koster, 2007). Therefore, relative longevity of the species is determined by general response to storage conditions.

Lipid peroxidation on other hand seems to be the most important reason for early loss of seed viability (Sattler *et al.,* 2004). Apart from high temperature and relative humidity which control seed moisture content, several other environmental stresses directly or indirectly hasten up the lipid peroxidation process leading to early loss of seed viability (Priestley, 1986; Wilson and McDonald, 1986; McDonald, 1999) these authors have reported that, continual accumulation of free fatty acids culminated in a reduction of cellular pH and was detrimental to normal cellular metabolism. Furthermore, it denatures enzymes resulting in their loss of activity.

As seed quality declined, there is a concurrent increase in the levels of free fatty acids. Elevated levels of free fatty acids which are toxic to cells were not found in healthy seed tissues while, it was indeed found in deteriorated seeds (Trawatha *et al.,* 1995). During ageing peroxidative changes may be the major cause of seed deterioration (Goel *et al.,* 2003). The longevity of seeds has been shown to vary across species in relation to a number of factors such as taxonomy, seed structure and the climate of the geographical origin. Across 276 species stored under seed bank conditions, Walters *et al.,* (2005) predicted that, the time required for viability to fall to 50 % ranged from 7 years in Bromussitchensis to 633 years in *Trifolium campestre*. Analysis of these results shows that, seed life-span is also determined at the plant family level as well. According to Robert *et al.,* (2009) endospermic seed species from cool wet environments are predicted to be shorter lived in dry storage compared to non-endospermic seed species from hot dry environments.

The seed ability to withstand dehydration is an essential prerequisite for enhancing longevity and vigour which ensures successful germination of seeds (Ellis *et al.,* 1992). Seed germination also controlled by multiple factors such as biotic and abiotic

stresses, mechanical damage as well as physiological conditions. Seed moisture content (MC), temperature and oxygen concentration are the major factors which contribute for germination in the storage environment conditions. Most often, seed longevity is linked to seed water content which is regulated by relative humidity of the storage conditions (Walters *et al.,* 1998). The longevity of seeds increases in a quantifiable way with decrease in moisture content. An exponential increase in longevity with decreasing water content for all grain types until seeds are dried to storable level was noticed (Ellis *et al.*, 1992). The mode by which the seed moisture regulates ageing process is highly dependent on cell composition and structure. Even a small difference in water content has larger effects on seed deterioration rate (Walters and Koster, 2007). However, with molecular constituents such as sugar and LEA proteins, the moisture content effects on seed viability can be minimized and it is likely that, these molecules behave differently at different relative humidity conditions (Berjak *et al.,* 1997). Ellis *et al.,* (1992) have found a 12-fold increase in longevity in rape (*Brassica napus*) seeds when stored at 3 % MC instead of 5 %. Groundnut seeds can be safely dried to very low levels of MC of 2–6 % equivalent to a water potential of −350 Mpa (Roberts and Ellis, 1989). Several arguments demonstrate that nature has evolved complex systems in order to optimize seed life span.

Seeds possess a wide range of systems (protection, detoxification, repair) allowing them to survive in the dry state and to preserve a high germination ability (Fig. 1). Therefore, the seed system provides an appropriate model to study longevity and ageing.

**2.2 Role of reactive oxygen species on seed deterioration process**

The reactive oxygen species (ROS) comprising $O_2$-, $H_2O_2$, Triplet state of Chl (3Chl*), Superoxide ($O_2$−), Singlet oxygen ($^1O_2$*), Hydroxyl radical (•OH, $HO_2$, ROOH, ROO-, and RO-, are highly reactive and toxic and cause damage to proteins, lipids, carbohydrates, DNA which ultimately results in cell death. In plants, the most important of these are driven by or associated with light dependent events. Photosynthetic cells are prone to oxidative stress because they contain an array of photosensitizing pigments and they both produce and consume oxygen. The photosynthetic electron transport system is the major source of active oxygen species in plant tissues has the potential to generate singlet oxygen ($^1O_2$) and superoxide radical ($O^-$). Generation of reactive oxygen species (ROS) is a characteristic feature for hypoxia and especially for re-oxygenation. Hydrogen peroxide ($H_2O_2$) and superoxide ($O^-$) are produced in a number of cellular reactions including the iron-catalyzed Fenton reaction and by various enzymes such as lipoxygenases, peroxidases, NADPH oxidase and xanthine oxidase. The main cellular components susceptible to damage by free radicals are lipids (peroxidation of unsaturated fatty acids in membranes), proteins (denaturation), carbohydrates and nucleic acids. Consequences of hypoxia induced oxidative stress depend on tissue and/or species (i.e. their tolerance to anoxia), on membrane properties, on endogenous antioxidant content and on the ability to induce the response in the antioxidant system. Effective utilization of energy resources (starch, sugars) and the switch to anaerobic metabolism and the preservation of the redox status of the cell are vital for survival.

ROS are key components of the seed signal transduction pathways and it has been hypothesized that, seed germination can be completed only when the ROS content is maintained low under a critical threshold (‘oxidative window for germination’) that

allows the induction of ROS mediated signaling mechanisms (Bailly *et al.*, 2011). There are several examples of the ROS involvement as signaling molecules in seed physiology such as the alleviation of sunflower seed dormancy associated with non-enzymatic ROS production. These ROS will lead to the formation of reactive carbonyl compounds (RCCs) (Bailly *et al.*, 1998).

The destruction of the lipid membranes can amplify cellular toxicity by formation of lipid hydroperoxides and their toxic aldehyde degradation products. In higher plants, the key role of ROS during abiotic and biotic stresses has been well documented. Lipid peroxidation compounds have been found to cause DNA damage. In dry seeds, ROS and non-enzymatic reactions such as lipid peroxidation cause lose in seed viability. The latter derives from lipid degradation in glyoxysomes and purine catabolism in peroxisomes, respiratory reactions in mitochondria, photosynthesis in chloroplasts etc. In addition, specific enzymes can produce ROS such as NADPH oxidase on the plasma membrane and peroxidases at the cell wall (Walters and Koster, 2007).

Scavenging of ROS is also known to be undertaken by glutathione, ascorbic acid (vitamin C) and peroxiredoxins which is called as first line of defense. However, if the metabolism of these systems presents major changes during seed desiccation and dry storage, their role in protection against seed ageing in the dry state remains to be ascertained. Stress-induced ROS accumulation is counteracted by enzymatic antioxidant systems that include a variety of scavengers, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX), glutathione-S-transferase (GST) which are the second line of defense and non-enzymatic low molecular metabolites, such as ASH, GSH, α-tocopherol, carotenoids and flavonoids (Mittler *et al.*, 2011). SOD has been proposed to be important in plant stress tolerance and provide the first line of defense against the toxic effects of elevated levels of ROS. Overexpression of a MnSOD in transgenic *Arabidopsis* plants also showed increased salt tolerance (Wong *et al.*, 2004). Cu/Zn-SOD overexpressing transgenic tobacco plants showed multiple stress tolerance (Badawi *et al.*, 2004).

Catalase (CAT) is tetramericheme containing enzymes with the potential to directly dismutate $H_2O_2$ into $H_2O$ and is indispensable for ROS detoxification during stress (Garg and Manchand, 2009). CAT is important in the removal of $H_2O_2$ generated in peroxisomes by oxidases involved in beta-oxidation of fatty acids, photorespiration and purine catabolism (Sree *et al.*, 2000). The *E. coli* CAT encoded by the katE gene overexpressed in O. sativa conferred tolerance to transgenic rice plants under salt stress (Nagamiya *et al.*, 2007). Ascorbate peroxidase (APX) is involved in scavenging of $H_2O$ in water-water and ASH-GSH cycles and utilizes ASH as the electron donor. The APX family consists of at least five different isoforms including thylakoid (tAPX) and glyoxisome membrane forms (GmAPX), as well as chloroplast stromal soluble form (sAPX), cytosolic form (cAPX) (Noctor *et al.*, 1998). Overexpression of APX in *Nicotiana tobacum* chloroplasts enhanced plant tolerance to salt and water deficit (Badawi *et al.*, 2004b). Transgenic *Arabidopsis* plants over-expressing OsAPXa or OsAPXb exhibited increased salt tolerance was shown (Lu *et al.*, 2007b). Similarly the presence of the mitochondrial succinic-semialdehyde dehydrogenase (SSADH) in the Arabidopsis proteome from dry mature and germinating seeds involved in protecting

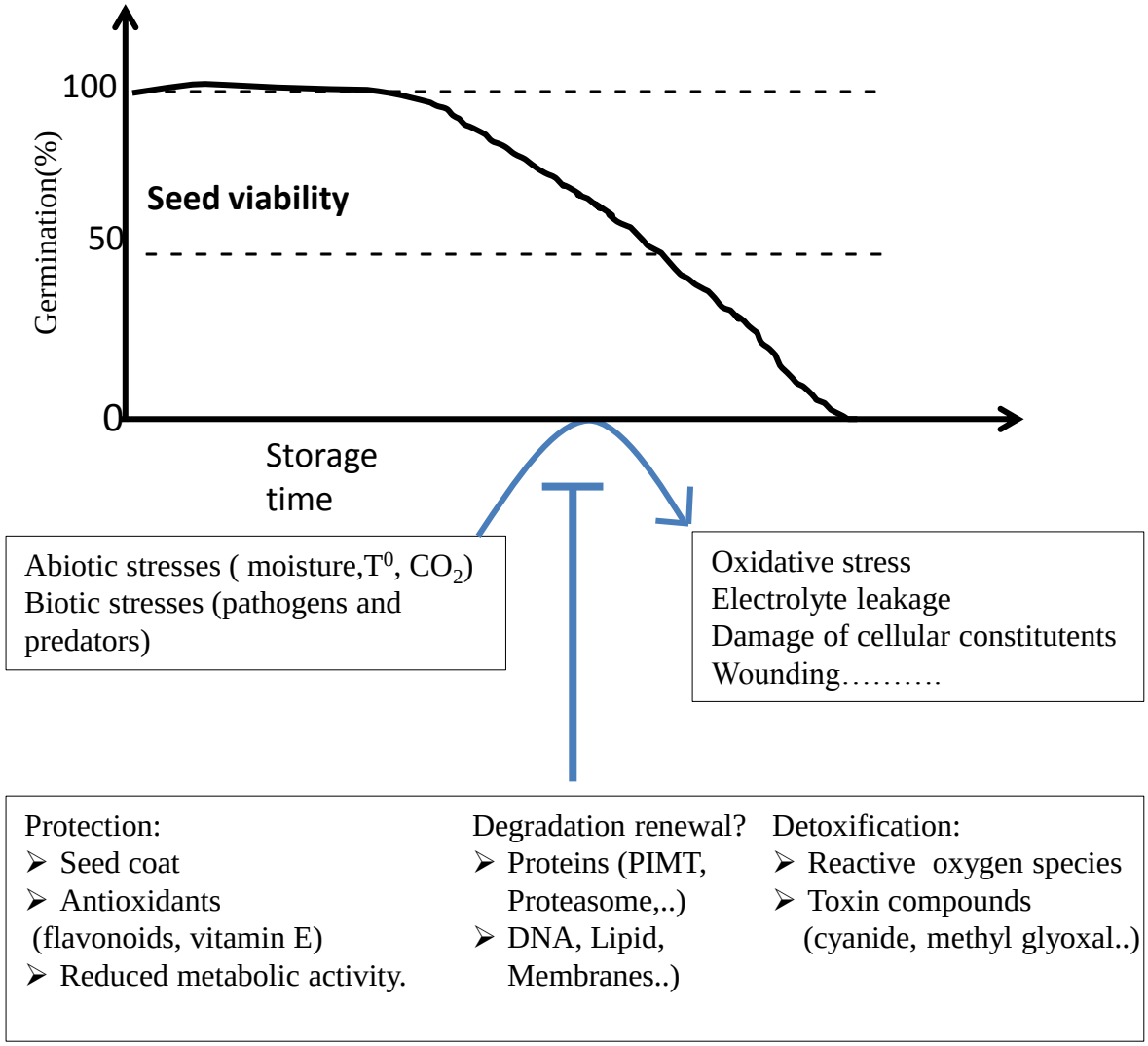



**Fig. 1. Schematic presentation of the main interactive parameters determining seed viability**

plant against oxidative stress and also involved in controlling seed longevity and germination (Rajjou *et al.,* 2008).

### 2.3 Reactive carbonyl compounds (RCCs) and their role in regulating seed deterioration process

Various environmental stresses lead to the production of reactive oxygen species (ROS) and reactive carbonyl compounds (RCCs) (Foyer *et al.,* 2003). The ROS produced oxidize various biomolecules to inactivate them in vitro. Seeds during storage are like any other dry desiccating tissue and hence expected to produce significant amount of reactive oxygen species and RCCs. Most often, the ROS found on the membranes are primarily scavenged by abundant antioxidants and non-enzymatic compounds such as caroteinoids, tocopherol, ascorbate and the reduced form of glutathione (Asada, 2006). In addition, scavenging enzymes such as super oxide dismutase (SOD), ascorbates, and peroxidase also scavenge the reactive oxygen species (ROS) as mentioned above. However, in spite of several scavenging mechanisms, a small fraction of ROS escape from the scavenging systems will oxidize surrounding molecules (Bailly *et al.,* 2011). Membrane lipids are abundant molecules in the membrane and hence, most probable target of ROS.

The oxidation of lipids with ROS results in a production of carbonyl species with a different length of carbon chain and various extents of unsaturation and oxygenation. Carbohydrates and lipids targeted by ROS increases the amount of RCCs that are eventually involved in the formation of advanced glycation end products (AGEs) and advanced lipoxidation end products (ALEs) (Mano, 2012). These RCCs are constantly produced by the metabolism of carbohydrates, lipids and amino acids all of which are abundantly present throughout the cell. These Carbonyl compounds like most other intermediates and by-products of metabolism are electrophilic, and thus are highly reactive with different cellular constituents majority of which are nucleophiles (Zimniak *et al.,* 2011). RCCs, e.g., glyoxal, methylglyoxal, HNE, arabinose, glycoaldehyde, 3-Deoxyglucosone, 3-Deoxyfructose, Acetaldehyde are formed from carbohydrates and ascorbate enzymatically. They react non-enzymatically with protein amino groups and eventually yield AGEs, e.g., Carboxymethyllysine (CML), pentosidine, pyrraline, imidazolone, glyoxal-lysine dimer and methylglyoxal-lysine dimer. Similarly, glyoxal, malondialdehyde, hydroxynonenal and acrolein are generated by lipid peroxidation of polyunsaturated fatty acids. RCS have a relatively long half life time and therefore higher stability, in contrast to ROS. In addition, RCCs, such as glyoxal, methyl glyoxal, acrolein and glycoaldehyde are also produced during the myeloperoxidase catalyzed by metabolism of amino acids. These RCCs react with proteins and form ALEs as well as AGEs (Anderson *et al.,* 1998). The Maillard reaction, a non-enzymatic process is initiated when proteins are exposed to glucose or other carbohydrates. It generates first reversible Schiff base adducts and subsequently more stable Amadori rearrangement products. Through a series of oxidative and non-oxidative reactions, it eventually yields the irreversible advanced glycation end products (AGE) linked with amino groups, e.g., lysine residues, of several proteins (Miyata *et al.,* 2001).

Reactive carbonyls, especially α, β-unsaturated carbonyls produced through lipid peroxidation damage biomolecules such as proteins and nucleotides. Since they are highly electrophilic and react with nucleophilic molecules react on cellular and tissue

proteins to form adducts which alters the cellular responses. Oxidation of polyunsaturated fatty acids generates RCCs which includes highly reactive α, β-unsaturated alkenals. Hence, these, β-unsaturated carbonyls are highly cytotoxic which includes MG, MDA, glyoxal, and deoxyglucosone.

Methyl glyoxal is an important reactive carbonyl compound ubiquitously present in all living organisms. Methyl glyoxal (MG) is a potent protein glycating agent and a cytotoxic compound mainly produces from triose phosphates. It also forms 3-aminoacetone, which is an intermediate of threonine catabolism as well as through lipid peroxidation and most important through glycolysis. Recently, it has been shown that MG is also produced by the Calvin cycle and that sugar-derived RCCs production is inevitable during photosynthesis. MG concentration increases linearly with prolonged illumination in presence of 3-PGA. Further, more MG production is enhanced under high-$CO_2$ conditions, high temperature, high light conditions in illuminated wheat leaves (Takagi *et al.,* 2014).

### 2.4 Role of Amadori and millard products on seed deterioration process

Deterioration of seeds during storage also seems to be influenced by Amadori and the Miallard reactions. In fact, some of these reactions found to be active in dry systems and play an important role in deterioration of dried food products (Feeney *et al.,* 1982; Fourie *et al.,* 1990). The Amadori reaction involves a simple, non-enzymatic attack on amino groups by reducing sugars to form fructosyl derivatives or glycated proteins. The Maillard reaction represents subsequent complex interactions between the glycated Amadori products to form polymeric, brown colored products and hence, the term "browning reaction" (Hodge, 1993). The deterioration of seeds during storage can be considered to be an ageing event, in that, it is not a programmed developmental process. Therefore, it appears that, lot of physiological and biochemical changes occur during seed deterioration process leading to loss of early seed viability. In this context, it is imperative to investigate the physiological and biochemical changes to understand the seed deterioration process. This would not only help in identifying reasons for improving the storage life of seeds but also provide information that would enable incorporation of trait for better storability in the genetic background of high yielding variety. Amadori products accumulate in a relatively dry medium such as dry seeds in storage which contributes to the deterioration of dry seeds in storage. Attempts to measure the products of such reactions in soybean seeds have yielded evidence that such products do in fact occur during accelerated ageing (Wettlaufer and Leopold, 1991).

In many species the loss of seed vigour or viability linearly associated with the initial accumulation of Maillard products in seed axes which decrease the storage life of seed. Accumulation of Maillard products which also occurs in dry seeds shows correlation with the loss of germinability likely through chemical modification of macromolecules during storage, which gradually reduces metabolic capability, the ability of the metabolic events to limit the free radical damage and to repair the damage during germination. As a result, they lead to a decreased seed vigour during storage and eventually seed death. Non enzymatic glycosylation was also associated with DNA damage (Lee and Cerami, 1989). A wide range of seed water contents and storage temperatures was used to determine the relationship between advances in seed ageing and several possible primary biochemical deterioration processes including lipid peroxidation

and sugar hydrolysis (i.e., glucose accumulation). At low moisture contents, enzymatic reactions are expected to play little role in seed ageing (Priestley, 1986). However, even at low moisture contents (<0.08 g ±1 DW), mung bean seeds contained a considerable amount of compounds (e.g. reducing sugars and aldehydes) that were able to initiate the Maillard reactions during storage. Although the content of such compounds did not increase significantly, seed ageing during storage could be due to the slow Amadori and Maillard reactions. Some antioxidant enzymes such as glutathione reductase, ascorbate peroxidase and catalase are sensitive to Maillard reactions, which causes a decline in the antioxidant capacity and an inability to limit oxidative damage during germination resulting in impaired seed vigour and loss of viability (Murthy *et al.,* 2003). All these evidences, indicates that Amadori and Maillard reactions seem to be the possible contributors to the deteriorative sequence in seeds.

The following flow chart (Fig. 2) depicts effects of ROS and cytotoxic RCCs which are generated under stress or during seed storage/ageing (as the seeds are like a dry desiccating tissue experiencing stress during stress) leading to cell death or loss of seed viability.

### 2.5 Genetic enhancement of seed viability and seedling vigour

#### 2.5.1 Role of Aldo-Keto Reductase in detoxifying reactive carbonyl compounds

Scavenging and detoxification of ROS as well as RCCs are essential to protect the cells from the oxidative damages. Of these two compounds, RCCs are found to be more reactive and cytotoxic as they have high electrophilic and readily react with nucleophilic molecules to α, β unsaturated bond leading to the formation of Michel adduct (Mano, 2012). Therefore, it is essential to scavenge RCCs to protect the cells from the damage. Three enzymatic reactions for detoxifying RCCs and other carbonyls are known for plants. These are Aldehyde dehydrogenases (ALDH) that oxidize carbonyl group to carboxyl group; reduction of carboxyl group to an alcohol which is catalyzed by aldo-keto reductases (AKRs) and aldehyde reductases, Alkanol reductases (AER) which catalyse the reduction of c-c double bond of RCCs to form saturated carbonyl.

Higher level accumulation of MG is toxic to the cell as it inhibits cell proliferation and results in a number of adverse effects such as increasing the degradation of proteins by modifying Arg, Lys, and Cys residues, adducting with guanyl nucleotide in DNA, and inactivating antioxidant defense system. In addition to the enzymatic reactions, the conjugation of RCCs especially MG is reduced to lactate form by the action reduced glutathione (GSH) forms which is the another important critical detoxification reaction (Mano *et al.,* 2012). Glyoxalase system comprises of two enzymes, glyoxalaseI (gly I) and glyoxalase II (gly II), which catalyze the detoxification of MG. GSH is essential for detoxification of methyl glyoxyl in the glyoxalase 1 reaction. Transgenic tobacco plants overexpressing gly I and gly II genes individually or together confer their role in salt tolerance (Pearce *et al.,* 1980). There is every possibility that MG could therefore act as a signal for plants to respond to stress. Detoxification of MG occurs mainly through the glyoxalase pathway, at least during the early phase of seed germination. In addition to gly I, GSH may also be a limiting factor affecting the level of MG. The beneficial roles of carbonyl detoxifying enzymes such as ALDHs, AKRs and AERs have been shown in a number of systems. Over expression of these genes regulating the detoxifying enzymes

has been shown to improve the tolerance of transgenic plants against several environmental stresses (Mano, 2012). Of these enzymes, AKRs are known to play a pivotal role in detoxifying RCCs in plants.

Aldo–keto Reductases (AKRs) are widely distributed in nature and play numerous roles in the metabolism of steroids, sugars and other carbonyls. They have also frequently been implicated in the metabolism of exogenous and endogenous toxicants including those stimulated by stress. The Aldo–Keto Reductase (AKR) super family comprises a range of generally monomeric 34 to 37-kDa proteins that are NADPH-dependent and share a common (α/β) 8-barrel structural motif. They are widely distributed from prokaryotes to eukaryotes and typically catalyze the reduction of a number of carbonyl compounds to corresponding alcohols or the reverse oxidation reactions. It has been argued that, in some cases, their ability to accept multiple substrates is linked to a function in alleviating stress, in that, some are capable of detoxifying toxic carbonyls including both endogenous stress-induced aldehydes such as 4-hydroxy-2-nonenal (HNE), malondialdehyde and methyl glyoxal derived from lipid or sugar oxidation and xenobiotic toxicants (Oberschall *et al.,* 2000).

The majority of AKRs studied in plants so far have belonged to the AKR4 family with more belonging to the AKR4C subfamily. Several AKR4C members have been associated with tolerance to environmental stresses (Simpson *et al.,* 2009). For example, in barley embryos, synthesis of AKR4C1, the first member of the 4C subfamily was identified as a protein that conferred desiccation tolerance (Bartels *et al.,* 1991). An alternative role of AKR4C1–AKR4C4 could be the reduction of reactive electrophile species such as methyl glyoxal and HNE which are produced in cells under oxidative stress conditions. HNE and several other reactive aldehydes are generated as a result of lipid peroxidation in cells due to an increase in reactive oxygen species. In plants, reactive oxygen species are produced by the chloroplasts and under normal conditions are effectively scavenged. However, under abiotic stress conditions such as drought, their concentration can increase.

AKR has a role in scavenging Methyl glyoxal (MG) in chloroplast and cytosol; MG is also recognized as cytotoxic aldehydes. MG can react with biomolecules such as proteins and DNA and can potentially destroy their functional activity. It is produced as a non-enzymatic by-product of glycolysis and lipid peroxidation and also enzymatically either through stress-induced production from triose phosphates or from dihydroxyacetone phosphate in a reaction catalyzed by MG synthase in prokaryotes as well as in eukaryotes. Whenever there is an increase in cellular methyl glyoxal levels, there is always an oxidative damage. For example, in rice the level increases in response to drought, high salinity and cold stresses. Both HNE and methyl glyoxal can covalently modify proteins and DNA via their reactive carbonyl groups. The reduction of the reactive aldehyde groups on these molecules is an important step in clearing HNE and methyl glyoxal from stressed cells and this role has been suggested for an aldose reductase from *Medicago* (Oberschall *et al.,* 2000). Expression of this *Medicago* AKR is induced by various stresses, notably drought, heavy metals and ABA and over expression in bacteria produced a protein that could metabolize several substrates including the stress induced aldehyde HNE, although the affinity for these substrates was relatively low.

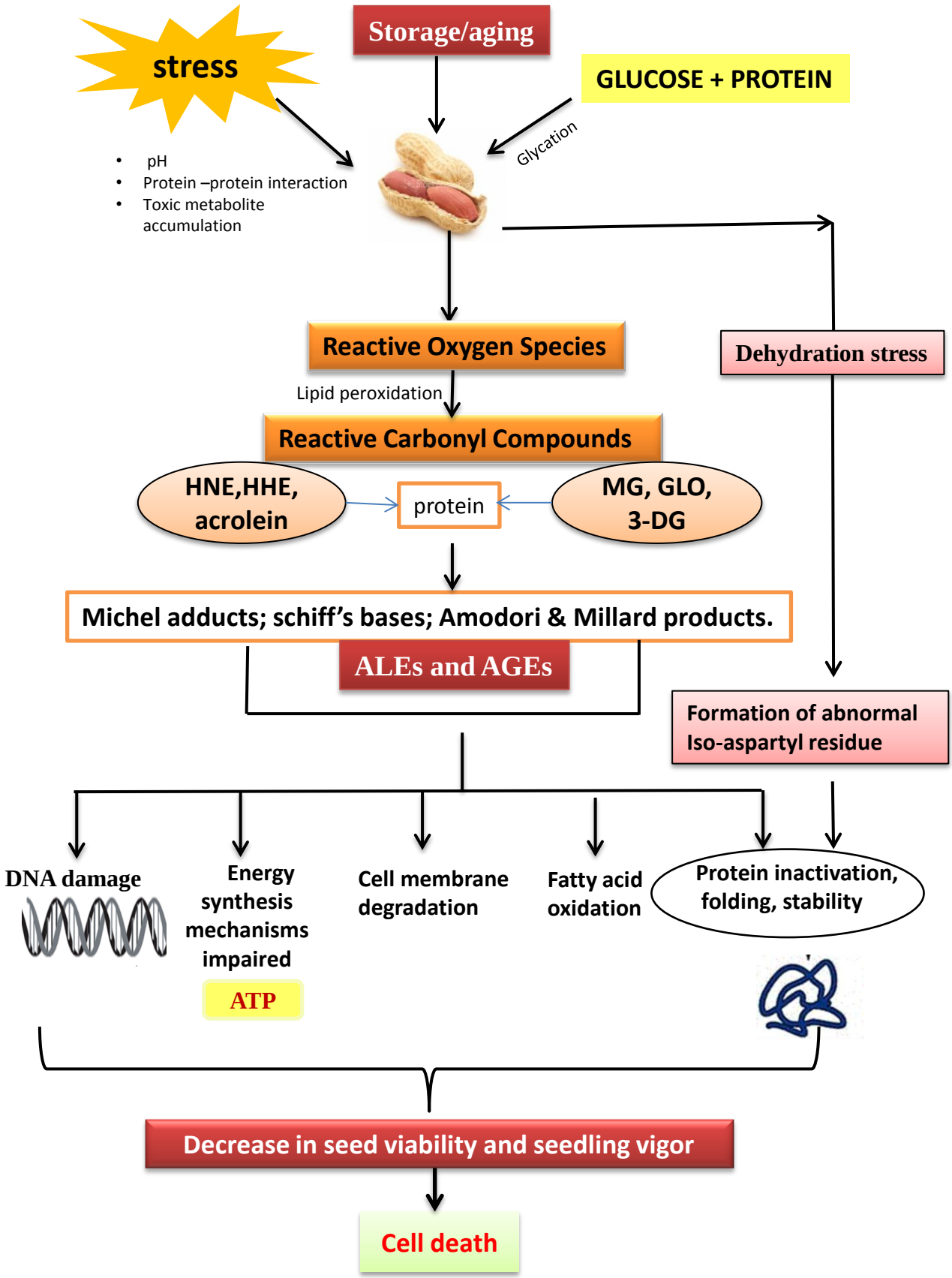



**Fig. 2: Schematic representation of ROS and RCCs production and their effects on cell viability**

Based on the chromosomal localization and on their homology with other stress-induced aldo-keto reductase (AKRs) Turoczy *et al.,* (2011) identified three rice AKR genes. The transcription level of OsAKR1 was greatly induced by abscissic acid and various stress treatments; the other two AKR genes tested were moderately stress inducible. The OsAKR1 recombinant protein exhibited a high catalytic activity to reduce toxic aldehydes including glycolysis derived methyl glyoxal (MG) and lipid peroxidation originated malondialdehyde (MDA). Presence of OsAKR1 gene in transgenic tobacco plants resulted in increased tolerance against oxidative stress generated by methyl viologen (MV) and improved resistance to high temperature. In these plants, lower levels of MDA were detected both following MV and heat treatment due to the activity of the OsAKR1 enzyme. These data therefore confirm the role of OsAKR1 in detoxifying the RCCs and help in reducing stress effects.

A flow chart depicting how AKRs detoxify the cytotoxic compounds is given below (Fig. 3).

Aldo-keto reductase (AKR) catalyzes the reduction of aldehyde to alcohol. In plants, the scavenging of α, β-unsaturated carbonyl compounds are essential for maintaining their viability. Nevertheless, knowledge of α, β-unsaturated carbonyl compound scavenging enzymes and especially of the NAD(P)H dependent pathway is very limited. The NAD(P)H-dependent reduction of the aldehyde group is known to be mediated by AKR.

### 2.5.2 DNA REPAIR

DNA gets damaged when plants get exposed to ROS is one of the primary causes of DNA decay in eukaryotes. In plants, in spite of respiration and photosynthesis, ROS is also generated due to environmental stresses. Oxidative attack on DNA generates both altered bases and damaged sugar residues that undergo fragmentation and lead to strand breaks. Hence, DNA repair in higher plants has significant role removing oxidized bases and other oxidative DNA lesions (Rolda *et al.,* 2009; Prakash *et al.,* 2005).

Plants are well equipped to cope with oxidative damage to cellular macromolecules, including DNA. Oxidative attack on DNA generates both altered bases and damaged sugar residues that undergo fragmentation and lead to strand breaks. Recent advances in the study of DNA repair in higher plants show that they use mechanisms similar to those present in other eukaryotes to remove and/or tolerate oxidized bases and other oxidative DNA lesions (Batty *et al.,* 2000) Besides direct oxidation, DNA get damaged via lipid peroxidation, caused by attack of oxygen radicals to the polyunsaturated fatty acid residues of membrane phospholipids and the major cytotoxic compounds products of lipid peroxidation. Thus, when the plants cell are exposed to such damage deteriorates the cell hasten up results in decreases the longevity of species (Britt *et al.,* 1996). Therefore, plants represent a valuable model system for the study of DNA oxidative repair. The maintenance of a functional DNA repair complex appears therefore an essential condition for long-term survival in the dry state.

### 2.5.3 Protein synthesis and repair

Seed longevity was measured as germination ability after seed dry storage at ambient conditions, which is referred to as “natural ageing”. Proteins and enzymes are

also one among the factors which determine seed longevity. Heat stress transcription factor-over accumulating seeds of transgenic Arabidopsis displayed enhanced accumulation of Heat Stress Protein and improved tolerance to ageing (Prieto-Dapena *et al.,* 2006). Seed germination has a direct relation with protein synthesis. Thus, cycloheximide, an inhibitor of protein translation, induces a complete inhibition of Arabidopsis seed germination (Rajjou *et al.,* 2004). Pillay *et al.,* 1997 have shown that, translation capacity has a direct role in seed capacity to germinate during ageing process in soybean. *De nova* protein synthesis take place in mRNA which allows the renewal of non-functional proteins altered during storage and that are essential to initiate metabolism during germination (Rajjou *et al.,* 2008). Hence, protein repair appears to play a key role in the long term survival of seeds in the dry state.

Under stress condition /ageing/ during storage several storage proteins are inactivated due to spontaneous formation of isoapartyl residue. Hence, a protein repair plays a crucial role in the maintaining long-term survival of seeds in the storage condition. Protein damage can be of two general types which includes conformational change to three-dimensional structure and covalent change to primary structure (Galletti *et al.,* 1995; Visick and Clarke, 1998). In addition, the germination process induces an increased synthesis of several enzymes involved in methionine metabolism, namely, methionine synthase, S-adenosyl methionine synthetase, and S-adenosyl homocysteine hydrolase. It has also been shown that the cellular activity in germinating seeds owing to the general importance of methionine and S-adenosyl methionine (AdoMet) in plant metabolism (Ravanel *et al.,* 1998) which indicates germinating seeds have a special requirement for methionine and/or AdoMet.

Like other organisms, plants particularly seeds suffer from spontaneous damage to their proteins due to ageing or environment stresses. One among several damaged proteins is the formation of abnormal and non-functional iso-aspartyl residue. Verma *et al.,* (2013) have reported the formation of iso-aspartyl in protein can negatively affects the functionality of proteins including catalytic proficiency of enzymes. In this context, it is worth noting that seeds contain a very active protein L-isoaspartyl O-methyltransferase (PIMT), an AdoMet-dependent enzyme playing a role in repairing age related damaged iso-aspartyl and asparaginyl residues in proteins.

#### 2.5.3.1 PIMT (Protein L-isoaspartyl Methyltransferase)

Deamidated, isomerized and racemized aspartyl and asparaginyl residue represents a significant part of the spontaneous damage to proteins that results from ageing process. The accumulation of these altered residues can lead to loss of proteins function and consequent loss of cellular function. However, almost all the cells in nature contain a methyl transferase that can recognize this damage protein. One such enzymes is PIMT which is ubiquitously present in all organisms and has a role in reverting/limits or repairs age-damaged aspartyl and asparaginyl residues in proteins (Galleti *et al.,* 1995). However, naturally aged barley seeds show decreased level of PIMT activity during storage (Mudgett *et al.,* 1997).

#### 2.5.3.2 Mode of action of PIMT

Over time / under stress conditions, protein may undergo modification or damage (Fang *et al.,* 2010). Among the known covalent damage that occur spontaneously to

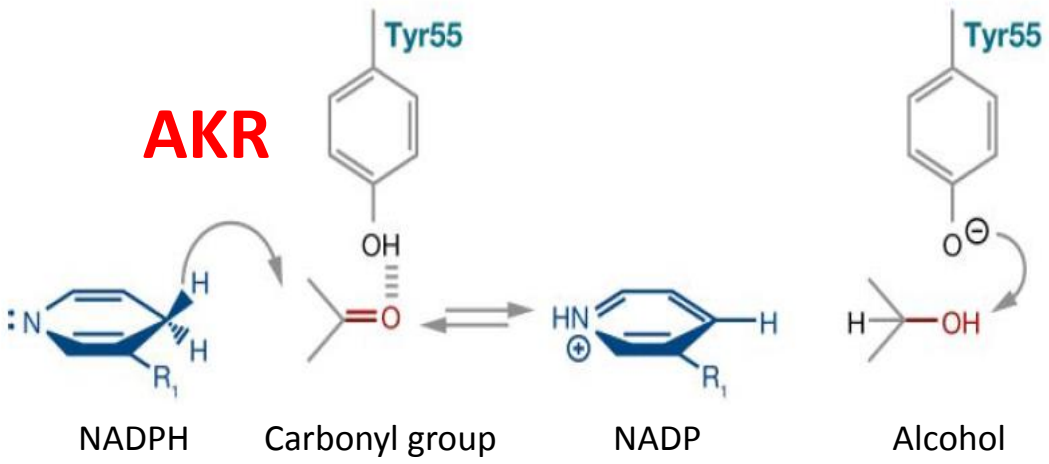



**Fig . 3: Action of AKRs in reducing aldehydes and ketones to their respective alcohols**

proteins, the formation of abnormal L-isoaspartyl residue is age related protein damage in seeds. The protein L-isoaspartyl methyl transferase (PIMT) combats protein misfolding perform protein repair mechanisms by converting abnormal L-isoaspartyl residue into normal asparate residue by first transferring the methyl group of protein from S-adenosyl-L-methionine (SAM) to form methyl ester on the α-carboxyl group of an isoaspartyl residue. The non-enzymatic reaction then results in the rapid formation of succinimide intermediates. The succinimide is unstable and is quickly converted into normal aspartate residue with a possibility of about 15-30 % upon hydrolysis. It requires several enzymatic cycles to fully repair such protein damage. Mechanism of L-isoaspartyl formation and enzymatic repair by PIMT as shown in Fig. 4.

PIMT belongs to super family SAM Adomet, which is involved in methyl group transfer. The addition to methyl group to lipid, protein and nucleic acid results in physiochemical change in these biologically active molecules further detoxification, protein sorting and repair, and nucleic acid processing are also carried out. Also, AdoMet metabolism seems somehow implicated in plant growth. The author Ravanel *et al.,* 1998; Gallardo, *et al.,* 2002 has published the data which suggests that germination process as increased in several enzymes involved in methionine metabolism/Adomet and demonstrated the requirement of Adomet in Arabidopsis seedling establishment which indicates seed contain PIMT, an Adomet dependent enzyme playing a role in limiting or repairing age damaged aspartyl and asparaginyl residues in proteins. Therefore, it necessitates a sustained production of AdoMet.

#### 2.5.3.3 The PIMT repair enzyme required in seeds to limit protein damage by IsoAsp accumulation

Iso-aspartyl is a deleterious protein alteration that occurs during ageing. The role of PIMT in repairing a damaged protein has been shown in Arabidopsis which is vital mediates for seed longevity. Impaired PIMT activity would hinder protein function, possibly resulting in poor seed performances (Chen *et al.,* 2010). Oge *et al.,* 2008 studied the level of iso-aspartyl in seed protein from Arabidopsis PIMT over expressing lines and PIMT knockout mutants accumulate high level of iso-aspartyl residues. However, PIMT repair activity this at least a substantial part of the damaged seeds proteins are repaired via PIMT-mediated process (Kim *et al.,* 1997; Visick *et al.,* 1998).

#### 2.5.3.4 PIMT is required for Seed Longevity

Seed longevity is a major trait for both ecological and agronomic aspects. PIMT has been associated with greater seed longevity because, it is highly accumulated in sacred lotus seed (*Nelumbo nucifera*), one of the world's longest living seeds (1,300 years; Shen-Miller, 2002). In plants, PIMT activity is localized in seeds which are always prone to non-enzymatic protein damage which occur during ageing of seeds. The role of these enzymes has been emphasized during the stress adaptation (Clarke, 2003). Overexpression of PIMT1 in Arabidopsis enhanced both seed longevity and vigour whereas, reduced PIMT1 expression led to increased sensitivity to ageing treatments and loss of seed vigour under stressful conditions (Ogé *et al.,* 2008). The PIMT repair pathway likely works in concert with other anti-ageing pathways to actively eliminate deleterious protein products thus enabling successful seedling establishment and strengthening plant proliferation in natural environment.

### 2.5.3.5 PIMT improves germination and seedling establishment in stress condition

Seeds assayed for change in damage and degradation of cellular protein was observed in age-induced seed deterioration. Kester *et al.,* (1997) have emphasis on change in L-isoaspartyl Methyltransferase activity during accelerated ageing in tomato seeds suggesting accelerated ageing reduced the germination percentage, increase the time of germination. During ermination L-isoaspartyl Methyltransferase activity remains constant for 48 h post-imbibition and then declined, suggesting the enzyme was active at dry state of seeds. However, it is possible, that during imbibition, PIMT target specific proteins that must be repaired for success germination (Vigeswara *et al.,* 2006; Zhu *et al.,* 2006, 2007; Dinkins *et al.,* 2008). In addition, PIMT accumulation and activity in seedling are generally induced by water and salt stress (Mudgett and Clarke, 1996; Thapar *et al.,* 2001; Xu *et al.,* 2004). Oge *et al.,* (2008) observed phenotypic variation, physiological quality of seeds, enhanced seed vigour and germination and tolerances towards NaCl stress in Arabidopsis seeds over expressing PIMT1 and suggesting the importance of protein stored in dry mature seeds and of *de nova* protein translation for the success of seed germination (Rajjou *et al.,* 2004, 2008). The enzyme activity found to increase in germinated maize subjected to osmotic stress (Thapar *et al.,* 2001).

PIMT is one such enzyme which can withstand and perform repair mechanisms even at 100 °C which indicates PIMT has a role in heat tolerance (Shen-Miller *et al.,* 1995, 2013). It has also shown that examined caPIMT2 has a role in seed vigour and longevity by performing protein repair mechanisms, predominantly in nuclear proteins upon seed-specific expression in Arabidopsis. Seeds exposed to accelerated ageing show a significant reduction in germination percentage coupled with significant increase in abnormal nonfunctional protein and a substantial decrease in PIMT activity (Verma *et al.,* 2013). These evidences clearly showed the role of PIMT in improving seed viability.

As explained above, detoxifying of Reactive Carbonyl compounds, DNA repair, the maintenance of a functional protein repair mechanism appears to be a key condition for long-term survival of seeds in the dry state and critical for seed cell maintaince under stressful condition.

In conclusion, the dry quiescent seeds when exposed at any stress condition or as ageing, decreases seed longevity and germination ability. Though seed coat, antioxidants contribute primarily towards seed longevity by maintaining metabolic activity, protection against various environment stresses and by counteracting the free radicals. When these antioxidant scavenging mechanisms get saturated, detoxification mechanisms might affect which indeed results in increasing cytotoxic compounds and substantially leads to decrease in longevity of seed and finally cause seed death. A better knowledge of DNA and protein protection and repair mechanisms seems promising to manipulate seed longevity. Due to this property, the seed system provides an appropriate model to study longevity and ageing, which is of paramount interest for human health.

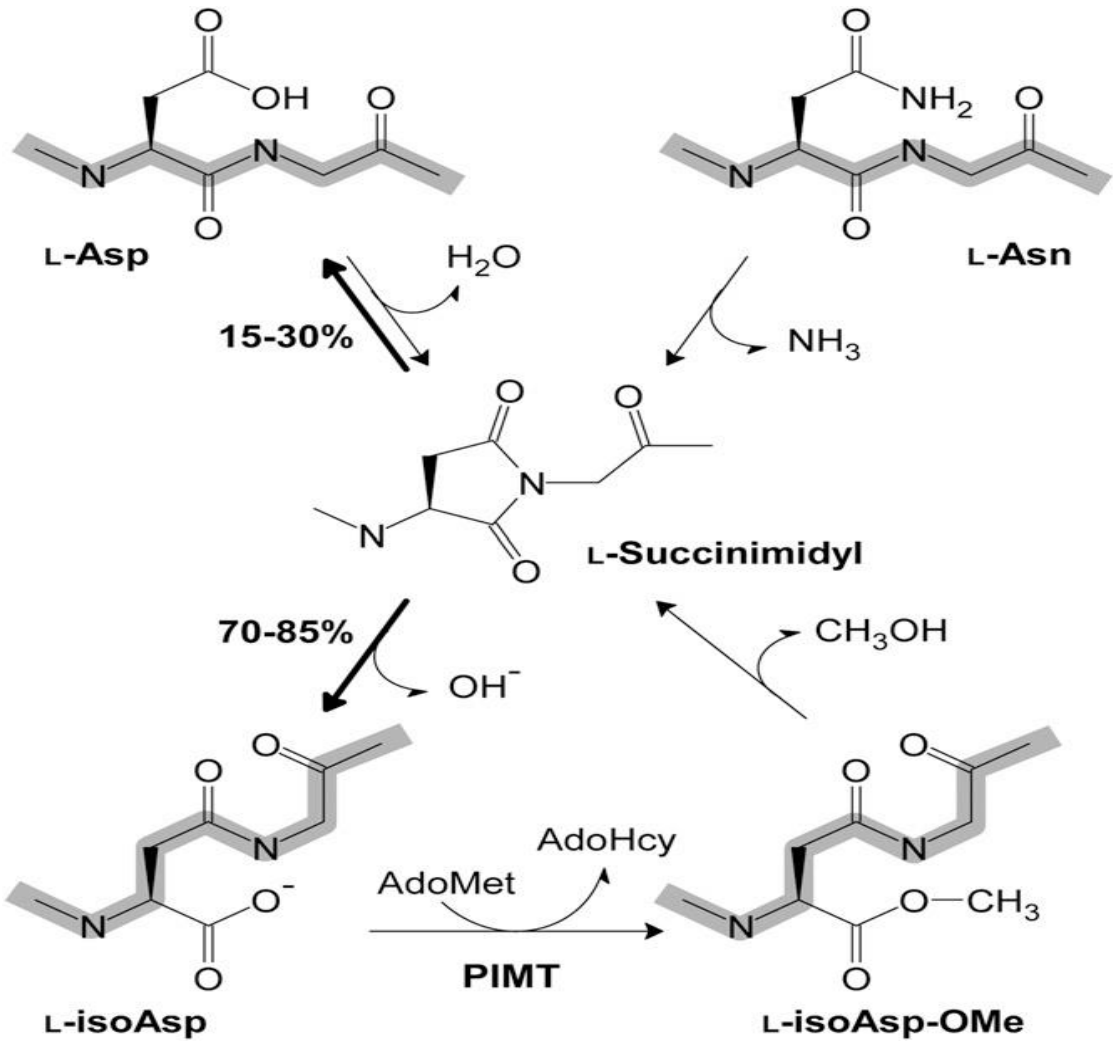



**Fig. 4 : Mechanism of abnormal L-isoaspartyl formation and enzymatic Repair by PIMT**

# III MATERIAL AND METHODS

During seed storage, seeds deteriorate, lose vigour and as a result become more sensitive to stresses during germination and ultimately die. The rate of ageing depends on the seed moisture content, temperature and initial seed quality. During seed deterioration, the accumulation of lipid peroxidation products, formation of reactive carbonyl compounds like methyl glyoxal, deoxyglucosone lead to the production of Amodari/ Millard products which ultimately lead to the production of AGEs (Advanced Glycation Endproducts) and ALEs (Advanced Lipoxidation Endproducts) in naturally aged seeds. Many of these reactions found to be active in dry system and play an important role in deterioration of dried food products as well. Seed deterioration is also due to protein inactivation by spontaneous formation of abnormal iso-aspartyl residue, which results in loss of seed viability and vigour. Plants have evolved many mechanisms to detoxify these cytotoxic compounds. Among them Aldo Keto Reductases (AKR's) scavenge RCCs and prevent oxidative stress damage, while Protein L-isoapartyl Methyltransferase (PIMT) converts abnormal isoaspartyl residues into normal aspartate residues, thereby maintains overall protein integrity.

Therefore, it is hypothesized that, co-expression of AKR1 and PIMT2 may reduce cytotoxic compounds and maintain overall protein integrity thereby, maintain the seed viability and vigour during storage. This hypothesis was initially tested in putative groundnut transgenics co-expressing AKR1 and PIMT2. The methodology followed to conduct several experiments is presented in this chapter.

## 3.1 Assessing genetic variability for seed viability across the selected groundnut genotypes

Although seeds deteriorate during storage, the time taken for complete deterioration/ loss of viability is generally longer (unless the seeds are exposed to adverse environmental conditions). Globally, researcher's employed accelerated ageing method efficiently to screen large number of genotypes to assess the genetic variability for cellular tolerance and seed viability upon ageing. Therefore, accelerated ageing technique was followed where the seeds were exposed to high temperature and relative humidity, to hasten up the deterioration process (Kester *et al.,* 1997). The protocol followed for accelerated ageing is given below.

Initially, accelerated ageing technique was standardized by exposing the groundnut seeds to varied temperature and relative humidity and the LD50 valve was taken to arrive at the duration and conditions for ageing treatment. Following the standardized protocol, selected groundnut genotypes were screened for seed viability.

**Imposition of accelerated ageing treatment for groundnut genotypes:**

Seeds samples of 20 groundnut genotypes of same age were subjected to accelerated ageing (6 days, 45 °C and 100 % RH).To achieve this,

1. The uniform sized groundnut seeds were selected and placed in small paper covers bags (blotting papers covers are better).

2. Air tight paper bags with seed samples were placed on a mesh in a desiccator filled with distilled water (water should not be in contact with seed. Here water is used to maintain 100 % RH).
3. Desiccators were placed inside the incubator for defined period of time (6 days) by maintaining the temperature of 45 °C.
4. At the end of the specified duration, seeds were taken out from desiccator and exposed to normal room temperature and relative humidity overnight.
5. These seeds were then used for assessing the seed viability, germination and for quantification of cytotoxic compounds.
6. For comparison, one set of seeds were kept inside the desiccators but maintained at room temperatures and ambient humidity which served as control.

### 3.1.1 Assessment of Seed viability in groundnut genotypes through 2, 3, 5-Triphenyl Tetrazolium Chloride test (TTC) upon accelerated ageing

Seeds sample of 20 groundnut genotypes of same age which were subjected to accelerated ageing for 6 days with respective control seeds were soaked in distilled water separately for four hours and end of which, the seeds were transferred to container containing 1 % Triphenyl Tetrazolium Chloride solution (TTC) and incubated overnight in dark. Later, the TTC solution was decanted and seeds so collected were observed for staining. The pink coloration which occurs due to reduction of TTC to formazan, an indication of seed viability was assessed based on the number of seeds stained to that of number of seeds treated in TTC solution.

Further to assess the extent of staining, approximately 100 mg of seed embryos that were incubated in TTC solution were ground in 1 ml of 2-methoxy ethanol and was centrifuged at 8000 rpm for 15 minutes. Later, the supernatant was collected and the absorbance was read at 485 nm using spectrophotometer. The seed viability was assessed based on the OD values as there is a direct relationship between absorbance values and seed viability.

### 3.1.2 Assessment of germination percentage (%) and seedling vigour in seeds of different groundnut genotypes upon ageing treatment

As mentioned in 3.1 seeds of 20 different groundnut genotypes were subjected for accelerated ageing treatment for 6 days and end of which, seeds were tested for seed germination. Ten seeds from each of the groundnut genotypes were soaked in distilled water for 4 hours and later sown in a sterilized petriplates having moistened blotting paper. The petriplates were then kept at room temperature and after 4 days, the number of seeds germinated was counted and percent seed germination was arrived as:

Germination percentage = (Number of seeds germinated/ Number of seeds taken) x 100

In order to assess seedling vigour, seedlings were maintained in petriplates for 5 more days and end of which, the root length as well as shoot length were measured and with the data of seed germination, the seedling vigour index was determined and compared with the seedlings of control treatment.

Seedling vigour index (SVI) = Germination percent (%) x (shoot length + root length)

### 3.1.3 Assessment of cytotoxic compounds in seeds of different groundnut genotypes upon ageing treatment

Dry seeds in storage are like desiccating tissues and are expected to produce a number of cytotoxic compounds. With this background, seeds of different groundnut genotypes subjected for accelerated ageing treatment along with control seeds were used to quantify cytotoxic compounds/ reactive carbonyl compounds such as Melondialdehyde (MDA), Methyl Glyoxal (MG) and Amadori products. The protocols followed to quantify these compounds are given below.

**a) Estimation of Melondialdehyde (MDA)**

Melondialdehyde is a product of lipid peroxidation of unsaturated fatty acids in phospholipids and is responsible for cell membrane damage. Excised embryos of about 100 mg from 20 different groundnut genotypes upon 6 daysof ageing treatment and control seeds were homogenized in 5 ml of 5 % (W/V) trichloroacetic acid and the homogenate was centrifuged at 12000 rpm for 15 min at room temperature. The supernatant was mixed with an equal amount of thiobarbutiric acid (0.5 % in 20 % (W/V) trichloro acetic acid) and the mixture was boiled at 100 °C for 25 minutes followed by centrifugation for 5 minutes at 7500 rpm. The absorbance of the supernatant was measured at 532 nm and 600 nm and corrected for nonspecific turbidity by subtracting the A600. The Pure MDA from Sigma Aldrich was used to develop the standard graph. The OD values were converted based on the standard graph. By substituting the OD values against standard graph, the MDA content was quantified and expressed as µM/gFW of tissue.

**b) Estimation of Methyl Glyoxal (MG)**

100 mg of embryos from different groundnut genotypes subjected for 6 days of accelerated ageing treatment along with control seeds were collected and ground in 0.5 M perchloric acid. These were further centrifuged at 11000 rpm for 10 minutes at 4 °C and later, supernatant was collected. If supernatants are coloured (which happens when leaf and other plant extracts are used), charcoal at the rate of 10 mg/ml was added to decolorize the supernatant. Once decolorized, the supernatants were neutralized by keeping in saturated solution of potassium carbonate at room temperature for 15 minutes and centrifuged again at 11000rpm for 10 minutes. The neutralized supernatant so collected was used for MG estimation. In order to quantify the Methyl Glyoxal content, 250 µl of 7.2 mM of 1,2-diaminobenzene(1,2-phenylene diamine), 100 µl of 5 M perchloric acid and 650 µl of the neutralized supernatant were added in the same order. After mixing the contents thoroughly, the absorbance was read at 336 nm using spectrophotometer. Against MG standards, the OD values were substituted to arrive at the MG content found in seeds of each treatment.

**c) Estimation of Amadori reaction products**

100 mg of excised embryos of different groundnut genotypes subjected for 6 days of accelerated ageing treatment and embryos from control seeds were ground in 1.2 ml of 50 mM phosphate buffer (pH 7.2). Later, 200 ml of 10 % streptomycin sulphate dissolved

in 50 mM HEPES with a pH of 7.2 was added to the homogenate to precipitate the nucleic acids. After vortexing and centrifuging at 15000 g for 15 min, another 200 ml streptomycin sulphate was added and the suspensions were centrifuged again. Proteins in the supernatant were precipitated with ammonium sulphate (0.55 g/ml±1). After centrifugation, the pellet was redissolved in 3.3 ml of phosphate buffer (50 mM, pH 7.2).

Extracted proteins were used to measure the Amadori reaction products. The content of Amadori reaction products was measured using the Nitro Blue Tetrazolium (NBT) method (Wettlaufer and Leopold, 1991). One ml of NBT reagent (0.5 mM NBT in 100 mM sodium carbonate with a pH of 10.3) was added to 0.2 mg of extracted axis proteins and incubated at 40 °C in water bath. The absorbance at 550 nm was recorded after 10 and 20 min of incubation. The absorbance (OD) was used to express the content of Amadori reaction products.

### 3.2 Relevance of AKR1 and PIMT2 to improve seed viability through transgenic approach

NAPIN:PIMT2:TRBCS gene cassette in pCAMBIA2301 binary vector, which is under seed specific napin promoter was procured from Dr.Manoj Mazee, NIPGR, New Delhi. Similarly, PRBCS:AKR1:TRBCS gene cassette in pi12GW binary vector ,which is codon optimized and custom synthesized by Dept. of Crop Physiology, UAS, GKVK, Bengaluru was procured. These binary vectors were further confirmed and used for sub-cloning to develop PRBCS:AKR1:TRBCS::NAPIN:PIMT2:TRBCS and also single gene cassette with compatible restriction enzymes. The Binary vector expressing these genes constructs were transformed to *E-coli* (strain) and plated on a selection media. The positive colonies which were survived on selection media were used to isolate plasmid and confirmed with PCR and restriction digestion with suitable restriction enzymes. The confirmed plasmid from *E-coli* cells was used to transform into *Agrobacterium* by electroporation method and the positive colonies which showed growth on selection media colonies were used to isolate plasmid and confirmed for the presence of both genes by PCR as well as restriction digestion.

#### 3.2.1 Preparation of competent *E.coli* cells

The *E.coli* strain DH5α, XL-1 Blue and DB3.1 cells were used to prepare competent cells for routine transformation experiments to multiply different plasmids used in the present study. From a freshly streaked LB plate (Luria Bertani: Bacto Yeast extract (5 g/L); Bacto-Tryptone ( 10 g/L); NaCl ( 10 g/L); pH-7.0; Agar (1 %), a single colony was brothed and grown overnight at 37 °C on a rotary shaker at 180 rpm. The grown culture (1 ml) was inoculated to a sterile 250 ml conical flask containing 100 ml of 2X-YT medium (Bacto Yeast extract (10 g/L)); Bacto-Tryptone (16 g/L); Nacl (10 g/L); pH-7.0; Agar(1 %) and was grown on a rotary shaker at 180 rpm. The growth of the culture was monitored every 30 minutes by measuring the OD at 600 nm (spectronic Genesys II). When the OD reached to around 0.6, the culture was cooled on ice bath for 30 minutes. The cells were pelleted by centrifuging at 3000 rpm for 10 minutes at 4 °C. The pellet was resuspended in 4 ml of pre-chilled TSB (Transformation and storage Buffer: 10 % PEG, 5 % DMSO, 10 mM $MgCL_2$ , 10 mM MgSO4, 10 % (v/v) Glycerol, 50 % (v/v) LB medium, pH-6) and centrifuged at 3000 rpm for 10 minutes at 4 °C.The

pellet was resuspended in 4 ml of TSB and incubated on ice for 10 minutes. The cells were further aliquot into pre-chilled sterile microfuge and shock frozen in liquid nitrogen and stored at -80 °C until further use.

### 3.2.2 KCM method of transformation

100 µL of competent cells + 20 µL KCM buffer + 100 ng of DNA were added to a sterilized centrifuge tube and volume was made up to 200 µL with sterile water.

↓

This mixture was kept on an ice for 20 minutes.

↓

Later shifted to room temperature for 10 minutes.

↓

800 µL of LB (Luria bertan) was added and mixture was gently incubated at 37 °C with 180 rpm for 1 hr. Cells were pelleted by spinning at 4000rpm for 5 minutes.

↓

Supernatant was discarded, pellet was resuspended. Suspension was then spread on LB plates with the respective antibiotic.

↓

These plates were incubated at 37 °C overnight.

↓

Colonies were confirmed by colony PCR with gene specific primers.

### 3.2.3 Modified alkaline lysis method of plasmid isolation

About 1.5 mL of bacterial cultures grown in 3 ml of L-broth containing appropriate antibiotics was transferred to microfuge tubes and spun at 12,000 rpm for 60 seconds to pellet down the cells. The 1.5 ml culture was transferred to the same tube after discarding the supernatant and centrifuged to collect the cells. The supernatant was carefully removed with affine-tip pipette and the pellet was thoroughly resuspended in 200 µl of solution I (Sterile distilled water and RNAse A solution – (2.5 mg/ml), stored at 4 °C) by vortexing. To the reaction mixture, 200 µl of solution II (Alkaline SDS solution – 1 % NaOH and 2 % sodium dodecyl sulfate (SDS), stored at room temperature) was added and the tube was gently inverted to get a clear suspension. To the suspension, 350 µl of solution III (3M Guanidine HCL (sigma Aldrich Chemical Company, USA) prepared in water and stored at room temperature) was added and the content of the tube were gently mixed by inverting for a few seconds. The tube then was centrifuged for 5-10 min at 12,000 rpm and the clear supernatant was transferred to afresh centrifuged tube. To the reaction mixture, equal volume of absolute alcohol was added and incubated at

room temperature for 10 minutes (generally any DNA would get precipitated if the alcohol concentration is above 66 % and hence, alcohol twice the volume of the supernatant is generally added). On the other hand isopropanol at a final concentration of 30-50 % could be conveniently used. The DNA pellet from the reaction mixture was collected by centrifugation (10 minutes at 12000 rpm) and the supernatant was removed by pipetting. The pellet was washed with chilled ethanol (70 %, v/v) by centrifuging at 12000 rpm for 5 minutes. The DNA pellet was dissolved in 30-50 µl of Tris or TE Buffer (10 mM, pH 8, according to the pellet size and used for analysis). The isolation DNA was quantified using spectrophotometer (UV-VIA, Simadzu, Japan) and also analyzed by agarose gel electrophoresis.

### 3.2.4 Restriction Endonuclease treatment

Plasmid DNA digestion was carried out by appropriate restriction endonucleases according to the criteria that the reaction buffer (10X) was $1/10^{th}$ of the end volume and 5 U of restriction enzymes were used per 1µg of DNA to be digested. A double digestion was possible per reaction only when both restriction enzymes used the same buffer (or any compatible buffer) otherwise, the digestions were performed chronologically. The DNA samples that were digested to completion were resolved on 0.8 % agarose gel.

### 3.2.5 Elution

DNA fragments of interest were purified from the gel by sigma gel elution kit method

Step : The DNA fragment of interest from agarose gel was excised with sterilized sharp scalpel.

Step 2: The gel slice was weighed.

Step 3: Three volume of gel solubilization solution was added to the gel slice.

Step 4: The gel mixture was incubated at 50-60 °C for 10 minutes or until the gel slice I completely dissolved.

Step 5: Binding column was prepared; 500 µl of the column preparation solution was added to each binding column and centrifuged for 1 minute, discarded flow through liquid.

Step 6: Once the gel mix is completely dissolved, equal volume of isopropanol was added and mixed until homogenized.

Step 7: Solubilized gel solution mixture was loaded from step 6 into binding column that is assembled in a 2 ml collection tube. Further, centrifuged for 1 min at 12000rpm after loading the column each time. Discard the flow-through liquid.

Step 8: 700 µl of wash solution was added to the binding column. Centrifuged for 1 min. Binding column from the collection tube was removed and discarded the flow through liquid.

Step 9: Transferred the binding column to a fresh collection tube. 50 µl of pre-warm elution solution (65 °C for 20minute) was added, centrifuged for 1 minute, 12000 rpm. Eluted DNA was resolved on 0.8 % agarose gel.

### 3.2.6 Ligation

Different DNA inserts were ligated into binary vector in various independent DNA recombination experiments. The ligation reaction mixture was brought to 15 µl (end volume), which comprised of 1.5 µl T4 ligase buffer (10X), digested plasmid DNA vector (dephosphorylated or not), 5 U of T4-DNA ligase (MBI-Ferments) and DNA insert. The mixture was brought to 15 µl with sterile distilled water and incubated at 16 °C for 16 hours. For a good ligation reaction, the amount of plasmid DNA vector must represent one third of the DNA insert in the ligation mixture.

### 3.2.7 Transformation of plasmid constructs into *E.coli*

One micro liter plasmid DNA (25 – 50 ng/µl) or ligated plasmid DNA mixture and 20 µl of filter sterilized 5X KCM buffer (0.5 M KCL, 0.15 M CaCL2 and 0.2 M $MgCl_2$) was made upto 100 µl using sterile distilled water and added to one aliquot of competent cells (100 µl) and carefully mixed. The mixture was placed in ice for 20min and then at room temperature for 10 min. Later, LB medium (800 µl) was added to the transformed cells and further incubated at 37 °C (180 rpm) for 1hour. Aliquot (100-200 µl) of the diluted cells were spread on appropriate selection plates and incubated at 37 °C overnight.

### 3.2.8 Screening of recombinant clones

The recombinant clones were initially identified based on blue/white selection ( if the vector had Lac Z interrupted by the multiple cloning site where the appropriate gene fragments were sub cloned) on the selection plate supplemented with X-gal (40 µl of IPTG (200 mg/ml in Dimethyl formamide) to each plate. The white colonies were further tested for their recombinant nature by colony PCR (instead of DNA as template, the entire white colony was used as the template (indirect template) in the PCR mixture) by using appropriate gene specific primers. A typical colony PCR was performed similar to that of a standard PCR except that the initial denaturation was carried out for 6-7 minutes. The PCR positive colonies were further confirmed for their recombinant nature by restriction digestion with appropriate endonucleases and observed for the expected fragments by agarose gel electrophoresis.

### 3.2.9 Preparation of electro-competent *Agrobacterium tumefaciens* cells

Electroporation competent *A.tumefaciens* (EHA 105) cells were prepared. A single colony of *A. tumefaciens* (EHA 105) was inoculated in 3 ml YEM medium, incubated at 28 °C for 16 h at 250 rpm and resuspended into fresh YEM medium (50ml) and further grown to an OD of 0.5. The cell culture was cooled on ice for 30 min and centrifuged (5000 rpm, 4 °C) for 5min. The pellet was washed successively in 1.0, 0.5, 0.2 and 0.1 culture volumes of cold 10 % (v/v) glycerol and resuspended in 0.01 volume of 10 % (v/v) glycerol. Aliquots (40 ul) of the last suspension were made, shock frozen in liquid nitrogen and stored at -80 °C as electro competent *A.tumefaciens* cells.

### 3.2.10 Transformation of plasmid into *Agrobacterium* via electroporation

The frozen *A. tumefaciens* competent cells (EHA 105) were thawed on ice and a 40 µl aliquot was transferred to a pre-cooled 2 mm electroporation cuvette (Bio-Rad Laboratories Ltd.). About 1 or 2 µl (100 ng/µl) of specific recombinant plasmid DNA was added to the competent cells and carefully mixed in a pre-cooled electro competent cuvette and an electric pulse was applied immediately using a Gene Pulser with Pulse controller unit (Bio-Rad). Good transformation efficiencies were obtained at field strength of 12.5 kV/cm, a capacitance of 25 µF and resistors of 400 W in parallel with the sample. 1 ml of YEM medium was then added to the transformed cells and further incubated in a glass tube and shaken at 250 rpm (28 °C) for 3 hours. Aliquot of 100 µl of the cells were finally plated on YEM agar medium containing appropriate antibiotics and incubated at 28 °C for 48 hours.

### 3.2.11 Extraction of genomic DNA

Genomic DNA was extracted from groundnut leaf as described by Doyle and Doyle (1989), homogenized in 7.5 ml of pre-warned (65 °C ) DNA extraction buffer [(4 % CTAB) (Appendix 2); 100 mM Tris-HCL, pH 8.0; 1.4 M NaCl; and 0.2 % β-mercaptoethanol (added *in situ* before DNA extraction)] and further incubated at 65 °C in water bath for 30 min. An aliquot volume (7.5 ml) of chloroform:isoamylalcohol (24:1) was added after cooling it to room temperature and centrifuged at 20 °C for 20 min at 11000 rpm. To the supernatant (10 ml) 2/3 volume (6 ml) of chilled isopropanol was added and incubated at 4 °C for 1-2 h. The mixture was later centrifuged at 8000 rpm for 15 min at 20 °C and the pellet resuspended in 5 ml of 70 % ethanol and centrifuged again (10 min, 8000 rpm, 20 °C). The supernatant was carefully discarded; the pellet air dried and then resuspended in 500 µl high salt TE buffer (10 mM Tris-HCL. 1 mM EDTA, pH 8.0) and stored at room temperature. To this mixture, 10 µl RNAase A (1 mg/ml) was added and incubated at 37 °C for 1 hour. An equal volume (500 µl) of cold phenol: chloroform: isoamylalcohol (25:24:1) was added and centrifuged for 5 min at 10,000 rpm. The supernatant was mixed with an equal volume of chloroform: isoamyalcohol (24: 1) and again centrifuged for 5 minutes at 10,000 rpm. To the resulting supernatant, twice the volume of absolute ethanol was added and incubated at 20 °C for 1-2 hours. The mixture was centrifuged (10 min, 13000 rpm, 4 °C) and the pellet washed twice in 70 % ethanol, air dried and resuspended in 100 µl TE buffer (1 mM) and stored at -20 °C.

### 3.2.12 Isolation of total RNA

Total RNA was extracted according to the protocol described by Dong *et al.,* (1986) with some modification. All care was taken to use RNase free material during RNA isolation (Sambrook *et al.,* 1989). All glasswares and plasticwares were treated with 0.1 % DEPC (diethyl pyrocarbonate) and solutions were prepared using DEPC treated water and they were autoclaved. About 1 g of frozen tissue was taken in autoclaved mortar and pestle and ground to a fine powder in liquid nitrogen. The tissue was homogenized with 10 ml of RNA extraction buffer ( 0.1 M Tris-HCL, pH 8.0; 0.25 M sucrose ; 0.2 M NaCl and 0.01 M $Mgcl_2$), 1ml of 0.5 M EDTA, 1ml of 20 % SDS followed by 10 ml water saturated phenol: chloroform (1:1) and β-mercaptoethanol was added to the final concentration of 0.2 %. The homogenized mixture was taken in

oakridge tubes and incubated at 4 °C for 20 minutes while shaking. The tubes were centrifuge at 16000 rpm for 30 min at 4 °C. The supernatant was transferred into a fresh tube and equal volume of chloroform: isoamylalcohol (49:1) were added, vortexed and centrifuged at 15,000 rpm at 4 °C. The aqueous phase transferred to another tube and 8 M $LiCL_2$ was added to the final concentration of 3 M and incubated at -80 °C overnight. The thawed tubes were centrifuged for 30 minutes at 15000 rpm at 4 °C. The pellet was then washed with 2 M $LiCL_2$ followed by one wash with 70 % ethyl alcohol. The resulting pellet was air dried at room temperature and dissolved in 50 µl RNase free water. The concentration and purity of the total RNA was ascertained spectrophotometrically (Nano drop, USA) at 260/280 nm. The quality was also checked on formaldehyde denaturing agarose (0.8 %) gel.

### 3.2.13 Synthesis of complementary DNA (cDNA) from RNA

cDNA was synthesized by oligo (dT) primers using Molony Murine Leukaemia Virus reverse transcription enzyme (MMLV- RT; MBI Fermentus. Hanover MD, USA). About 4 ug of RNA was reverse transcribed in a 20 µl reaction mixture containing 100U of MMLV, 2 ul of 20pico moles oligo dT primer and IX reaction buffer with 10 mM dNTPs mix. The reverse transcription was performed at 42 °C for one and a half hour

### 3.2.14 RT-PCR analysis

The cDNA was used as a template to perform RT-PCR analysis using gene specific primers and tubulin was used as a loading control. PCR analysis was carried out using standardized conditions as follows.

### 3.2.15 Polymerase chain reaction

The DNA fragments were amplified from various plasmid DNA/ genomic DNA/cDNA in a 20 µl reaction volume containing 100 ng of template DNA, 2 µl PCR buffer (10x), 2 µl dNTPs (2 mM), 1µ l forward primer (5 pmol/µl), 1 µl reverse primer (5 pmol/µl), 1 U *Taq* DNA polymerase and volume was made upto 20 µl with sterile water. PCR was performed in Master cycler Gradient (Eppendorf AG, Geramany). The optimal number of PCR cycles and the annealing temperature (TA) was determined empirically for each PCR. A standard PCR program followed is as follows:

Step1: 94 °C for 4 min (Initial denaturation)

Step 2: 94 °C for 1 min (denaturation)

Step 3: TA for 30 sec (primer annealing)

Step 4: 72 °C for 1min/kb fragment (DNA synthesis)

Step 5: Go to step 2: Repeat 25 cycles

Step 6: 72 °C for 10 min (Final extension)

Step 7: hold at 4 °C; END

The PCR samples were analyzed by agarose gel electrophoresis. The presence of the expected PCR products was determined with the help of DNA ladders (Gene Ruler DNA ladder, MB1-fermentus).

### 3.2.16 Agarose gel separation of DNA

Separation of DNA fragments was performed using horizontal gel electrophoresis. Depending on the requirement, 0.8 to 1.2 % agarose solutions were prepared in 1X TAE buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8.0) by heating in a microwave oven. After cooling to 50 °C, Ethidium bromide (10 mg/ml) was added to a final concentration of 0.5 µg/ml to the solution and poured into a mold with comb in the apparatus. The comb was carefully removed when a gel was completely set, and the gel was mounted into the electrophoresis tank containing 1X TAE buffer. DNA samples were mixed with 0.1 volume loading buffer (0.25 % Bromophenol blue, 0.25 % Xylene cyanol and 15 % glycerol) and loaded into the slots of submerged gel. The DNA fragments were electrophoretically separated (45-60 min) using a 1 kb ladder or 100bp DNA ladder or 1-DNA/HindIII digest as the refernce marker. The detection of the DNA fragments was carried out under UV-transilluminator (302 nm) and photographed using a gel documentation system (Herolab, GmbH Laborgerate, Germany).

## 3.3 Development of groundnut transgenic plants

### 3.3.1 Tissue culture independent modified *in planta* transformation adopted to develop groundnut transgenic

There are several *Agrobacterium* mediated transformation methods, among them *in planta* transformation method is one of the tissue culture independent transformation method which was adopted to develop groundnut transgenics. Further, to identify the putative transformants the seeds are used to screen selective agents depending on the selectable marker such as nptII( kanamycin $^{R}$), hptII ( hygromycin $^{R}$), bar gene ( Basta $^{R}$) and EPSPS Glyphosate $^{R}$(based on which, genes were used in transgenic development).

**Plant material:**

Groundnut variety KCG-6 was used to develop transgenic groundnut overexpressing AKR1 and PIMT2 separately and AKR1-PIMT2 co-expressing together by *Agrobacterium* mediated modified *in planta* transformation method.

### 3.3.2 Agrobacterium mediated modified *in planta* transformation of Groundnut

A single colony of *Agrobacterium* harboring the binary vector was grown in LB medium containing 50 µg/ml Spectinomycin/Kanamycin as selectable marker and 100 µg/ml of Rifamplicillin at 28 °C overnight. The bacterial cells were later resuspeded in Winan's AB medium (pH 5.2) and grown for 18 hours. For *vir* gene induction, wounded tabacco leaf extract (4 ml in 50 ml Winan's medium) (Roa *et al.*, 2008; Rohini and Rao, 2000) was added separately to the *Agrobacterium* suspension in Winan's AB medium, 5 h before infection.

### 3.3.3 Preparation of plant material

Groundnut seeds (KCG-6) were soaked in distilled water overnight and later surface sterilized with 0.1 % mercuric chloride for 5-7 minutes, followed by thorough rinses with sterile water and germinated on moistened petriplates at 30 °C in dark. Two day old seedlings were used as explant where cotyledons was cut off at the site of attachment to the primary axis and kept ready for *Agrobacterium* infection.

### 3.3.4 Infection and recovery of transformants

*In planta* transformation strategy was adopted to develop transgenic plants in groundnut following the earlier standardized protocol as given below

Seeds soaked overnight

↓

Put for germination for one day

↓

Embryo axes of germinating seeds pricked randomly with sterile needles and immersed in the suspension of Agrobacterium in Winan's AB medium added with wounded tobacco leaf extract.

↓

Incubated with gentle agitation at 28 °C for 1 hour

↓

Allowed to germinate for one day, the healthy *Agrobacterium* infected T0 transformants were screened on glyphosate/ kanamycin to identify the putative transformats.

↓

Glyphosate/ kanamycin tolerant plants were advanced for next generation.

### 3.3.5 Identification of putative transformants

Identification of stable transformants is crucial in advancing the transformants to next generation. To minimize the possible segregates in the subsequent generations, an efficient screening protocol is required. In this context, the antibiotic kanamycin based protocol and glyphosate based screening protocols were standardized to screen the transgenics.

### 3.3.5.1 Standardization of lethal concentration (LC) of glyphosate and kanamycin for groundnut variety KCG6

**Flow of events in standardizing the lethal dose of glyphosate and kanamycin:**

Imbibed the seeds (wild type) for 4 h and germinated on wetted blotting paper.

↓

Two day's old equally germinated seeds were used for screening.

↓

Pre-germinated seeds were placed on saturated sterilized quartz sand containing known concentration of glyphosate ( 0, 2, 10, 15, 20, 25 and 30 ppm) and known concentration of kanamycin ( 0, 100, 200, 300, 400 and 500 ppm of kanamycin).

↓

Water status was maintained by weighing the pots each day.

↓

After 15 days, the percent inhibition of growth was recorded and lethal dose was identified.

### 3.3.5.2 Screening of T0 transformants against glyphosate and kanamycin

*Agrobacterium* infected T0 seeds and wild type seeds were surface sterilized with 0.1 % (w/v) mercuric chloride for 5-7 minutes followed by thorough rinses with sterile water and germinated on petriplates. Two days old healthy seedlings were screened against 30 ppm of glyphosate (AKR1 overexpressing construct seedlings and AKR1-PIMT2 co-expressing seedling) and 400 ppm kanamycin (PIMT2 overexpressing seedling). After 10 days, the survived seedlings were considered as putative transformants and transplanted them to pots for recovery and establishment. The plants which survived were advanced for next generation.

### 3.3.5.3 Screening of putative $T_1$ transformants with glyphosate and kanamycin

The $T_1$ germinated seeds from transformants and wild type seeds were screened on 30ppm glyphosate (AKR-PIMT2 co-expressing gene construct seeds; AKR1 overexpressing gene construct seeds) and 400 ppm of kanamycin (PIMT2 overexpressing gene construct seeds). Based on root and shoot inhibition by glyphosate and kanamycin, the $T_1$ putative transformants were selected by comparing with wild type seedlings. Healthy and survived putative transformants were grown under controlled environmental condition and further subject them for molecular characterization.

### 3.3.6 Molecular characterization

To confirm the integration of AKR1 and PIMT2 gene in groundnut putative $T_1$ transformants the genomic DNA was extracted by CTAB method as described in the section 3.2.11 (Appendix II). From the genomic DNA, 100 ng of template DNA was used for PCR confirmation as outlined in 3.2.15. PCR was carried out using different set of primers : promoter forward + gene specific reverse primer and also with nested primer (Appendix I) which is performed in Mastercycler Gradient ( Eppendorf $^{R}$ AG , Germany) keeping plasmid DNA as a positive control. The PCR amplified products were separated by electrophoresis on agarose (1 %) gel.

## 3.4 Characterization of T2 putative groundnut transgenics for seed viability and cytotoxic compounds

AKR and PIMT have been shown to have relevance in reducing the seed deterioration process by scavenging RCCs compounds and maintaining protein integrity. The hypothesis was further tested in T2 putative groundnut transgenic plants co-expressing AKR1 and PIMT2.

### 3.4.1 Assessment of seed viability in seeds of wild-type and putative T2 groundnut transgenics co-expressing AKR1 and PIMT2 genes upon ageing treatment

Putative T2 groundnut transgenic plants co-expressing AKR1 and PIMT2 genes were developed following *in planta* transformation technique. The T2 generation seeds were used to assess the seed viability and for other experiments. Therefore, in order to hasten up the deterioration process, accelerated ageing technique was followed where the seeds were exposed to high temperatures and 100 % relative humidity. The hypothesis was tested whether such seed deterioration process is delayed in groundnut transgenic co-expressing AKR1 and PIMT2, where detoxifying enzymes are overexpressed was tested by exposing the seeds to accelerated ageing technique as outlined in 3.1.

### 3.4.2 Assessment of germination percentage and seedling vigour in seeds of wild type and T2 putative transgenic groundnut co-expressing AKR1 and PIMT2 genes upon ageing treatment

As explained in 3.1, both transgenic and wild type seeds were subjected for accelerated ageing treatment for particular durations and end of which, seeds were tested for seed germination and seedling vigour index. Ten seeds from each of the transgenic lines and wild type were soaked in distilled water for 4 hours and later, sown in a sterilized petriplates having moistened blotting paper. The petri plates were then kept at room temperature and relative humidity and after 2 days, the number of seeds germinated was counted and percent seed germination was arrived at:

Percent seed germination= (Number of seeds germinated/ Number of seeds sown) x 100

In order to assess the seedling vigour, root and shoot growth were measured in the young seedlings which were maintained in quartz sand for 5 more days and end of which, the root length as well as shoot length were measured and with the data of seed

germination, the seedling vigour index was determined and compared with the seedlings of control treatment.

Seedling vigour index (SVI) = Germination percent (%) x (shoot length + root length)

### 3.4.3 Assessment of cytotoxic compounds in seeds of wild type and putative T2 transgenic groundnut co-expressing AKR1 and PIMT2 genes upon ageing treatment

Accelerated ageing being a sort of stress treatment, would induce the production of several cytotoxic compounds. Seeds of both wild type and T2 putative transgenic groundnut co-expressing AKR1 and PIMT2 were subjected for ageing treatment as outlined earlier were used for quantification of cytotoxic compounds such as melondialdehyde, methyl glyoxal, and Amadori products. Following the procedure outlined in 3.1.4 the cytotoxic compounds so quantified were correlated with seed viability as well as seed germination.

### 3.4.4 Assessing the stress tolerance of transgenic co-expressing AKR1 and PIMT2

Several kinds of stresses have been shown to increase the production of ROS and RCC's in plants. Similarly, stress is also known to damage protein structure and affects their functions. The genes used in the present study have been reported to have some function in imparting stress tolerance. In these contexts, the groundnut transgenics co-expressing AKR1 and PIMT2 were tested for salinity induced stress tolerance.

One day old and uniformly germinated transgenics and wild type seedling were induced with 100 mM of NaCl for 1 day followed by transferring them to a lethal concentration of 300 mM NaCl for 2 days. Later, these seedlings were kept for 2 days under recovery by transforming them to petriplates having moistened blotting paper. At the end of recovery period, seedling survival was recorded and the survived and tolerant seedlings were transplanted into pots for further establishment.

# IV RESULTS

Seed viability and seeding vigour are important for plant development and uniform establishment of crops. Seeds deteriorate during storage as the storage conditions vary. Seed moisture content, temperature, relative humidity forms the major determining factor in seed longevity. During storage, when storage conditions are not congenial induces the production of ROS, RCCs and other cytotoxic compounds like advanced lipoxidation end products (ALEs) and advanced glycation end products (AGEs) derived from lipid peroxidation and glucose metabolites respectively which inhibit the seed germination, vigour and establishment. In addition, formation of abnormal non-functional iso-aspartyl residue which is spontaneous age related protein damage also contributes to early loss of seed viability and vigour. Hence, it is important to detoxify these cytotoxic compounds and reduce the production of abnormal iso-aspartyl residue to protect the seeds from deterioration and hence, enhance seed viability in the context of crop improvement.

AKR1 from aldo-keto reductase super family has a role in detoxifying the cytotoxic compounds such as MG, HNE, MDA etc. whereas, PMT2 which belongs to SAM-Adomet family has a role in converting abnormal iso-aspartyl residue to normal asparate residue. Therefore in the present study, the role of AKR1 and PIMT2 in detoxifying the cytotoxic compounds and protein repair mechanisms respectively was examined through assessing seed viability, seedling vigour and growth. The results of the various experiments conducted are presented in this chapter.

## 4.1 Assessment of genetic variability for seed viability across the selected groundnut genotypes

To study the effect of ageing on seeds, a controlled environmental condition was stimulated in laboratory which is generally known as accelerated ageing technique. Groundnut genotypes harvested at the same season were subjected to accelerated ageing technique (Plate 1) to study the genetic variability for seed viability across groundnut genotypes and also to study the relevance of reactive carbonyl compounds on seed viability, seedling vigour and growth of groundnut seedlings.

### 4.1.1 Tetrazolium (TTC) test to assess seed viability across groundnut genotypes

Initially, 6 days of ageing treatment maintaining a temperature of 45 °C and 100 % RH was standardized for groundnut seeds. This standardized protocol was followed to impose the ageing treatment across 20 groundnut genotypes. The aged (6 days of AA) and control seeds (0 days of ageing) were assayed for seed viability by TTC test. The visual observation of seeds for extent of formazan staining indicate the viability of seeds and the absorbance values measured in the stained solution extracted in 2-methoxy ethanol at 485 nm by spectrophotometer indicate the extent of seed viability.

It was observed that, there is a significant genetic variability for seed viability across groundnut genotypes. With ageing treatment, the seed viability drastically decreased in all the genotypes and it was significantly lower compared to the seeds not exposed to accelerated ageing treatment (control). Some of the genotypes such a KCG6

and ICGV9114 showed a very least seed viability whereas, genotypes such as SB3 and SB15 showed high seed viability compared to other genotypes after 6 days of accelerated ageing (Fig. 5). The percent reduction in seed viability was higher in KCG6 and ICGV9114 to indicate that these genotypes are highly susceptible to ageing (Fig. 6).

### 4.1.2 Assessment of germination percentage and seedling vigour in seeds of different groundnut genotypes upon ageing treatment

Upon 6 days of accelerated ageing treatment, germination percentage was reduced significantly across groundnut genotypes when compared to seeds which are not exposed to ageing treatment (control seeds) where, the majority of the genotypes showed nearly 100 % seed germination (0 days of accelerated ageing). However, some genotypes showed better germination than others. Accordingly, genotypes such as SB3, SB15 and VB1, even after 6 days of accelerated ageing, showed nearly 80 % seed germination whereas genotypes such as KCG6 and ICGV9114 recorded very low seed germination percentage of approximately 60 % upon accelerated ageing conditions. It appears that genotype KCG6 and ICGV9114 were highly susceptible for ageing treatment and lose viability when seed storage condition are altered even to a less extent (Table 1). When seed viability and germination data put for correlation studies, a significant positive relationship was found indicating longer the viability of seed, greater the germination percentage (Fig. 7).

Based on germination percentage, root length and shoot length of seedlings from accelerated aged seeds of groundnut genotypes, seedling vigour index was estimated. Accordingly, some of the genotypes such as KCG6, ICGV9114, SB21 and VB11 showed least seedling vigour upon ageing treatment for 6 days. The low vigour was due to less or failure of seed germination in those species. As expected, with ageing treatment, the seedling vigour decreased in all genotypes (Fig. 8).

### 4.1.3 Assessment of cytotoxic compounds in seeds of groundnut genotypes upon ageing treatment

The cytotoxic compounds were quantified in control and accelerated aged seeds of 20 groundnut genotypes. Although there is no significant differences in production of cytotoxic compounds such as Melondialdehyde, Methyl glyoxal and Amadori products under controlled conditions (0 days of ageing), with exposed to 6 days of accelerated ageing, induces the accumulation of cytotoxic compounds. In fact, there is a significant increase in production of various cytotoxic compounds in aged seeds over their controlled seeds (0 days of ageing) (Fig. 9).

It was observed that, genotypes such as SB3, SB15 showed lower level of Melondialdehyde and Methyl glyoxal production upon 6 days of accelerated ageing compared to KCG6 and ICGV9114 which showed significantly higher level of cytotoxic compound upon ageing. However, rest of the genotypes showed no significant variation in accumulation of MDA and MG (Fig. 9).


**Plate 1:** Experimental set up for accelerated ageing

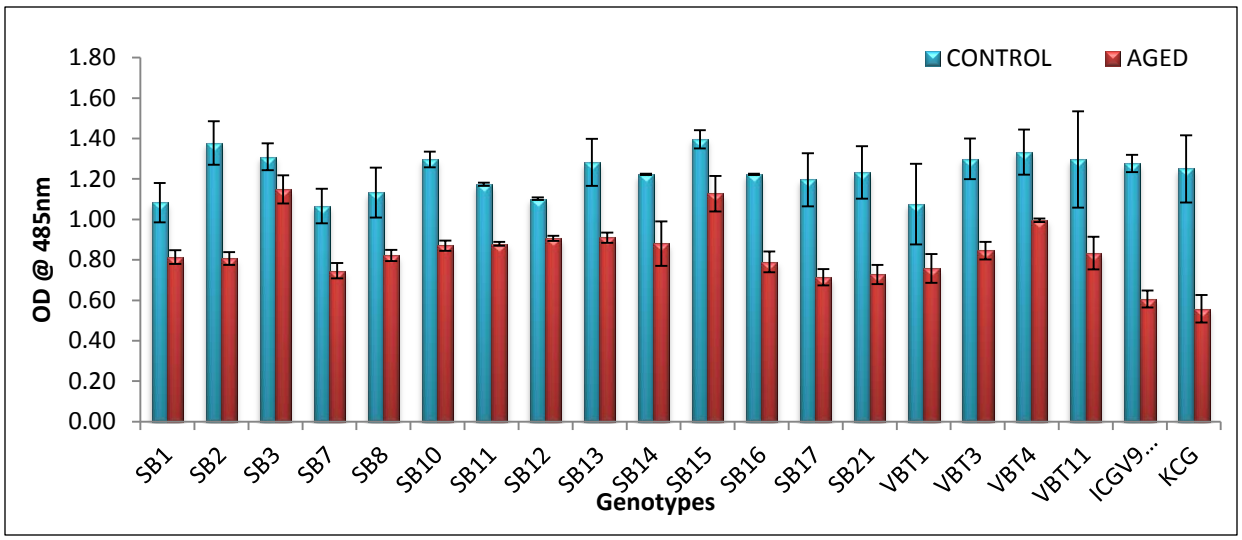



**Fig. 5**: Genotypic variability for seed viability across groundnut genotypes upon ageing treatment

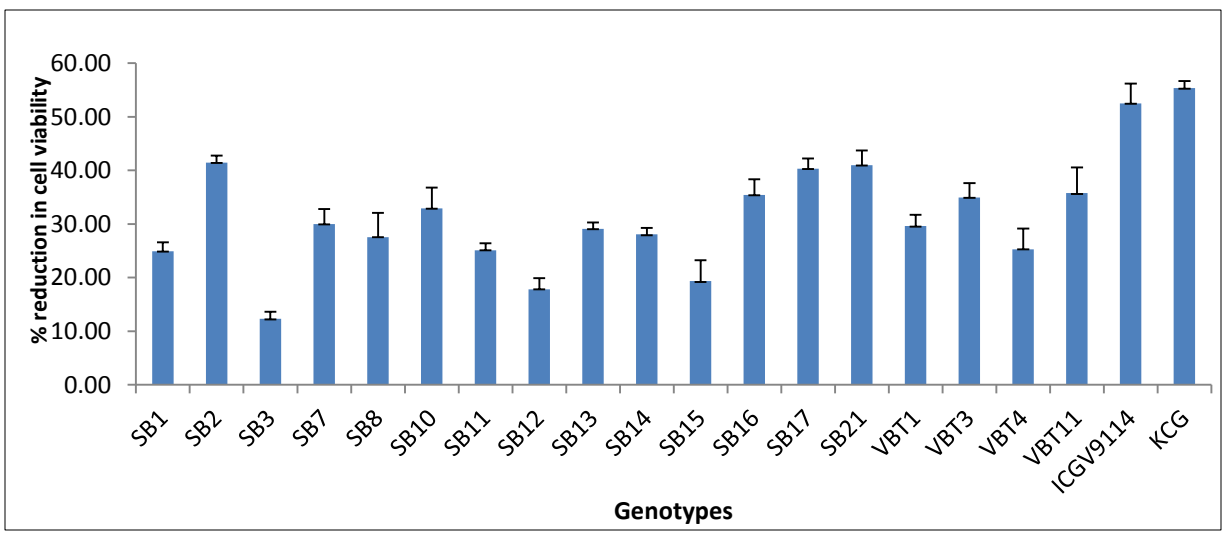



**Fig. 6**: variation in percent reduction in seed viability in 20 groundnut genotypes upon 6 days of accelerated ageing treatment

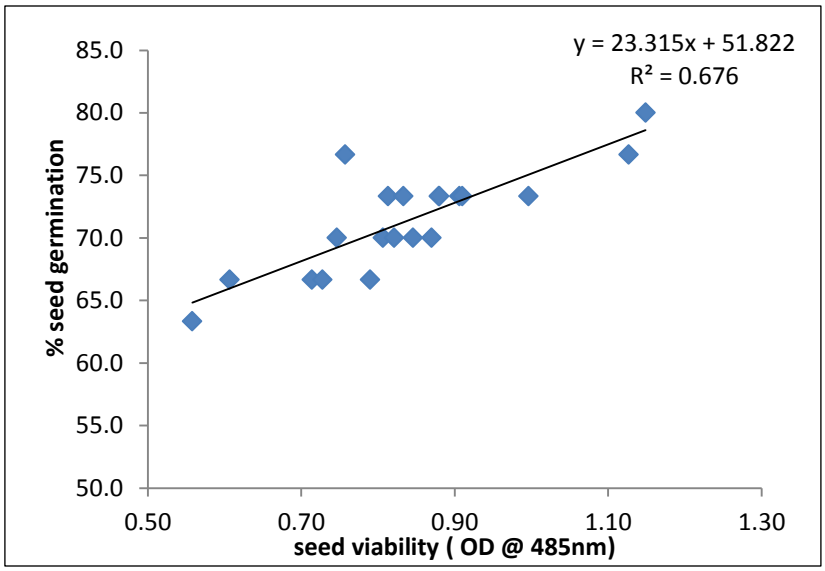



**Fig. 7:** Relationship between germination percentage and seed viability in 20 different groundnut genotypes

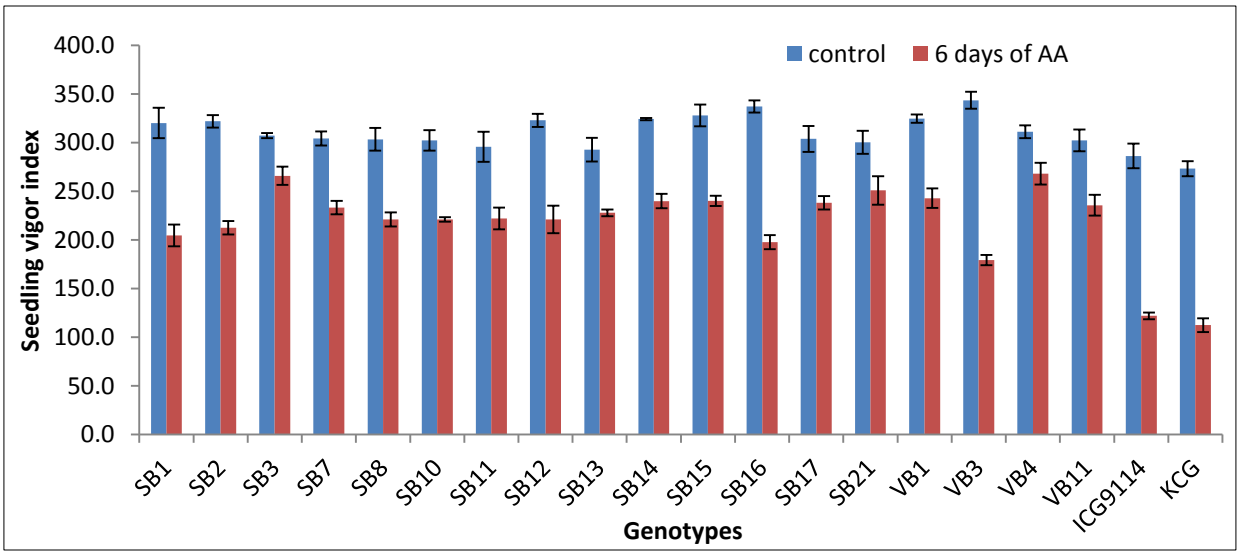



**Fig. 8 :** Effect of Accelerated ageing on seedling vigor index in 20 groundnut genotypes

**Table 1: Genotypic variability for seed germination as influenced by ageing treatment in ground genotypes**

| Genotypes | Control | 6 days of AA |
|---|---|---|
| SB3 | 93.3 ± 6.7 | 73.3 ± 3.3 |
| SB2 | 100 ± 0 | 70 ± 5.7 |
| SB3 | 100 ± 0 | 80 ± 0 |
| SB7 | 93± 6.7 | 70 ± 0 |
| SB8 | 93 ± 6.7 | 70 ± 0 |
| SB10 | 96 ± 3.33 | 70±0 |
| SB11 | 96 ± 3.33 | 73.3 ± 3.3 |
| SB12 | 100 ±0 | 73.3 ± 3.3 |
| SB13 | 96 ± 3.33 | 73.3 ± 3.3 |
| SB14 | 100 ±0 | 73.3 ± 3.3 |
| SB15 | 96.1 ± 3.33 | 76.6 ± 3.3 |
| SB6 | 100 ±0 | 68 ± 6.6 |
| SB17 | 90 ±5.7 | 68 ± 6.6 |
| SB21 | 93 ±6.7 | 68 ± 6.6 |
| VB1 | 96 ±3.3 | 76.6 ± 3.3 |
| VB3 | 100 ±0 | 70 ± 0 |
| VB4 | 96±3.3 | 73.3 ± 3.3 |
| VB11 | 96±3.3 | 73.3 ± 3.3 |
| ICGV9114 | 90 ±5.7 | 66 ± 6.6 |
| KCG6 | 90±5.7 | 63.3 ± 6.6 |
| CD@ 0.05 | 7.01 | 5.2095 |
| CV (%) | 7.12 | 1.8546 |

(each valve represents mean ± S.E. for three individual replicates)

As far as production and accumulation of Amadori products are concerned, significantly lower level of this compound was observed in controlled condition (0 days of ageing) as compared to aged seeds (6 days of accelerated ageing). Genotypes such as SB3, SB15, SB8 and VB3 showed significantly lower level of accumulation of Amadori products compared to other genotypes such as KCG6 and ICGV9114 which showed high level of production of Amadori products when exposed to accelerated ageing treatment (Fig. 9).

When percent seed germination and seed viability was compared with that of cytotoxic compounds, a strong and significant inverse relationship was observed which indicates the cytotoxic compounds effects on seed germination (Fig. 10) and seed viability (Fig. 11).

Based on TTC test (seed viability), percent seed germination, seedling vigour and level of cytotoxic compounds in 20 groundnut genotypes upon accelerated ageing treatment two genotypes each representing short and long seed viability among 20 groundnut genotypes were identified. Accordingly, genotypes like SB3, SB15 which showed long seed viability and KCG6 and ICGV9114 found to have shorter seed viability upon ageing (Fig. 12) were selected and used for further characterization. These genotypes were again subjected to ageing treatment and later, examined for seed viability, percent seed germination, cytotoxic compounds accumulation upon ageing. The results confirmed, the genotypes such as SB3 and SB15 with longer seed viability showed significantly low level of cytotoxic compounds like MDA, MG, Amadori products hence, consider as longer viability genotypes whereas, the genotypes KCG6 and ICGV9114 with shorter seed viability showed high accumulation of cytotoxic compounds (Fig. 13 and Plate 2). Further, the seed germination was also significantly higher for longer seed viability genotypes compared to the shorter seed viability genotypes (Table 2).

## 4.2 Relevance of AKR1 and PIMT2 to improve seed viability through transgenic approach

### 4.2.1 *In-silico* analysis of CaPIMT2 sequences:

The revised amino acid sequences of CaPIMT2 were used to compare the amino acid composition and conserved domains across PIMT2 from different crop species (NCBI gene bank). The analysis using SMS Software (sequence manipulation suite) revealed a high homology at C-terminal region across plant species and variable N-terminal end. Further, the amino acid sequence of PIMT2 was analyzed for the presences of five highly conserved domains which were reported earlier in various plant species (Kagan *et al.,* 1997). The five conserved domains are pre-region I (ATISAPHMHA), region I (IALDVGSGTG), region II (APYDHIHVG), region III (QLKPGGRMV) and post region III (VRYVPLTS) which are essential for PIMT function and substrate specificity were detected ( Fig. 14).

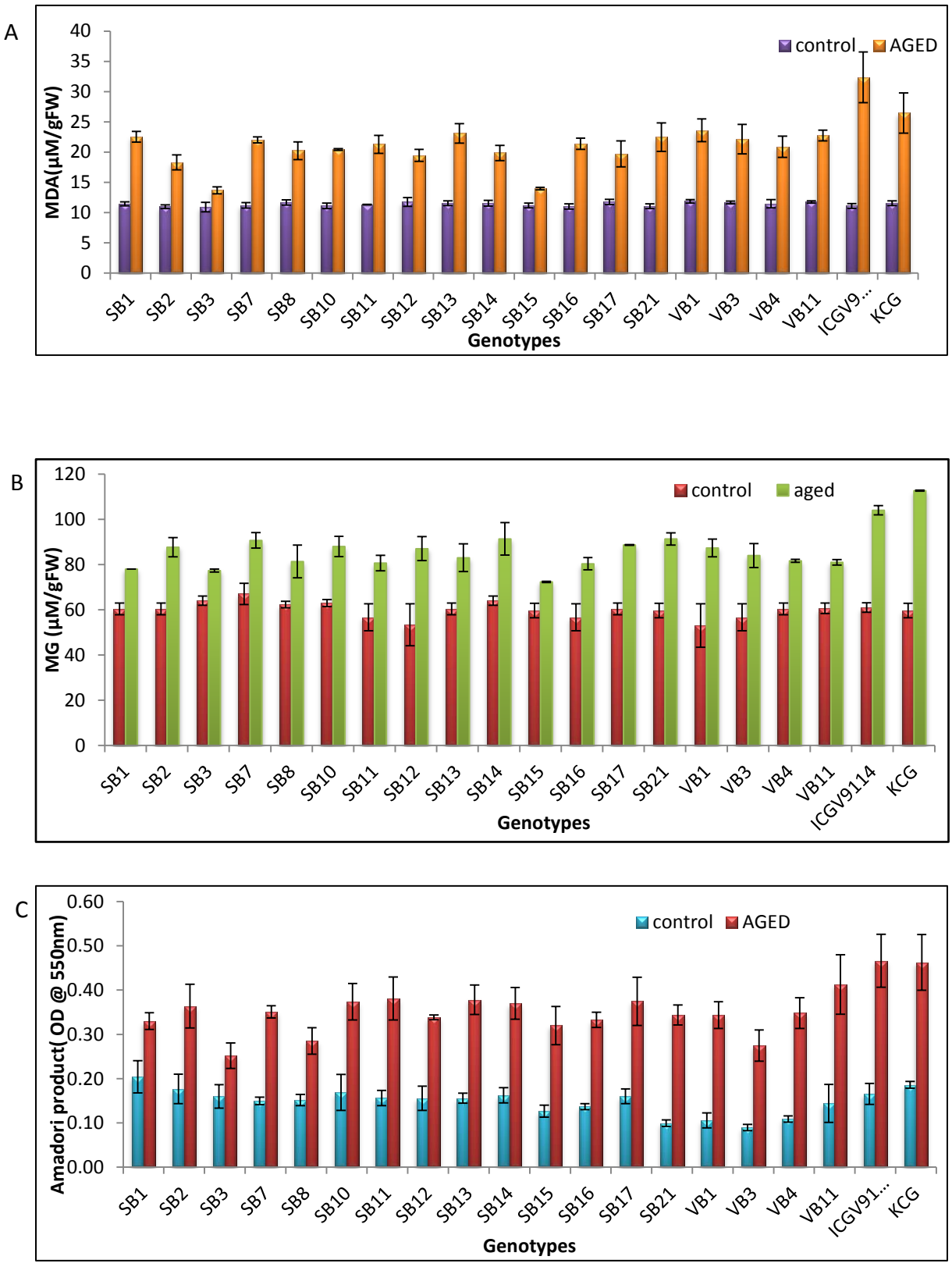



**Fig. 9:** Variation in reactive carbonyl compounds in 20 different groundnut genotypes subjected for accelerated ageing treatment

(A) Melondialdehyde

(B) Methyl glyoxal

(C) Amadori products

### 4.2.2 Homology analysis through BLAST

**Identity of CaPIMT2 across the crop species**:

The phylogenetic analysis of CaPIMT2 with other orthologous gene showed high identity with *Medicago trunkatula* (KEH35014.1) distinctly related to *Nelimbo nucifera* (XP010284719.1) and *Vitis vinifera* (XP02278792.2) (Fig. 15).

### 4.2.3 Development of plant transformation vectors-

1. PRBCS:AKR1:TRBCS ( AKR1 overexpressing gene construct)
2. NAPIN:PIMT2:TRBCS (PIMT2 overexpressing gene construct)
3. PRBCS:AKR1:TRBCS::NAPIN:PIMT2:TRBCS (Co-expression of AKR1 and PIMT2).

NAPIN:PIMT2:TRBCS gene cassette in pCAMBIA2301 binary vector, which is under seed specific napin promoter was procured from Dr.Manoj Mazee, NIPGR, New Delhi. Similarly, PRBCS:AKR1:TRBCS gene cassette in pi12GW binary vector ,which is codon optimized and custom synthesized by the Dept. of Crop Physiology, UAS, GKVK, Bengaluru were used for transformation. The single gene cassette were initially confirmed in respective binary vector and further sub-cloned into marker free binary vector pmf12GW to develop double gene cassette (PRBCS:AKR1:TRBCS::NAPIN:PIMT2:TRBCS) and also single gene cassette were used.

#### 4.2.3.1 Confirmation of AKR1 and PIMT2 genes in binary vector

The first step in the process of developing single gene cassette is to confirm the chosen set of genes in their respective binary vector system for the presence of entire cassette of gene of interest along with their promoter and terminator region.

(a) The presence of NAPIN:PIMT2:TRBCS gene cassette was confirmed by restriction digestion which was performed by using *EcoR1* restriction enzymes that released expected 2kb fragment from NAPIN:PIMT2:TRBCS gene cassette. The digested and undigested plasmid was electrophoresed on agarose (0.8 %) gel stained with ethidium bromide (Plate 3).

(b) The presence PRBCS:AKR1:TRBCS gene cassette was confirmed by restriction digestion which was performed using *Asc1* and *Pac1* restriction enzymes which released the entire 3.6kb fragment consisting of PRBCS:AKR1:TRBCS gene cassette. The restriction digested product of 3.6 kb was resolved on agarose (0.8 %) gel (Plate 4).

*E-coli* (DH5α) colony PCR was performed to confirm the presence of PRBCS:AKR1:TRBCS and NAPIN:PIMT2:TRBCS gene cassette in binary vector using gene specific primers (Appendix 1) which amplified 583 bp and 644 bp respectively (Plate 5 and 6) and amplified product resolved on agarose (0.8 %) gel stained with ethidium bromide.

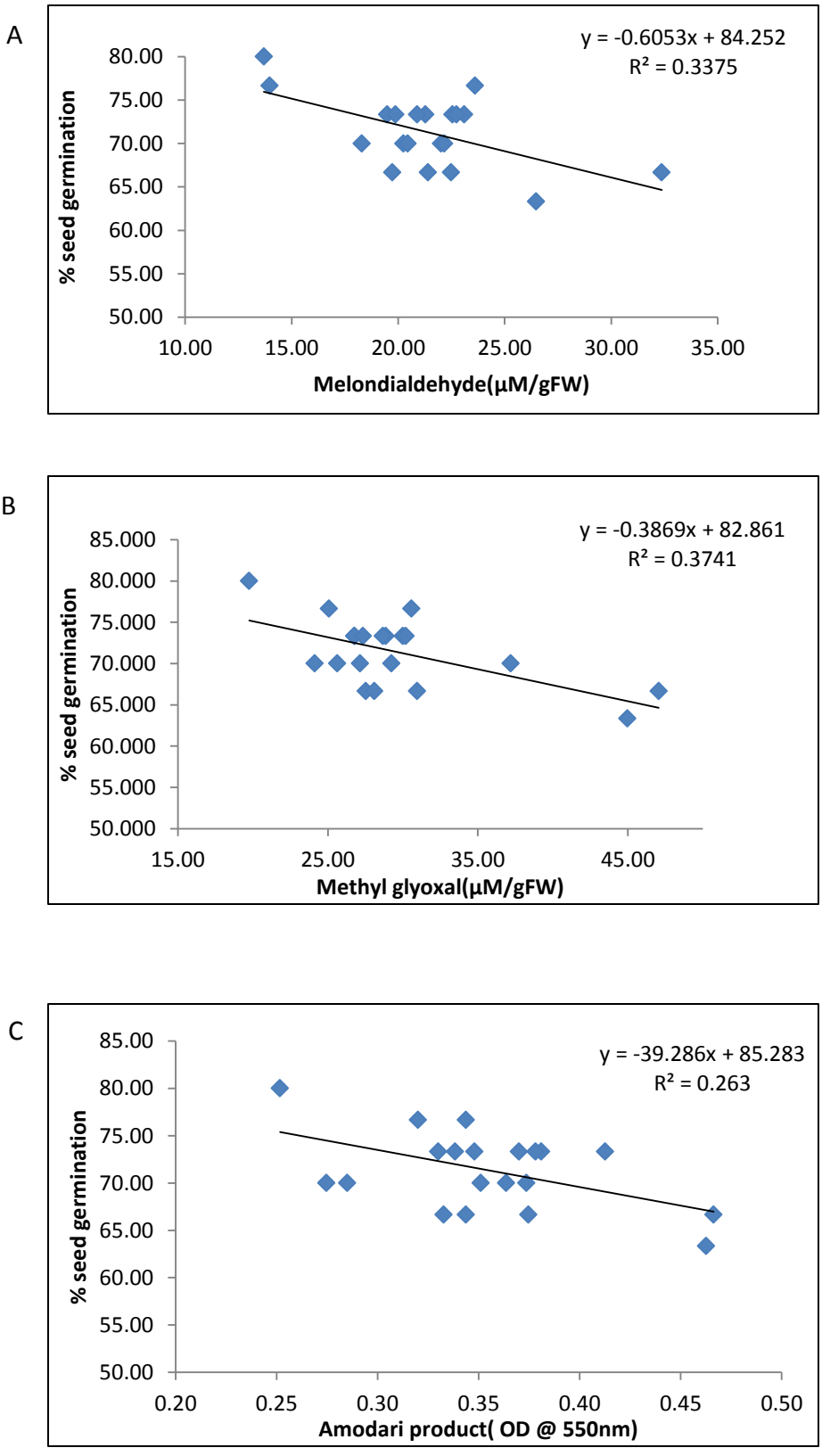



**Fig. 10:** Relationship between reactive carbonyl compounds (RCCs) and seed germination in 20 groundnut genotypes

(A) Melondialdehyde v/s Seed germination.

(B) Methyl glyoxal v/s Seed germination.

(C) Amadori product v/s Seed germination.

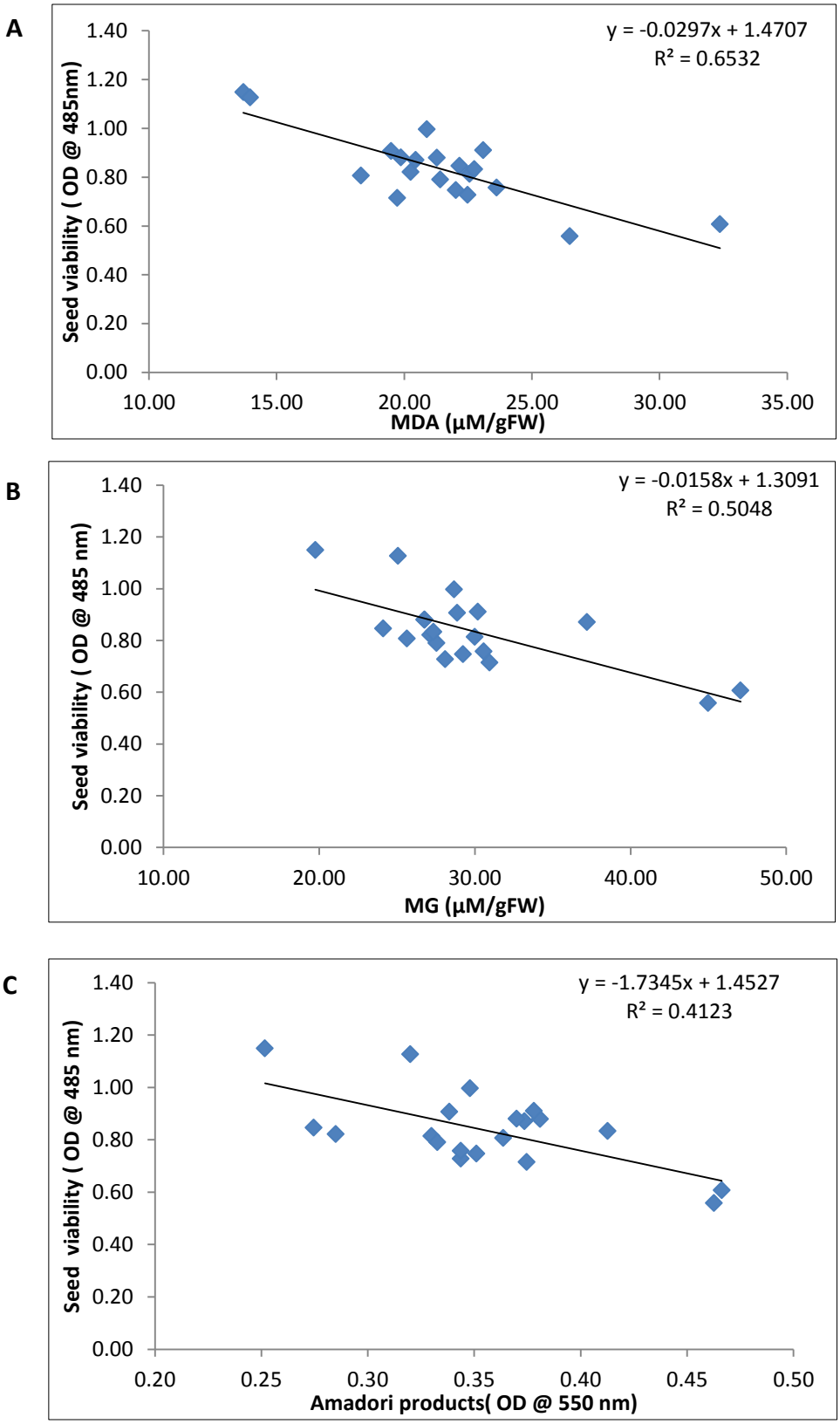



**Fig. 11:** Relationship between reactive carbonyl compounds (RCC's) and seed viability in 20 groundnut genotypes
(A) Melondialdehyde v/s seed viability.
(B) Methyl glyoxal v/s seed viability.
(C) Amadori products v/s seed viability.

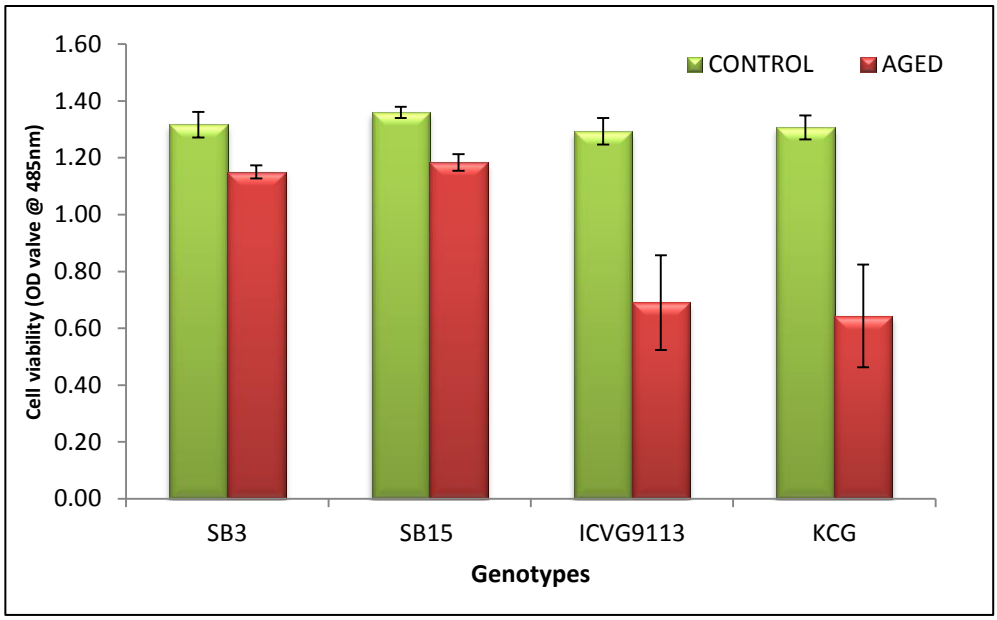



**Fig. 12:** Genotypic variability in seed viability in contrasting groundnut genotypes upon ageing

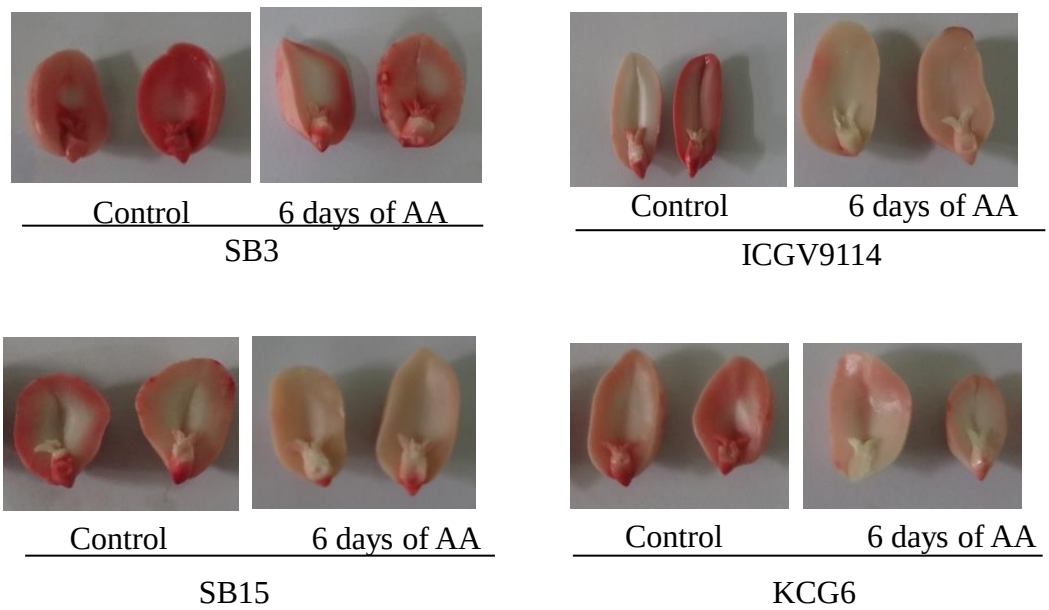



**Plate 2:** TTC staining of groundnut genotypes subjected to accelerated ageing treatment for 6 days

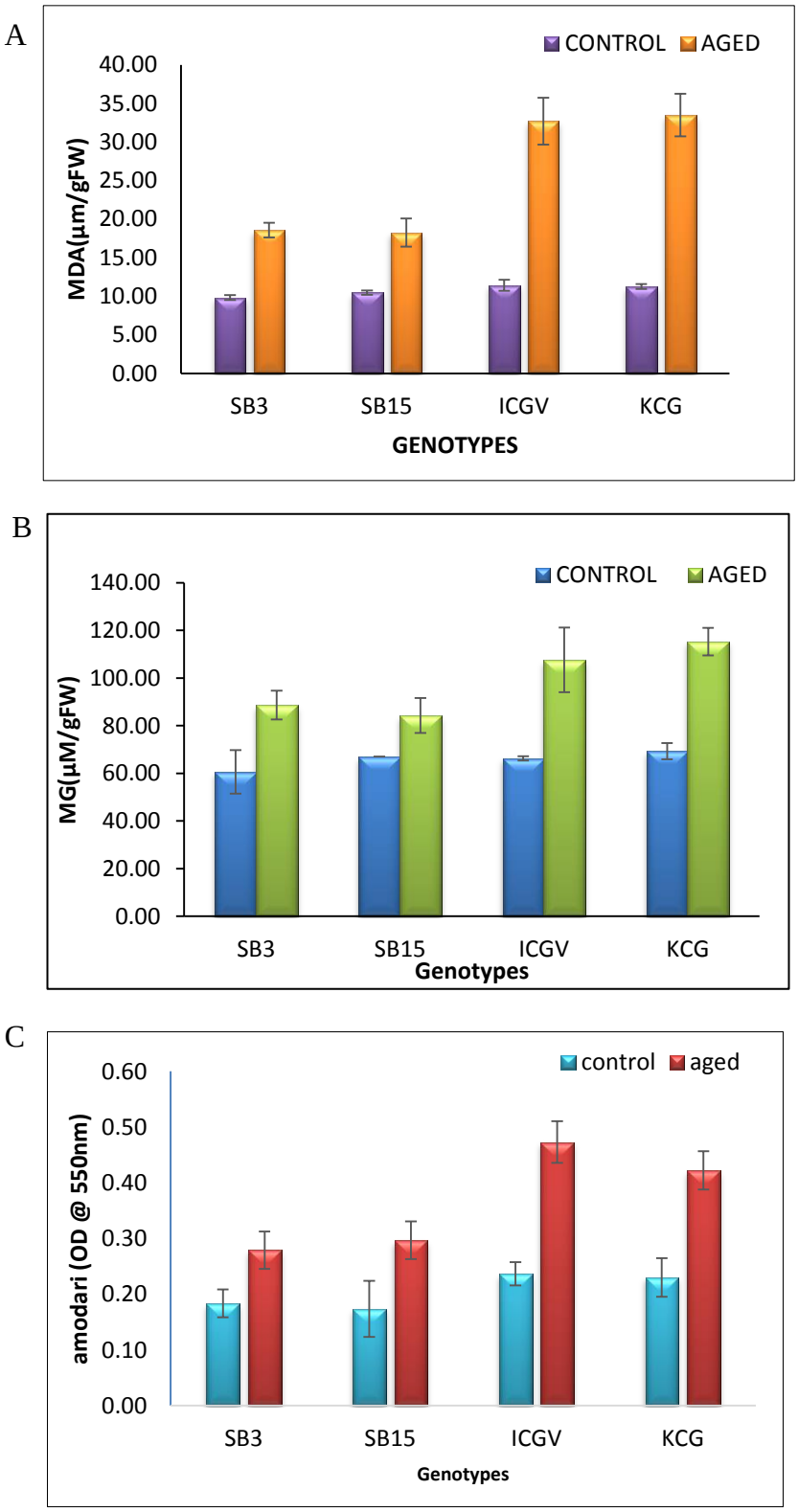



**Fig. 13**: Variation in reactive carbonyl compounds in 4 contrasting groundnut genotypes subjected for accelerated ageing treatment

(A) Melondialdehyde.

(B) Methyl glyoxal.

(C) Amadori products.

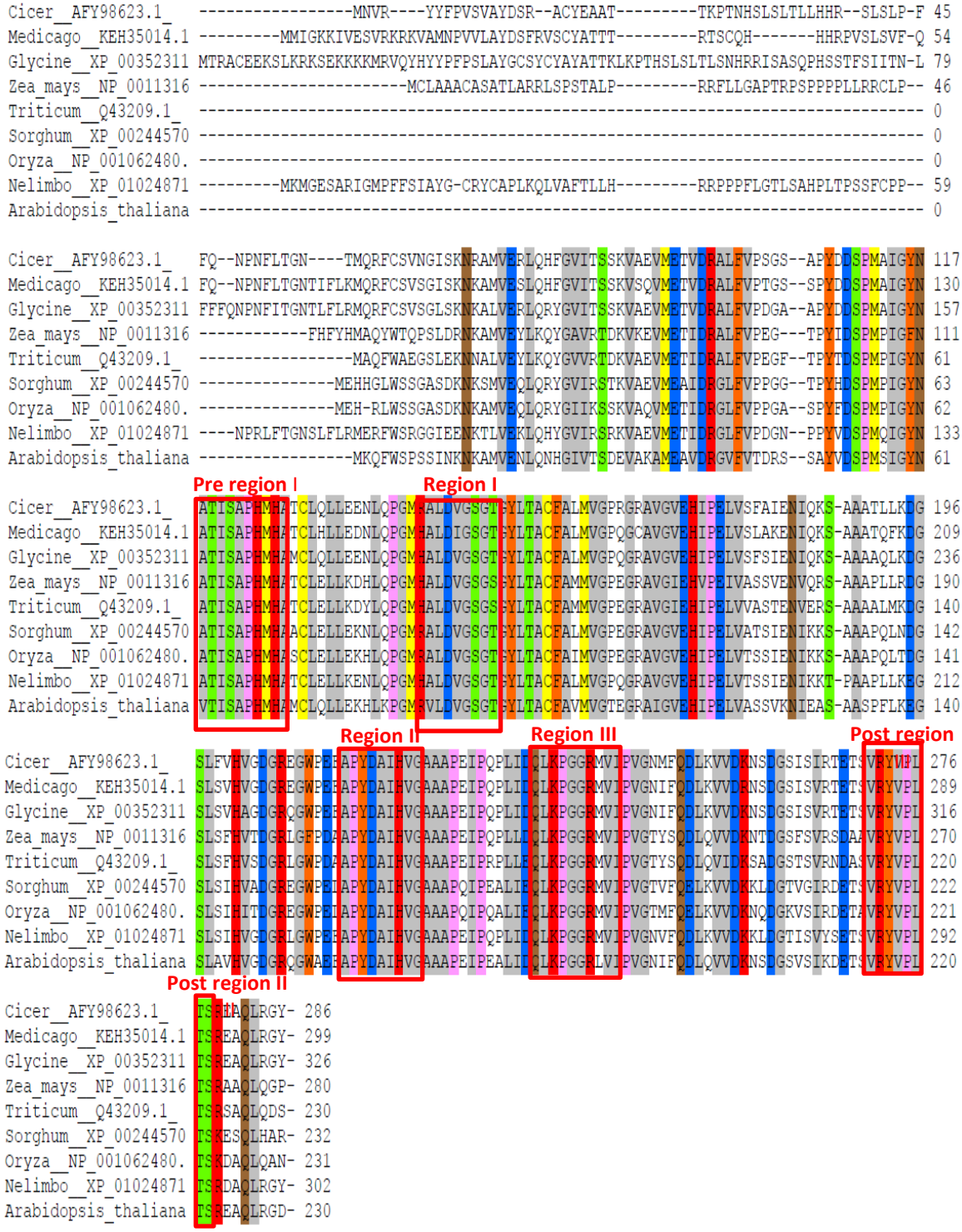



**Fig. 14:** Alignment of amino acid sequence of PIMT2 across crop species and amino acid residues were identical across the species are indicated in different colors

**Table 2: Seed germination as influenced by ageing treatment in contrasting genotypes**

| | Percent seed germination | |
|---|---|---|
| Genotypes | Control | 6 days of AA |
| SB3 | 88 | 70 |
| SB15 | 89 | 70 |
| ICGV9114 | 83 | 50 |
| KCG6 | 83 | 50 |
| CD @ 0.05 | 9.32 | 6.0 |
| CV (%) | 3.09 | 11.67 |

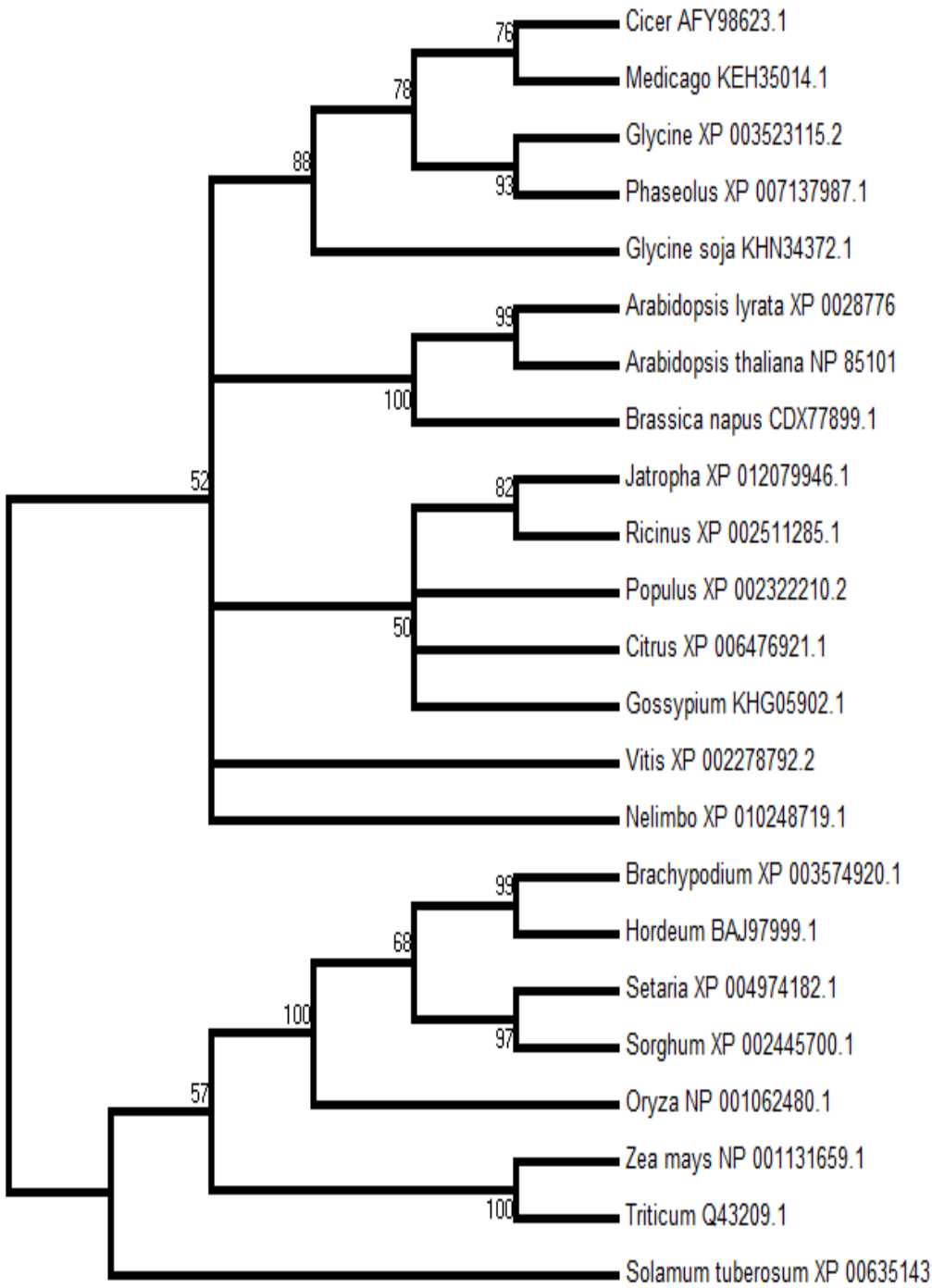



**Fig. 15: Phylogenetic analysis of revised amino acid sequences of PIMT2 across different plant species**

Amino acid sequence of caPIMT2 along with other orthologous PIMT2 amino acid sequences were used to derive phylogenetic tree by using MEGA5.

(for sources- number following species name indicates gene bank accession number from NCBI data base)

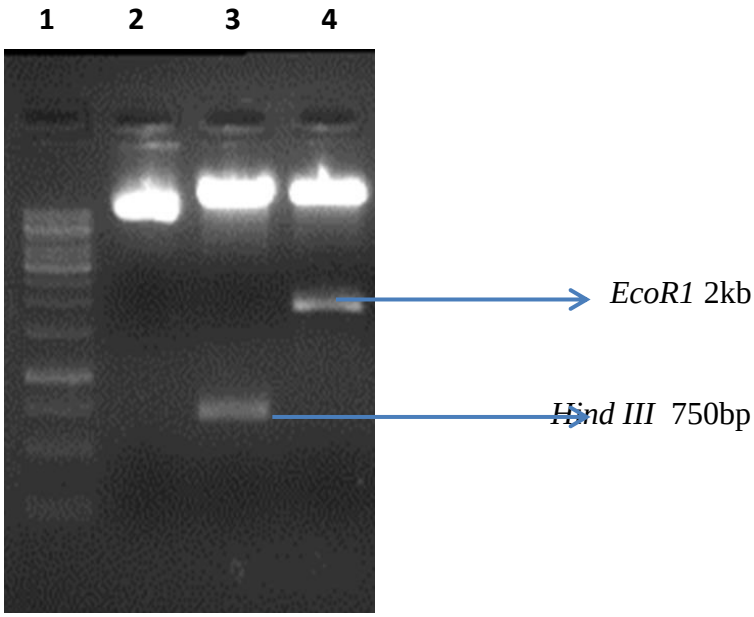



**Plate 3:** Restriction digestion of pCAMBIA2301 harboring NAPIN:PIMT2 gene cassette.. Lane 1: 1 KB ladder. Lane 2: pCAMBIA uncut PIMT2. Lane 3: pCAMBIA-PIMT2 digested with *HindIII*; Lane 4: pCAMBIA-PIMT2 digested with *EcoR1*

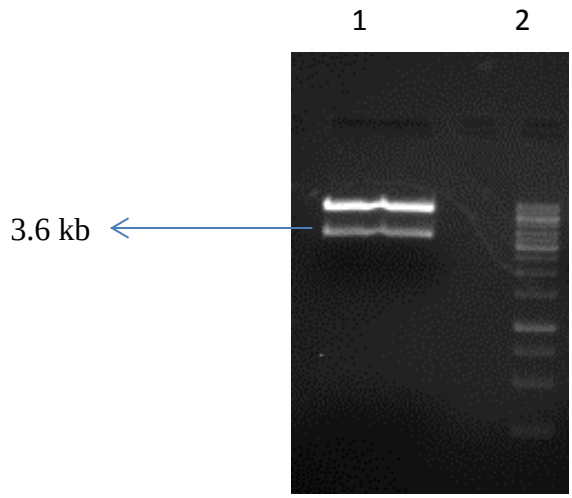



**Plate 4:** Restriction digestion of pi12GW harboring PRBCS:PsAKR1:TRBCS. Lane 1: Restriction digestion of PsAKR1 with Asc1 and Pac1. lane 2: 1 Kb ladder

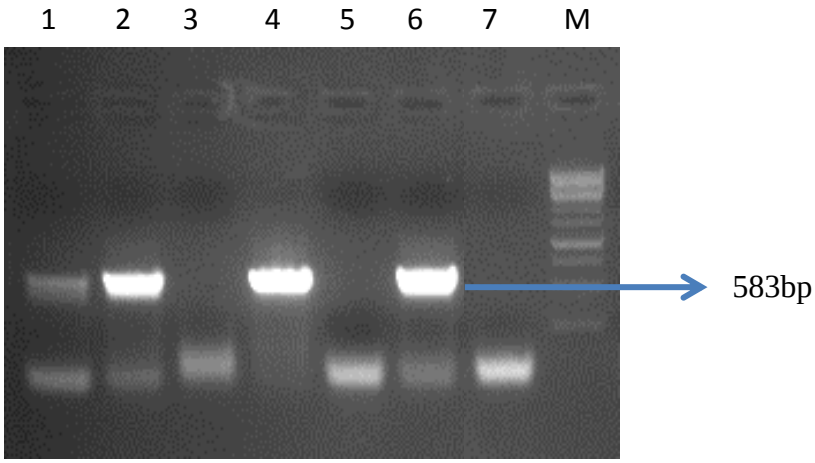



**Plate 5: *E-coli* colony PCR amplification of pi12GW//RBCS:AKR1:TRBCS gene cassette using gene specific primers**

Lane 1-5: PCR product of independent *E-coli* colonies.
Lane 6: reaction positive (plasmid).
Lane 7: reaction blank.
M: 1 KB ladder.

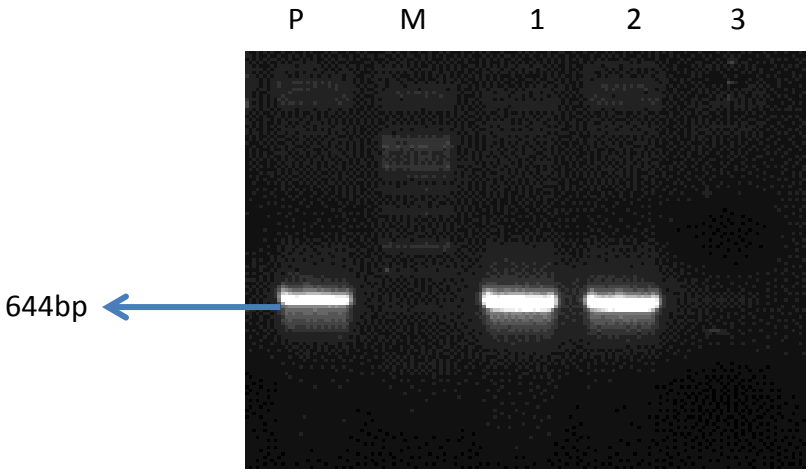



**Plate 6: *E-coli* colony PCR amplification of pCAMBIA2301//NAPIN:PIMT2 gene construct using gene specific primers**

Lane P: reaction positive (plasmid).
Lane 1 and 2: PCR product of independent colonies
Lane 3: reaction blank.
M: 1 KB ladder.

#### 4.2.3.2 Development double gene cassette co-expressing PsAKR1 and CaPIMT2 into suitable binary vector

pmf12GW a marker free binary vector which was developed at Dept. of Crop Physiology, UAS, GKVK, Bengaluru was selected for development of double gene cassette of PRBCS:AKR1:TRBCS::NAPIN:PIMT2:TRBCS.

Pmf12GW binary vector and NAPIN:PIMT2 gene cassette was digested sequentially with *EcoR1* restriction enzymes which releases an expected 8 kb and 2 kb respectively. The digested product were resolved on agarose (0.8 %) gel (plate 7) followed by blunting of *EcoR1* digested NAPIN:PIMT2 gene cassette and pmf12GW binary vector (Plate 7). Eluted products were purified and used for ligation reaction. The product of ligation mixture was introduced into the competent host cell (DH5α) and the transformants were confirmed by colony PCR using CaPIMT2 gene specific primers (Appendix1) (Plate 8). Further, Sub-cloning of PRBCS:AKR1:TRBCS to pmf12GW marker free binary vector harboring NAPIN:PIMT2:TRBCS gene cassette was performed. To achieve this, PRBSC:AKR1:TRBCS gene cassette and pmf12GW harboring PIMT2 was digested sequentially with *Asc1* and *Pac1* restriction enzymes which releases expected 3.6 kb and 10 kb respectively. The digested product was resolved on agarose (0.8 %) gel (Plate 9). The digested product was eluted and purified further and used for ligation reaction.

#### 4.2.3.3 Confirmation of PRBCS:AKR1:TRBCS::NAPIN:PIMT2:TRBCS in pmf12GW marker free binary vector through *E-coli* colony PCR and restriction digestion

Transformation of ligated product to *E-coli* (DH5α) and confirmation by colony PCR using appropriate gene specific primers (PsAKR1 forward and reverse; CaPIMT2 forward and reverse) (Appendix 1) .The expected PCR amplified product of 583 bp and 644 bp fragments respectively was resolved on agarose (0.8 %) gel (Plate 10). Plasmid isolation from the confirmed colony followed by confirmation through restriction digestion using the appropriate restriction enzymes was done. Around 1-2 µg of plasmid DNA was used for restriction digestion. Plasmid was digested with *EcoR1, Xbal, Asc1 and Pac1* for 1 h at 37 °C. The digested product resulted in the expected band size compared with undigested plasmid by electrophoresis on 0.8 % agarose containing 0.5 µg/ml ethidium bromide using 1X TAE buffer at 90V to confirm the gene cassette (Plate 11).

#### 4.2.3.4 Confirmation of pi12GW//PRBCS:AKR1:TRBCS gene cassette, pCAMBIA2301//NAPIN:PIMT2:TRBCS and pmf12GW marker free vector harboring PRBCS:AKR1:TRBCS::NAPIN:PIMT2:NOS gene cassette in *Agrobacterium* cells

It is per-requisite for plant transformation to mobilize the binary vector harboring single gene cassette PsAKR1; CaPIMT2 and double gene cassette (AKR1 and PIMT2) into *Agrobacterium*. For which, all the three binary vectors were transformed to *Agrobacterium* competent cells, EHA105 and transformants were confirmed by colony PCR using appropriate gene specific primers (Appendix 1) for the presences of AKR1

and PIMT2 in single gene cassette and in double gene cassette. The amplified product was resolved in 0.8 % agarose gel to confirm the presence of gene. pi12GW//PRBCS:AKR1;TRBCS was confirmed in *Agrobacterium cells* (Plate 12). pCAMBIA2301//NAPIN:PIMT2:TRBCS was confirmed in *Agrobacterium* cells (Plate 13). pmf12GW//PRBCS:AKR1:TRBCS::NAPIN:PIMT2:TRBCS was confirmed in *Agrobacterium* cells (Plate 14).

#### 4.2.3.5 3-ketoglycosidase test for Agrobacterium cells

To test for *agrobacterium* cells, 3-Ketoglycosidase assay was adopted. This test works on metabolic differences between the *agrobacterium* genes and other bacterium such as *E-coli* and rhizobium species. Agrobacterium species grown on lactose makes an enzyme Called hexapyronosides cytochrome oxireductase that can convert into 3-ketolactos (yellow) with benedict reagent as an indicator. Accordingly, the construct turned yellow colour when react with benedict reagent, suggesting the gene cassette are in *agrobacterium* cells (Plate 15).

### 4.3 *Agrobacterium* mediated modified *in planta* transformation was adopted to develop gronundnut transgenic plants

Groundnut is difficult to regenerate through *in vitro* technique. Therefore, it is crucial to have an efficient standardized transformation protocol for development of groundnut transgenic.

#### 4.3.1 Selection of seed material for transformation:

While characterizing the selected groundnut germplam for seed viability, a groundnut variety, KCG6 one of the leading varieties showed a shorter seed viability with higher level of cytotoxic compounds. Being the leading variety, it is highly relevant to improve the seed viability of this variety. Accordingly, KCG6 was used as a seed material for transformation. Following the earlier standardized protocol (Sankara *et al.,* 2009) using embryonic cells infection, transgenics were developed with a single gene construct (overexpressing AKR1 and PIMT2 separately) and with double gene cassette (co-expressing AKR1 and PIMT2 together) following modified *in planta* transformation technique as described in material and methods (3.3.4).

#### 4.3.2 Identification of putative transformants through screening technique

The approach is to standardize the protocol in wild type and then subsequently screen the transformed seeds at particular concentration with selectable marker. This method was used to screen large number of transformants and technique help to identify promising transformants.

#### 4.3.2.1 Standardization of lethal concentration of glyphosate and kanamycin for selection

For the initial standardization and screening on glyphosate (AKR1 as screenable marker) and on kanamycin (npt2 as selectable marker) mature seeds of wild type KCG6 were sterilized in 4 % sodium hypochloride solution followed by soaking in for 6 h in

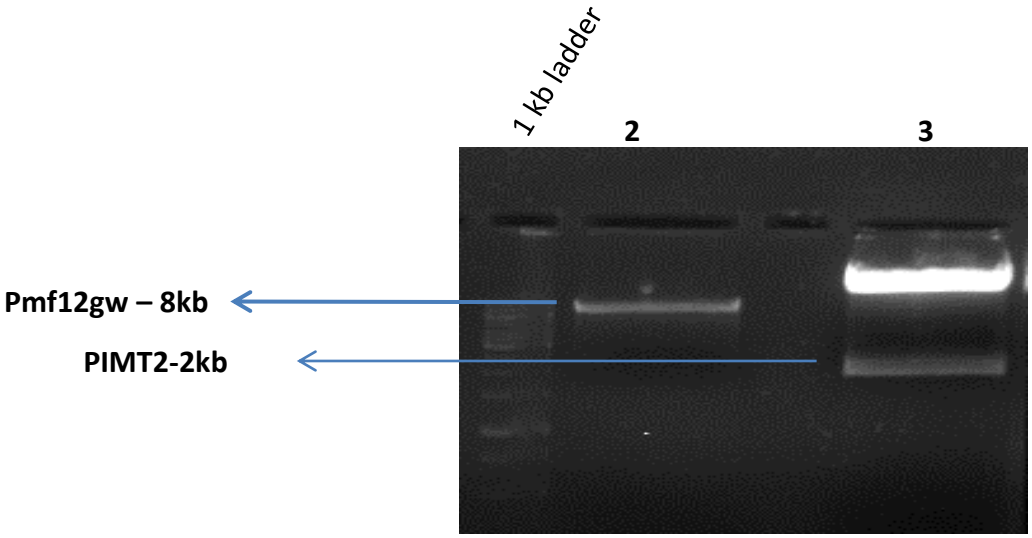



**Plate 7: Restriction digestion of pmf12GW binary vector and pCAMBIA2301//NAPIN:PIMT2 gene cassette**
Lane1: 1KB ladder.
Lane 2: Restriction digestion of Pmf12gw vector with *EcoR1*.
Lane 3: PIMT2 digested with *EcoR1*

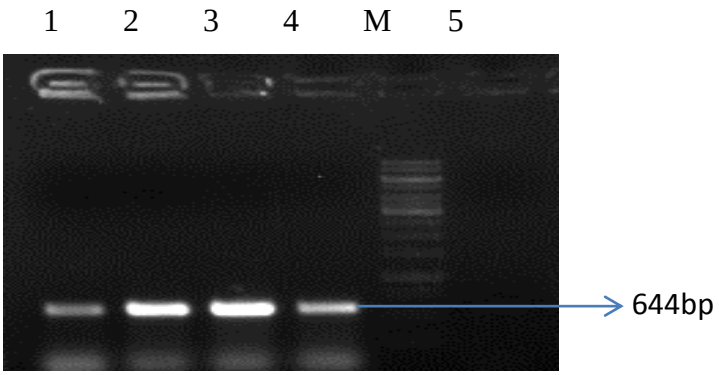



**Plate 8: *E-coli* colony PCR amplification of pmf12GW binary vector harboring NAPIN:PIMT2 using gene specific primers**
Lane 1-3: PCR product of independent colonies of PIMT2 in binary vector pmf12GW.
Lane 4: Reaction positive (plasmid).
Lane 5 : reaction blank.
M: 1 KB ladder.

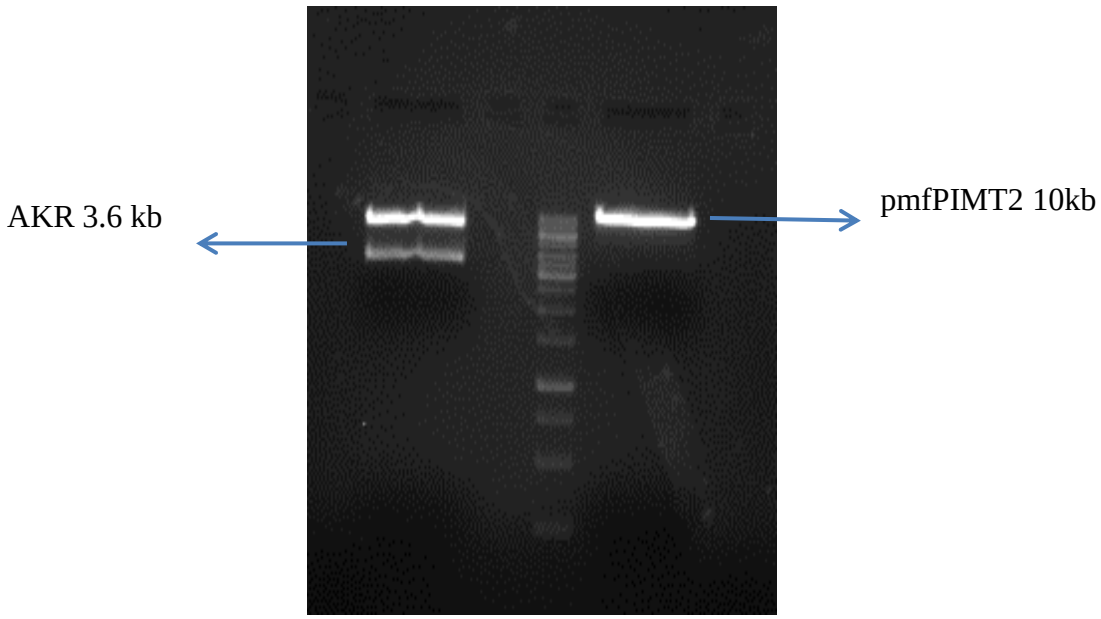



**Plate 9: Sub cloning of PRBCS:AKR1:TRBCS into pmf12GW harboring NAPIN:PIMT2:TRBCS**

Restriction digestion of AKR1 and PmfPIMT2 with Asc1 and Pac1.
Lane 1: Restriction digestion of AKR with Asc1 and Pac1.
Lane 2: Restriction digestion of PmfPIMT2 with Asc1 and Pac1.
M-1KB Ladder.

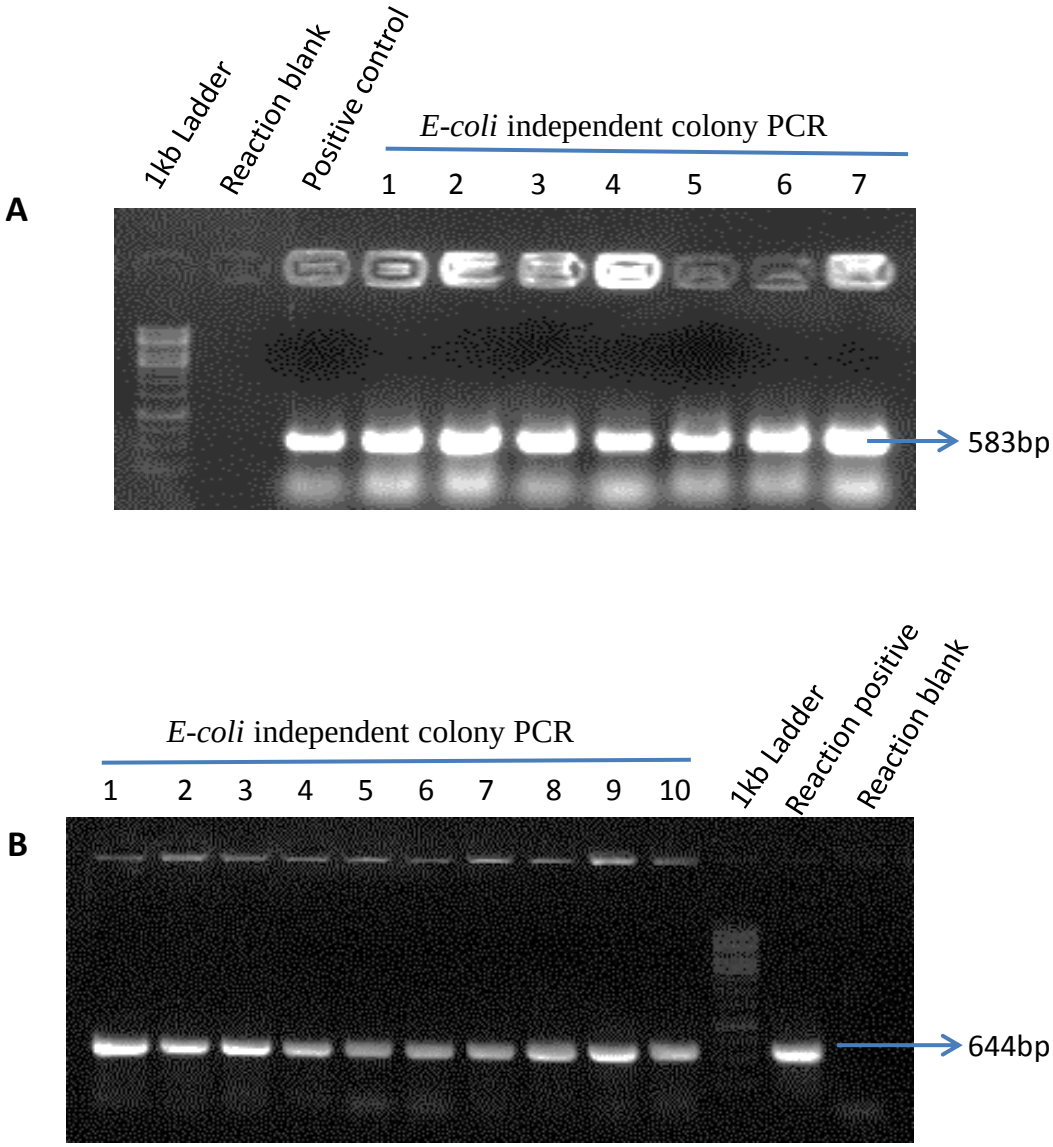
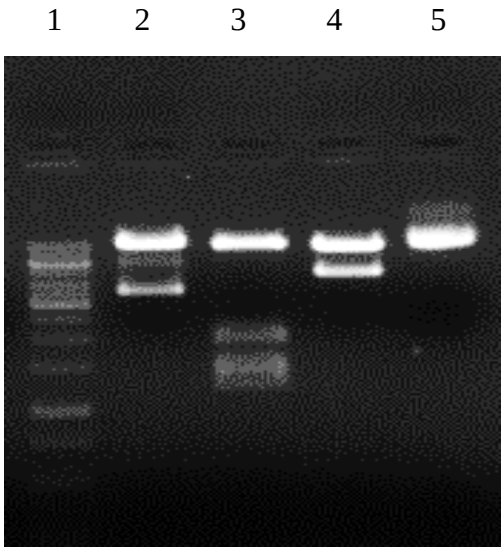



**Plate 10: *E-coli* colony PCR confirmation RBCS:AKR1:TRBCS::NAPIN::PIMT2:TRBCS in pmf12GW binary vector using gene specific primers**

**(A)** *E-coli* independent colony PCR for AKR1 using gene specific primers.

(B) *E-coli* independent colony PCR for PIMT2 using gene specific primers




Lane 1 - 1kb ladder

Lane 2 – Asc1 and Pac1 digested plasmid with 9.5 kb and 3.6 kb.

Lane 3 – EcoR1 digested plamid with 9.5 kb and 2.6 kb.

Lane 3 - Xbal digested plamid with 11 kb

Lane 4 – uncut plasmid

**Plate 11: Confirmation of the double gene cassette in pmf12GW binary vector (PRBCS:AKR1:TRBCS::NAPIN:PIMT2:TRBCS) by restriction digestion**

The gene cassette was confirmed in transformants by restriction digestion with *EcoR1,Xbal, Asc1* and *Pac1* restriction enzymes

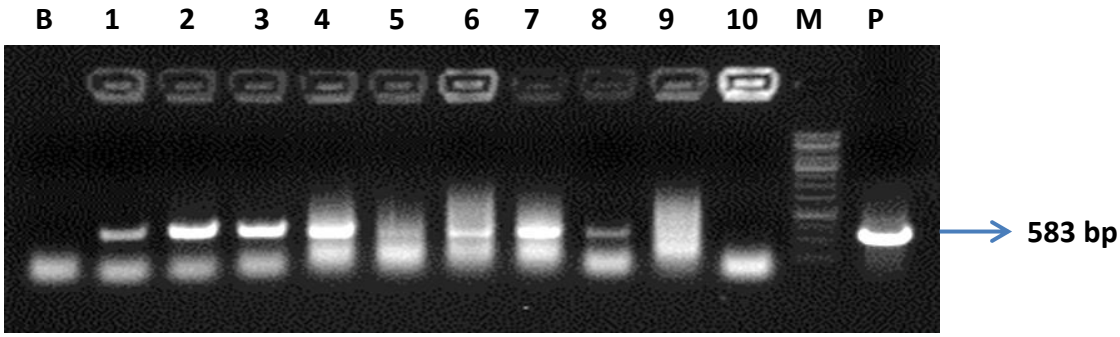



**Plate 12 : *Agrobacterium* colony PCR confirmation of PRBCS:AKR1:TRBCS in binary vector using gene specific primers**
Lane 1- 10: PCR product of independent *Agrobacterium* colonies.
M- 1KB ladder.
Lane B- reaction blank.
Lane P- Reaction positive(plasmid).

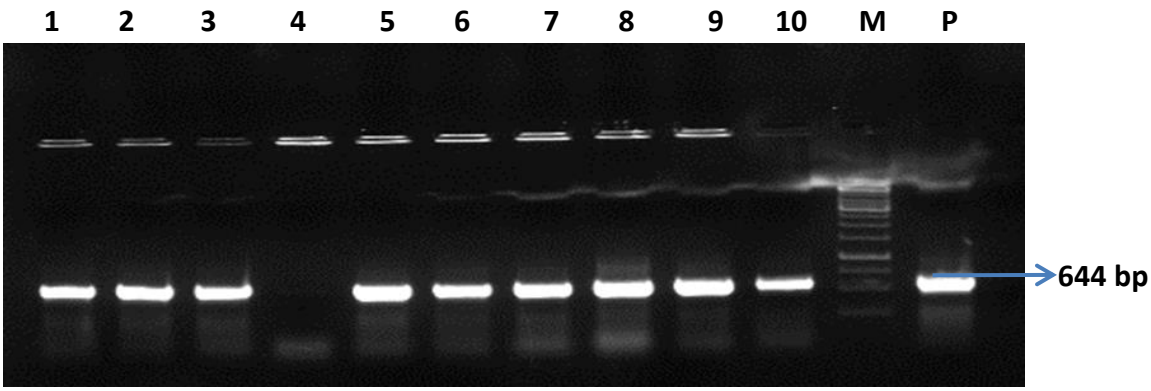



**Plate 13: *Agrobacterium* colony PCR confirmation of NAPIN:PIMT2:TRBCS gene cassette in pCAMBIA2301 binary vector using gene specific primers**
Lane 1-10: PCR product of independent *Agrobacterium* colonies.
M-1KB ladder.
Lane P- plasmid (positive**).**

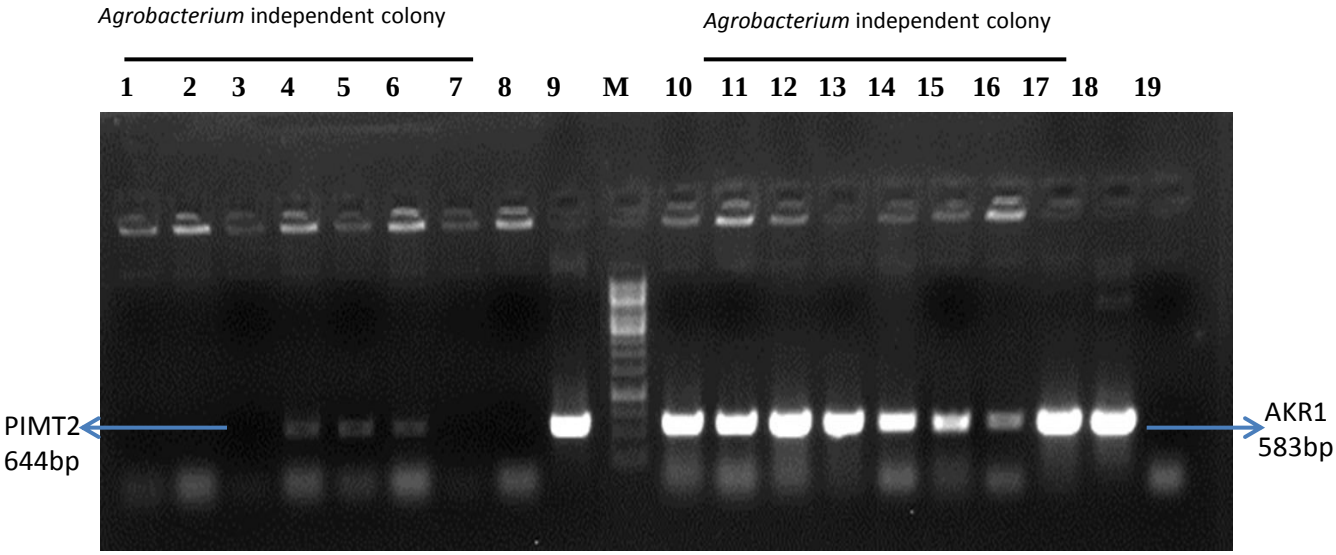
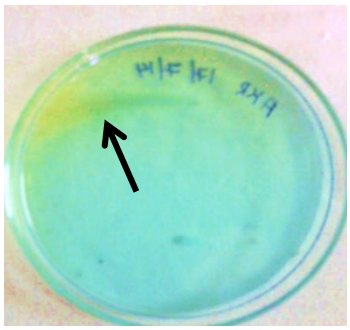



**Plate 14: *Agrobacterium* colony PCR confirmation of AKR1 and PIMT2 (PRBCS:AKR1:TRBCS::NAPIN::PIMT2:TRBCS) in pmf12GW binary vector**

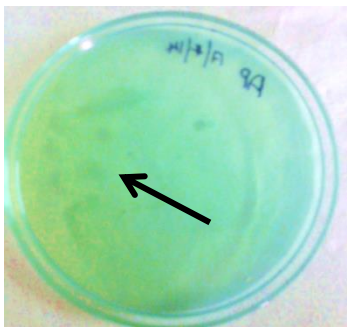
Lane 1-7: PCR amplification of independent *Agrobacterium* colonies of PIMT2 using gene specific primer.
Lane 8: reaction blank for PIMT2.
Lane 9: reaction positive (PIMT2 plasmid).
M-1KB ladder.
Lane 10-17: PCR amplification of independent *Agrobacterium* colonies of AKR1 using gene specific primer.
Lane 18: reaction positive(AKR1 plasmid).
Lane 19: reaction blank of AKR1.

(A)



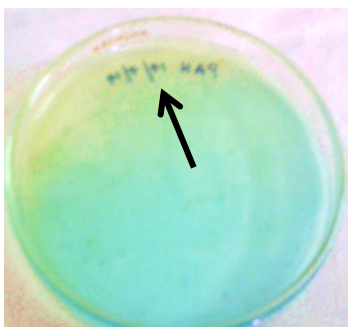
**pi12GW//PRBCS:AKR1:TRBCS**

(B)



**pCAMBIA2301// NAPIN:PIMT2:TRBCS**

(C)



**pmf12GW//PRBCS:AKR1:TRBCS::NAPIN:PIMT2:TRBS**

**Plate 15: 3-ketoglycosidase test for *Agrobacterium* cells**

distilled water and then germinated aseptically at 28 °C. One day old germinated seedlings of approximately 6 mm root length were placed on autoclaved quartz sand with different concentration of glyphosate (0, 10, 15, 20, 25, 30 ppm) and kanamycin (0, 100, 200, 300, 400, 400 ppm) separately. The seedling growth was inhibited with increasing concentration of glyphosate and kanamycin and also showed susceptibility at concentrations of 30 ppm for glyphosate and 400 ppm for kanamycin. On the basis of plant growth inhibitory response, 30 ppm of glyphosate and 400 ppm of kanamycin were selected as lethal dose to screen the putative T0 transformants (Plate 16 and 17).

#### 4.3.2.2 Screening of the $T_0$ putative transformants against 30 ppm of glyphosate and 400 ppm of kanamycin

$T_0$ transformants (*Agrobacterium* infected, one day germinated healthy seedling) were screened on 30 ppm glyphosate (PRBCS:AKR1:TRBCS gene construct, PRBCS:AKR1:TRBCS::NAPIN:PIMT2:TRBCS genes construct) (Plate 18) and 400 ppm of kanamycin (NAPIN:PIMT2:TRBCS gene construct) (Plate 19). Out of 500 infected seeds in each construct 31 plants (co-expressing AKR1 and PIMT2) showed and 24 plants (over expressing AKR1) showed tolerance and survived in 30 ppm of glyphosate, while 25 plants out of 500 infected seedling (overexpressing PIMT2) showed tolerance against 400 ppm of kanamycin with 6.2 %, 4.8 % and 5 % percentage recovery respectively in $T_0$ generation (Table 3). The glyphosate and kanamycin tolerant $T_0$ plants were grown under controlled environment condition and advanced to next generation (Plate 20).

#### 4.3.2.3 Screening of putative $T_1$ groundnut transformants against glyphosate and kanamycin

The $T_1$ seeds of putative transformants were screened on 30 ppm of glyphosate (Overexpressing AKR1 and Co-expressing AKR+PIMT2 transformants) and 400 ppm of kanamycin (Overexpressing PIMT2) (Plate 21). The wild type seedling were susceptible at a minimum concentration of 30 ppm of glyphosate and 400 ppm of kanamycin and showed severe growth reduction and unable to grow upon stress recovery whereas, the $T_1$ putative transformants showed tolerance even at 30 ppm of glyphosate and 400 ppm of kanamycin which showed good root and shoot growth. Totally, 950 seeds co-expressing AKR1 and PIMT2 and 720 seeds overexpressing AKR1 were screened on 30 ppm of glyphosate out of which 110 and 60 plants respectively showed tolerance with good root and shoot growth compared to wild type (Plate 22 & 24). Similarly, out of 750 seeds overexpressing PIMT2 gene, 70 plants survived and showed performed better than wild type at 400 ppm of kanamycin (Plate 26). The percent recovery in $T_1$ generation was significant (Table 3). The percentage of transformed plants in $T_1$ generation was significantly higher by adopting this modified *in planta* transformation protocol.

#### 4.3.2.4 Molecular characterization of putative $T_1$ transformants.

The glyphosate/ kanamycin tolerant $T_1$ putative groundnut transgenic plants grown in green house condition were subjected to molecular analysis. To confirm the integration of T-DNA in transformants obtained by modified *in planta* transformation technique, the genomic DNA was isolated from $T_1$ putative groundnut transgenic leaves

and used as template in the PCR reaction. Out of 110 putative $T_1$ transformants co-expressing AKR1 and PIMT2, 35 putative $T_1$ transformants lines were showed the presences of insert (Plate 23 & Table 3). Similarly, out of 60 putative $T_1$ transformants overexpressing AKR1, 12 putative $T_1$ transformants lines were confirmed the presence of AKR1 gene (Plate 25 & Table 3) and out of 70 putative $T_1$ transformants overexpressing PIMT2, 26 $T_1$ putative transformants lines showed the presence of PIMT2 (Plate 27, Table 3). However in all these cases, PCR products were amplified using promoter forward and gene specific reverse primers and also tested using nested primers (Appendix 1). All the survived and tolerant lines were advanced to $T_2$ generation (Plate 28).

#### 4.3.2.5 RT PCR analysis

To assess the expression of transgene in transgenic plant co-expressing PsAKR and CaPIMT2, the transcript level were analyzed by semi quantitative RT PCR. In the transgenic line, the expression of transgene was observed and the expression of AKR1 and PIMT2 found to be highly significant in seeds compared to leaf (Plate 29). This confirms the integration and expression of gene in transgenic plants.

Molecular characterization of glyphosate and kanamycin tolerant putative $T_1$ transformants suggest that, stable transformation can be obtained by adopting modified *in planta* transformation technique and following physiological screening (Table 3).

### 4.4 Physiological characterization of $T_2$ transgenic seeds co-expressing AKR1 and PIMT2

Transgenic groundnut developed adopting *in planta* transformation technique were advanced to $T_2$ generation. $T_2$ generation seeds were used to test the proof of concept whether or not the AKR and PIMT have any role in regulating seed viability, seedling vigour and growth of plants.

#### 4.4.1 Assessment of seed viability in wild type and putative $T_2$ transgenic groundnut co-expressing AKR1 and PIMT2 genes

The seeds of both wild type and $T_2$ transgenic groundnut co-expressing AKR1 and PIMT2 were subjected for accelerated ageing for 8 days maintaining an ambient temperature of 45 °C and 100 % RH then assessed for seed viability through TTC test. It was observed that, the transgenic seeds co-expressing AKR1 and PIMT2 showed enhanced seed viability compared to wild type. This is evident from the visual observation that, the extent of formazan staining was more in transgenic seeds compared to wild type (Plate 30).

Although ageing treatment reduced the number as well as intensity of staining, the extent of reduction in formazan staining was significantly higher in wild type seeds compared to transgenics. In fact, after 8 days of accelerated ageing, hardly few seeds of wild type groundnut showed TTC staining, while relatively more number of seeds showed staining in transgenic seeds indicating the role of AKRs and PIMT in improving the seed viability (Plate 30).

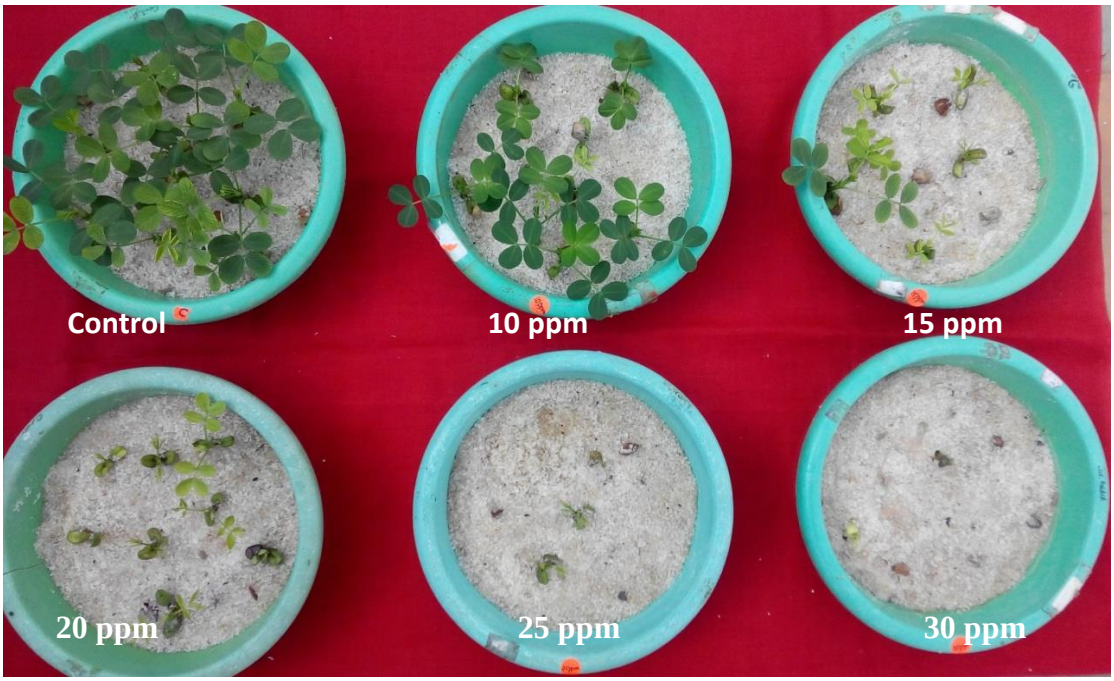




**Plate 16: Standardization of glyphosate lethal concentration for groundnut screening**

The groundnut variety, KCG-6 seeds were soaked overnight in distilled water and pre-germinated seeds were sown on different concentration of glyphosate on sterilized quartz sand. After 6-7 days of germination, the observation were recorded based on percent germination and recovery.

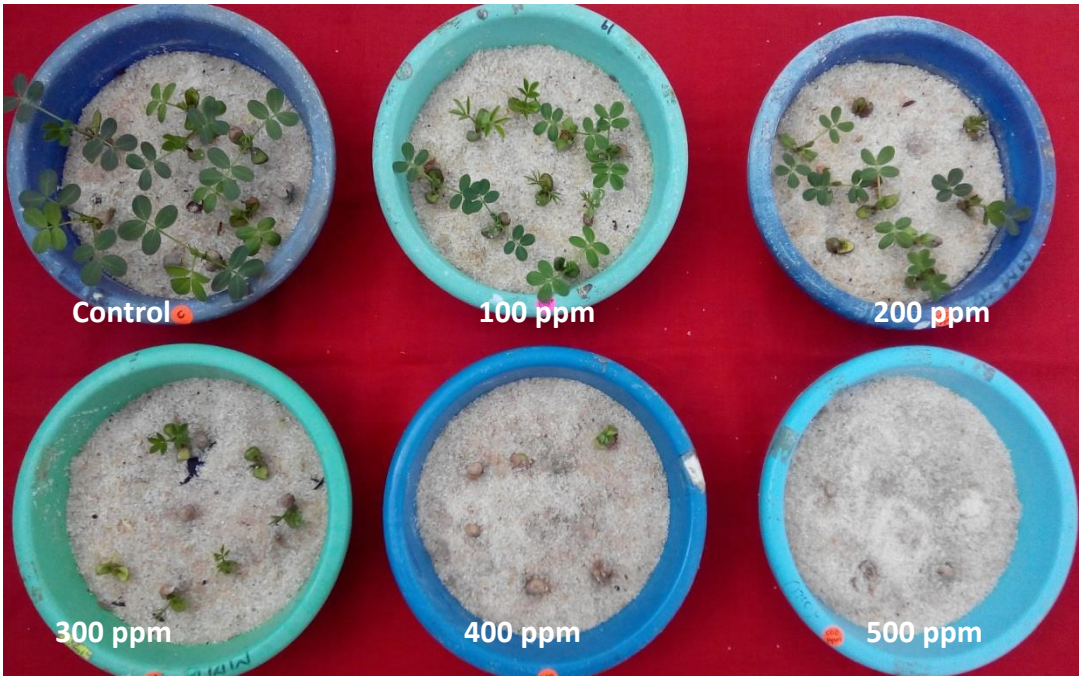
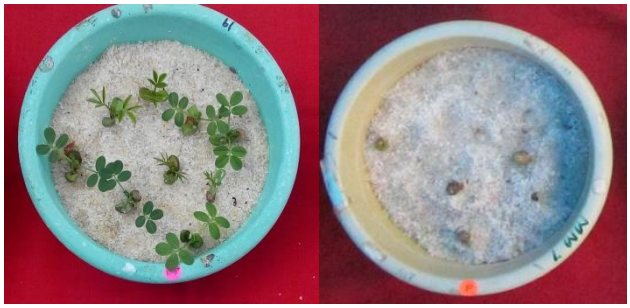




**Plate 17: Standardization of kanamycin lethal concentration for groundnut screening**

The groundnut variety, KCG-6 seeds were soaked overnight in distilled water and pre-germinated seeds were sown on different concentration of glyphosate on sterilized quartz sand. After 6-7 days of germination, the observation were recorded based on percent germination and recovery.



control WT

(A)

AKR1
$T_O$ Putative
transformants

(B)

AKR1-PIMT2
$T_0$ putative
transformants

control WT

(A)

AKR1 $T_O$ Putative transformants

(B)

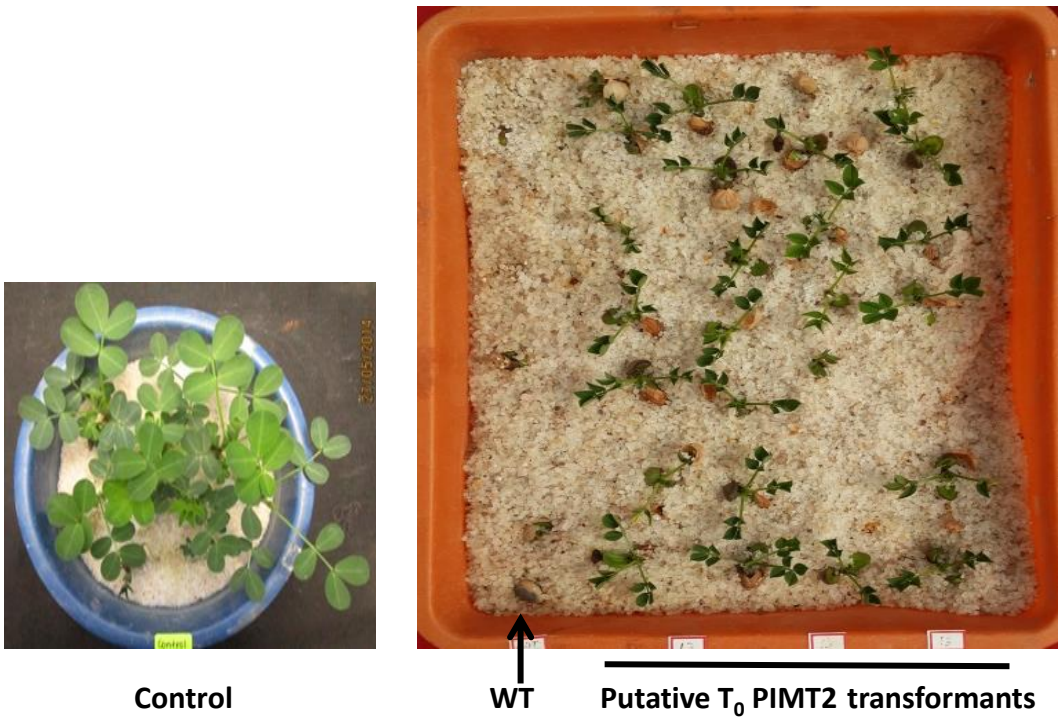
AKR1-PIMT2 $T_0$ putative transformants

**Plate 18: Screening of putative $T_0$ putative Groundnut transformants against 30 ppm of glyphosate**

*Agrobacterium* infected and 2 days old germinated seedlings along with wild-type were screened against 30 ppm glyphosate .

(A) Overexpressing AKR1 (PRBCS:AKR1:TRBCS) $T_0$ putative transformants.

(B) Co-expressing AKR1-PIMT2 (PRBCS:AKR1:TRBCS::NAPIN:PIMT2:TRBCS) $T_0$ putative transformants.



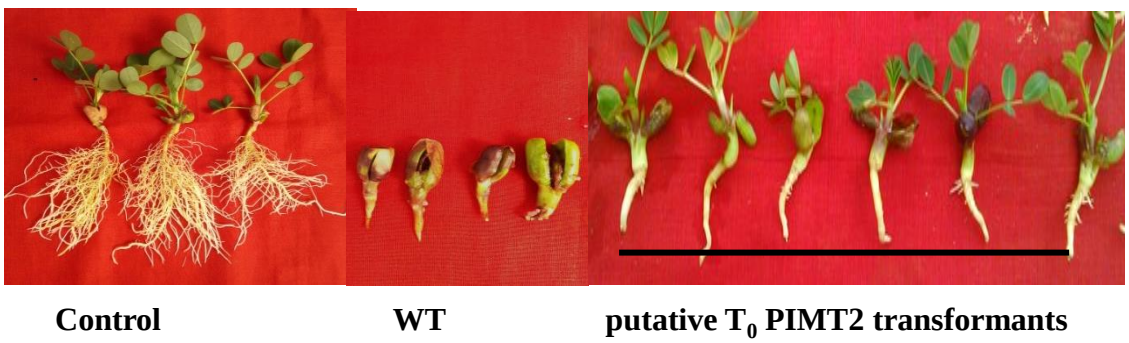
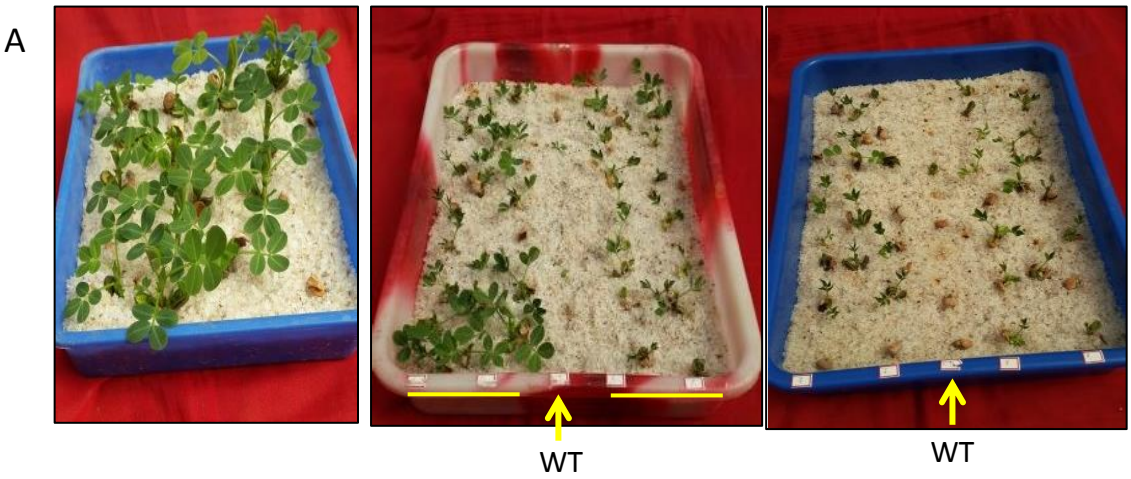




**Plate 19: Screening of putative $T_0$ Groundnut transformants overexpressing PIMT2 against 400 ppm of kanamycin**

*Agrobacterium* infected, 2 days old germinated seedlings along with wild-type were screened against 400 ppm kanamycin i.e, Overexpressing PIMT2 (NAPIN:PIMT2;NOS) transformants.

**Plate 20: Photograph showing $T_0$ transformants grown and maintained under green house conditions**



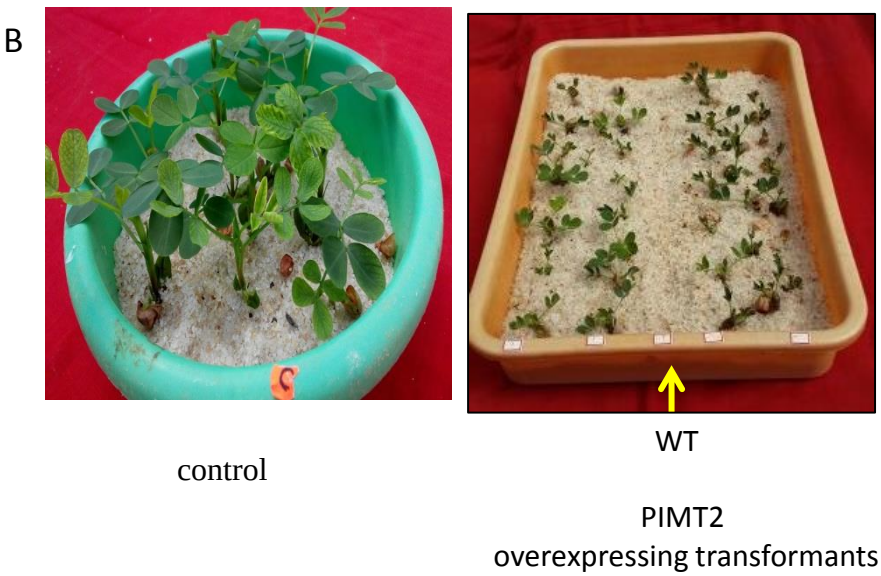




**Plate 21: Screening of putative $T_1$ putative Groundnut transformants against 30 ppm glyphosate and 400 ppm of kanamycin**

(A) Pre-germinated seeds of $T_1$ seeds along with wild-type were screened against 30 ppm glyphosate i.e, co-expressing AKR1-PIMT2 (double gene construct) and overexpressing AKR1 (single gene construct) plants.

(B) Pre-germinated seeds of $T_1$ seeds and wild-type were screened against 400ppm of kanamycin i.e Overexpression of PIMT2( single gene construct)

The plants which survived were advanced for next generation.

Control WT AKR1-PIMT2 putative transformants

**Plate 22: Identification of glyphosate tolerant putative $T_1$ groundnut transformants co-expressing AKR1 and PIMT2**

Pre-germinated seeds of $T_1$ transformants and wild type seeds were screened in 30 ppm glyphosate on autoclaved quartz sand. The distinctly growing seeds were compared with wild type seeds were selected for advancement.

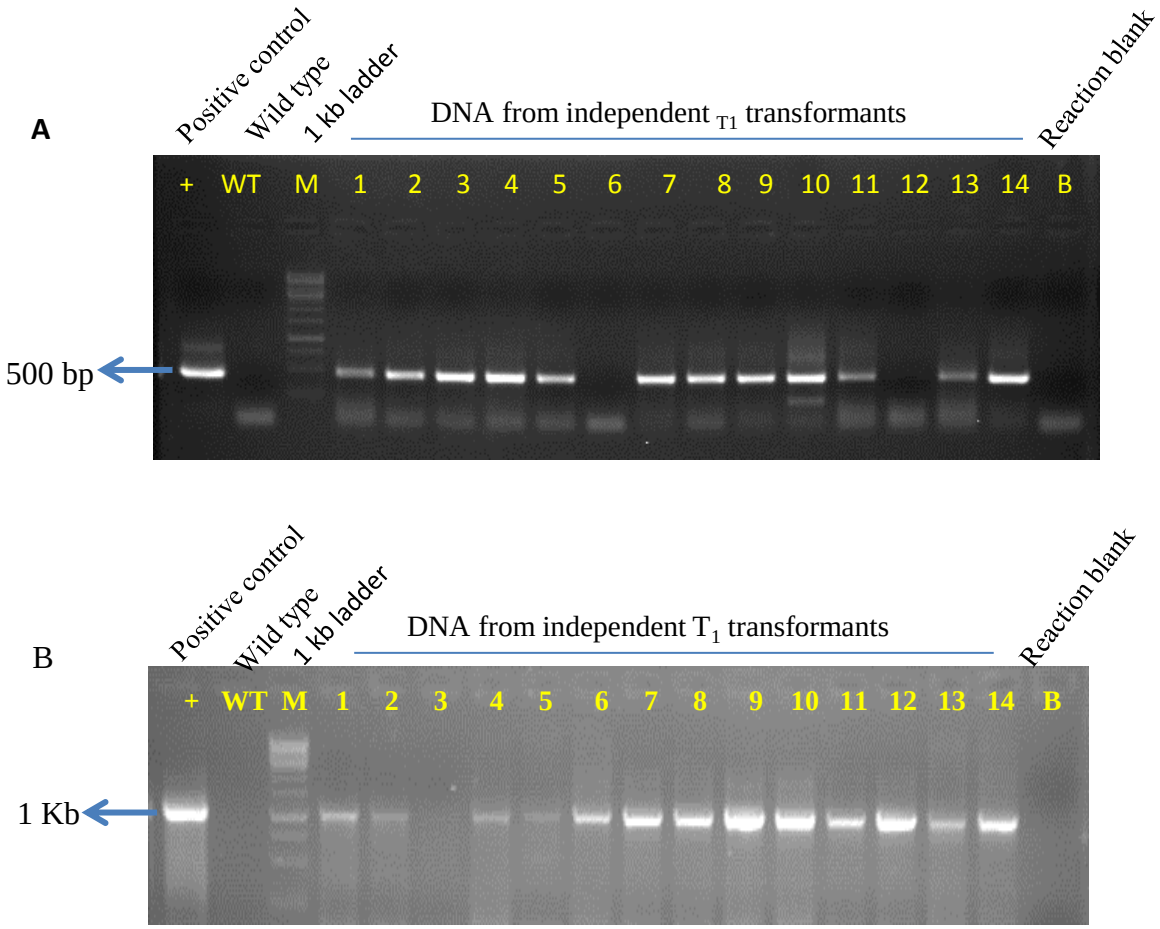



**Plate 23: Molecular characterization of glyphosate tolerant putative $T_1$ transgenic lines co-expressing AKR1 and PIMT2 through PCR**

Genomic DNA was isolated from transformants and used for amplification of PIMT2 (A) and AKR1 (B) by using promoter forward and gene specific reverse primer.


**Plate 24: Identification of glyphosate tolerant putative $T_1$ groundnut transformants overexpressing AKR1**

Pre-germinated seeds of T1 transformants and wild type seeds were screened in 30 ppm glyphosate on autoclaved quartz sand. Glyphosate resistant putative T1 transgenics selected based on shoot and root growth and advance for next generation.

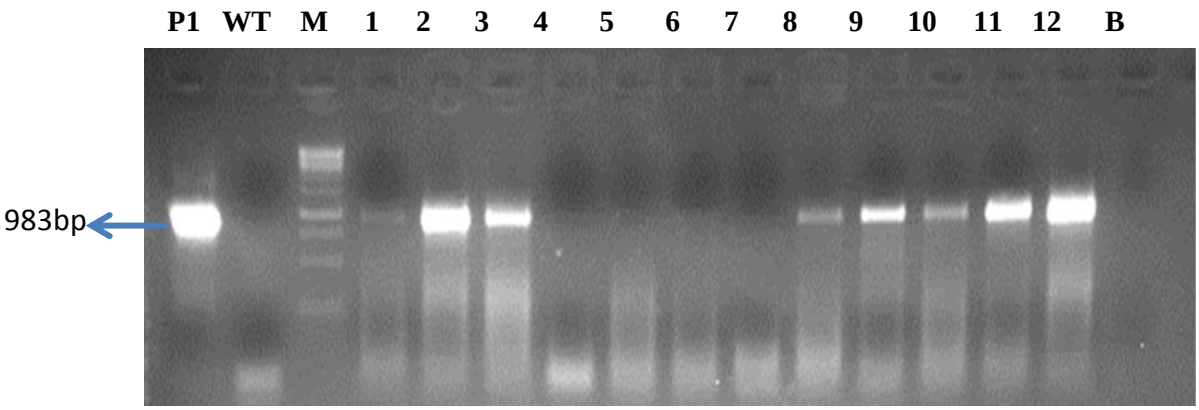



**Plate 25: Molecular confirmation of glyphosate tolerant $T_1$ putative transgenic lines overexpressing AKR1**

The genomic DNA from representative plants were extracted and amplified using promoter forward and gene specific primers. After PCR amplification the amplified product was separated using 0.8 % agarose gel electrophoresis. Lane P1-plasmid positive control, WT- wild type, Lane 1-12 –transformants, Lane B-reaction blank.

Control WT PIMT2 putative transformants

**Plate 26: Identification of kanamycin tolerant putative $T_1$ groundnut transformants overexpressing PIMT2**

Pre-germinated seeds of T1 transformants and wild type seeds were screened in 30 ppm glyphosate on autoclaved quartz sand. kanamycin resistant putative T1 transgenics selected based on shoot and root growth and advance for next generation.

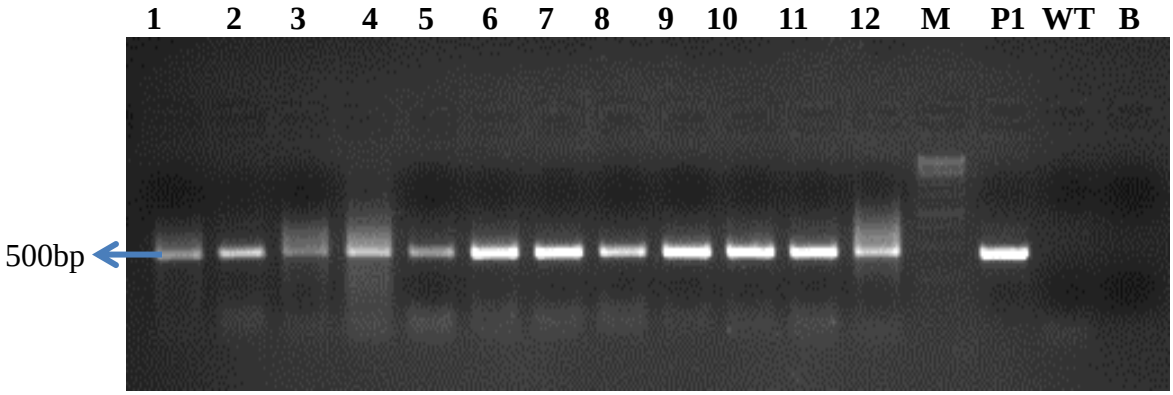



**Plate 27: Molecular confirmation of Kanamycin tolerant $T_1$ putative transgenic lines overexpressing PIMT2**

The genomic DNA from representative plants were extracted and amplified using promoter forward and gene specific primers. After PCR amplification the amplified product was separated using 0.8 % agarose gel electrophoresis. Lane P1-plasmid positive control, WT- wild type, Lane 1-12 – PIMT2 transformants, Lane B-reaction blank.


**Plate 28: Putative $T_1$ transformants grown under green house conditions**

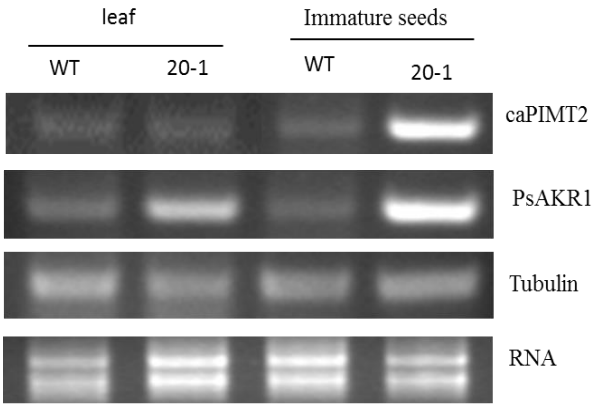
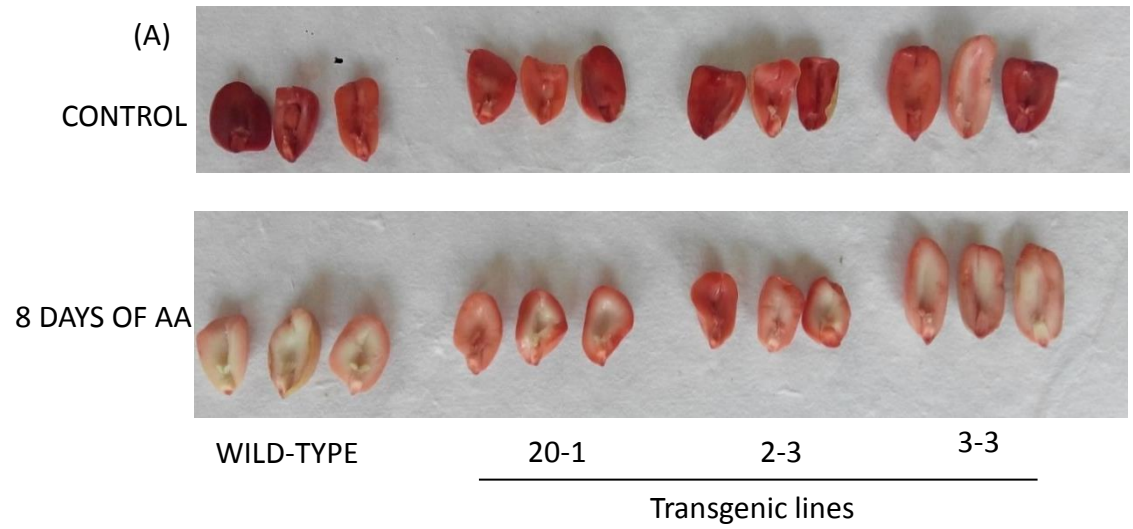



**Plate 29: Semi-quantitative RT PCR expression analysis of AKR1 and PIMT2 in transgenic and Wild type plants**

RT-PCR analysis of AKR1 and PIMT2 co-expressing groundnut transgenic plants along with wild type. Tubulin was used as loading control. Total RNA was isolated from leaf and seed of transgenic and wild type, reverse transcribed and first-strand cDNA was used as template for expression analysis



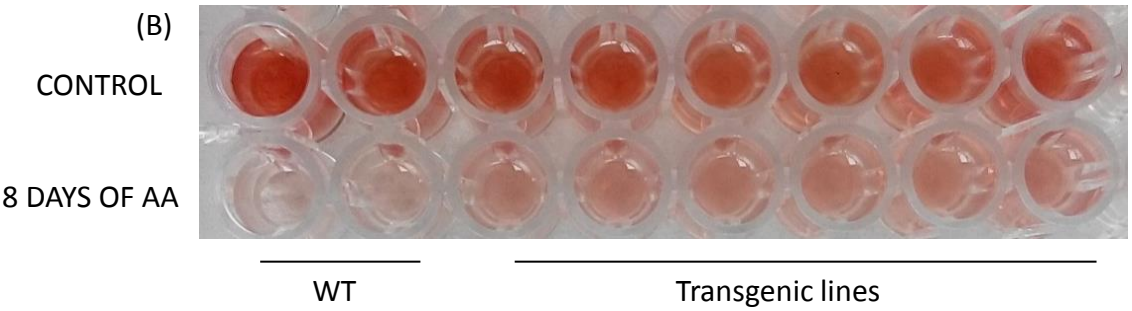




**Plate 30: Variation in color intensity (Formazan level) upon accelerated ageing in wild type and $T_2$ transgenic seeds co-expressing AKR1 and PIMT2 assessed by TTC staining**

**Table 3: Screening and survivability of putative $T_1$ transformants and their percent PCR positives**

(A) AKR1 overexpresses and AKR1+PIMT2 co-expressing transformants.

(B) PIMT2 overexpressing transformants.

**A**

| | AKR1-PIMT2 | AKR1 |
|---|---|---|
| Total number of seeds used for in planta transformation | 500 | 500 |
| Total number of $T_0$ seedling survived on screening on 30ppm of glyphosate | 31 | 24 |
| Percentage recovery of $T_0$ seedlings | 6.2 % | 4.8 % |
| Total number of $T_1$ seeds screened on 30 ppm of glyphosate | 950 | 720 |
| Total number of $T_1$ seedling survived on screening on 30 ppm of glyphosate | 110 | 60 |
| Number of $T_1$ plants with PCR positives | 35 | 12 |
| Percentage of PCR positives | 4 % | 3 % |

**B**

| | PIMT2 |
|---|---|
| Total number of seeds used for in planta transformation | 500 |
| Total number of $T_0$ seedling survived on screening on 400 ppm of kanamycin | 25 |
| Percentage recovery of $T_0$ seedlings | 5 % |
| Total number of $T_1$ seeds screened on 400 ppm of kanamycin | 750 |
| Total number of $T_1$ seedling survived on screening on 400 ppm of kanamycin | 70 |
| Number of $T_1$ plants with PCR positives | 29 |
| Percentage of PCR positives | 3. 8 % |

The absorbance value measured for stained solution extracted in 2-methoxy ethanol at 485 nm, an indication of seed viability was found to be higher in transgenic seeds compared to wild type. In fact, the OD values of wild type seeds were almost 1 to 1.5 times lower than that of transgenic seeds indicating the longer viability of transgenic seeds compared to wild type seeds (Fig. 16).

### 4.4.2 Assessment of seed germination and seedling vigour in seeds of wild type and putative $T_2$ transgenic co-expressing AKR1 and PIMT2 genes upon ageing treatment

The seeds of both wild type and putative $T_2$ transgenic subjected for ageing treatment were tested for seed germination and found that, at 8 days of accelerated ageing, the percent seed germination decreased in both wild type and transgenic seeds. However, the transgenic lines could able to germinate and establish as seedling when the wild type seeds failed to germinate. The transgenic lines recorded significantly higher percentage of seed germination compared around 70 % compared wild type (Table 4 and Plate 31).

Further, seedling vigour was also tested in wild type and putative $T_2$ groundnut transgenic lines. Accordingly, transgenic lines showed higher root and shoot length and hence, higher seedling vigour compared to wild type groundnut upon exposure to accelerated ageing treatment. The low seed vigour in wild type seeds were due to less or failure of seed germination (Plate 32 and Fig. 17). Similarly, it was also observed that, upon accelerated ageing the fresh weight in wild type plants was significantly low when to compared to $T_2$ transgenic co-expressing AKR1 and PIMT2 and these transgenics also showed more number of leaves at early stage than wild type (Table 5).

### 4.4.3 Assessment of cytotoxic compounds in seeds of wild type and putative $T_2$ transgenic groundnut co-expressing AKR1 and PIMT2 genes upon ageing treatment

Earlier attempts (Fig. 9, 10 and 11) showed the relevance of cytotoxic compounds such as MDA, MG and Amadori products on seed viability and seed germination upon ageing treatment prompted us to look at variation in accumulation of these cytotoxic compounds in transgenic groundnut seeds having relevant genes. Further, the variation in seed viability and seed germination as influenced by the cytotoxic compounds was also examined in wild type and putative $T_2$ groundnut transgenics.

The cytotoxic compounds quantified in seeds of both wild type and putative transgenic groundnut seeds upon accelerated ageing treatment showed increased production and accumulation of various cytotoxic compounds such as MDA, MG, and Amadori products. Although there is no significant difference in accumulation of MDA, MG and Amadari products in wild type and transgenics under controlled conditions (0 day of ageing), with ageing treatment, high accumulation of cytotoxic compounds was observed. Interestingly, although there is increased accumulation of MDA, MG and Amadori products in transgenic lines, the extent of accumulation was significantly high for wild type compared to transgenics (Fig. 18).

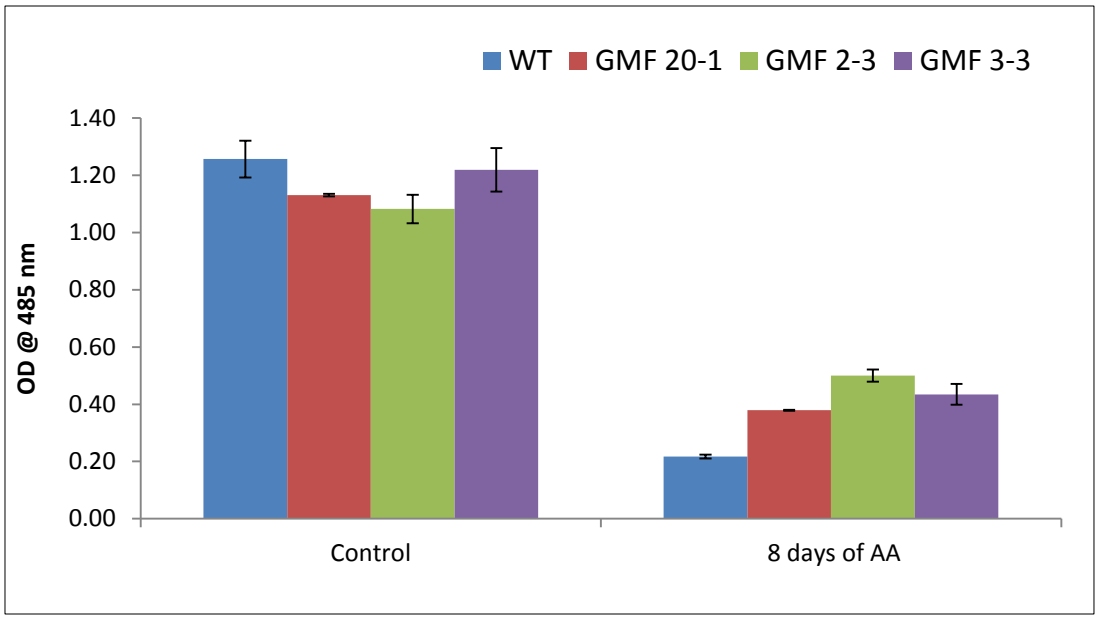



**Fig. 16: Effect of accelerated ageing on seed viability as assessed by TTC test in wild type and putative $T_2$ groundnut transgenic co-expressing AKR1 and PIMT2**


**Plate 31: Effects of accelerated ageing on seed germination and seedling growth in wild type and $T_2$ putative groundnut transgenic co-expressing AKR1 and PIMT2**

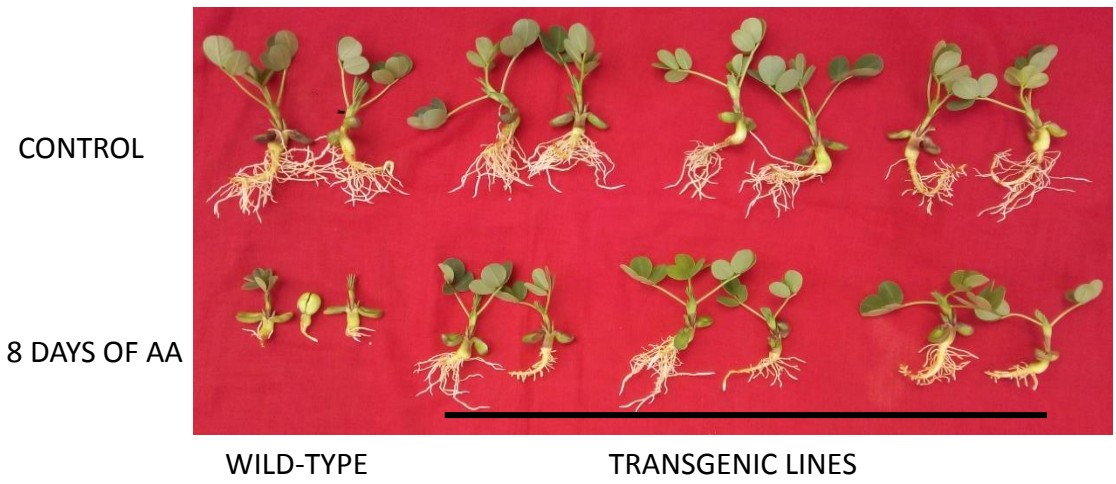



**Plate 32: Performances of seedling growth in $T_2$ transformants co-expressing AKR1 and PIMT2 and wild type upon ageing**

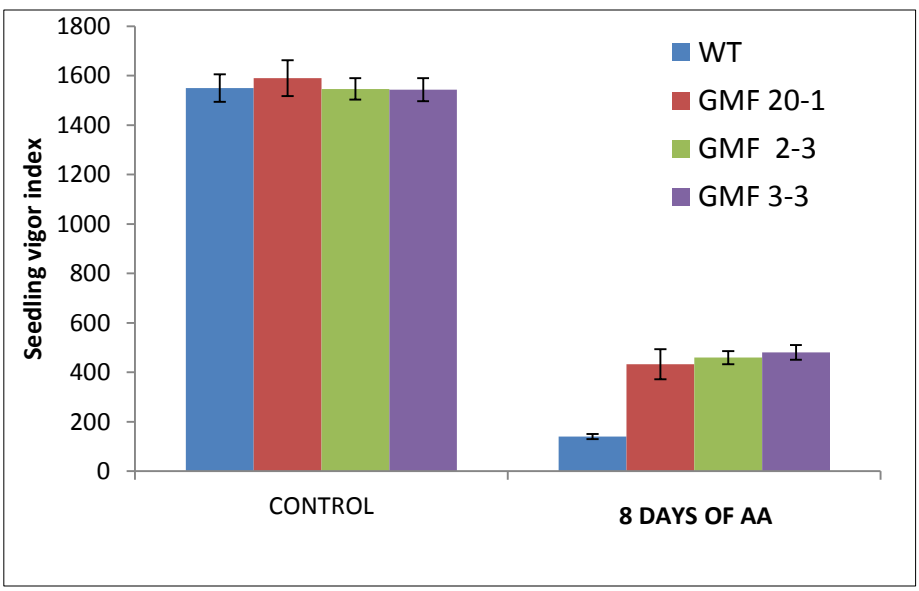



**Fig. 17: Effects of accelerated ageing on seedling vigor index in wild type and $T_2$ putative groundnut transgenic seeds co-expressing AKR1 and PIMT2**

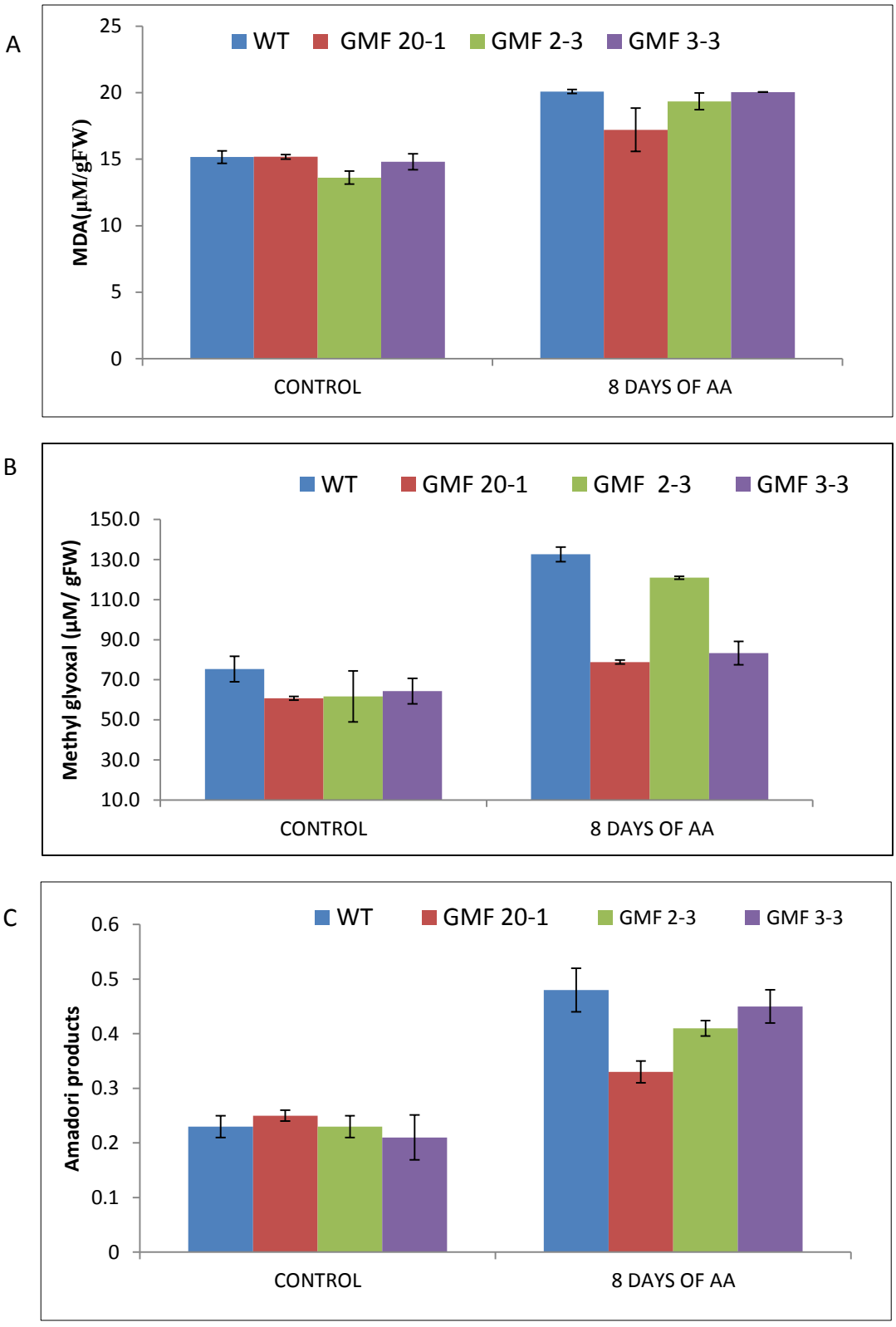



**Fig. 18: Effects of accelerated ageing on accumulation of reactive carbonyl compounds (RCC's) in wild type and putative $T_1$ transgenics lines co-expressing AKR1 and PIMT2**

(A) Melondialdehyde
(B) Methyl glyoxal
(C) Amadori products.

**Table 4: Effects of accelerated ageing on seed germination in wild type and putative $T_2$ transgenics co-expressing AKR1 and PIMT2**

| | CONTROL | 8 DAYS OF AA |
|---|---|---|
| WT | 100 | 40 |
| GMF 20-1 | 100 | 70 |
| GMF 2-3 | 100 | 60 |
| GMF 3-3 | 100 | 70 |
| CD @ 0.05 | 0 | 7.20 |
| CV (%) | 0 | 17.0 |

**Table 5: Effect of accelerated ageing on fresh weight in wild type plants and putative $T_2$ transgenics co-expressing AKR1 and PIMT2**

| | FRESH WEIGHT (g) | | NUMBER OF LEAFS PER PLANT | |
|---|---|---|---|---|
| | CONTROL | 8 DAYS OF AA | CONTROL | 8 DAYS OF AA |
| WT | 3.26 | 1 | 11.2 | 3.6 |
| GMF 20-1 | 3.52 | 2.78 | 10.4 | 9.6 |
| GMF 2-3 | 3.44 | 2.6 | 11.2 | 8.8 |
| GMF 3-3 | 3.46 | 2.56 | 11.2 | 9.6 |
| CD @ 0.05 | 0.11 | 0.83 | 0.4 | 2.92 |
| CV (%) | 0.01 | 0.04 | 0.2 | 0.14 |

### 4.4.4 Assessing the stress tolerance of $T_2$ transgenic groundnut co-expressing AKR1 and PIMT2.

To study the relevance of AKR1+ PIMT2 on stress response, the transgenic lines co-expressing AKR1 and PIMT2 were exposed to 300 mM of NaCl to study salinity induced response. It was observed that, transgenics showed higher percentage of germination and seedling survival and put on more recovery growth upon alleviation of stress compared to wild type induced (Plate 33). This clearly indicates the relevance of AKR1 and PIMT2 under stress condition.

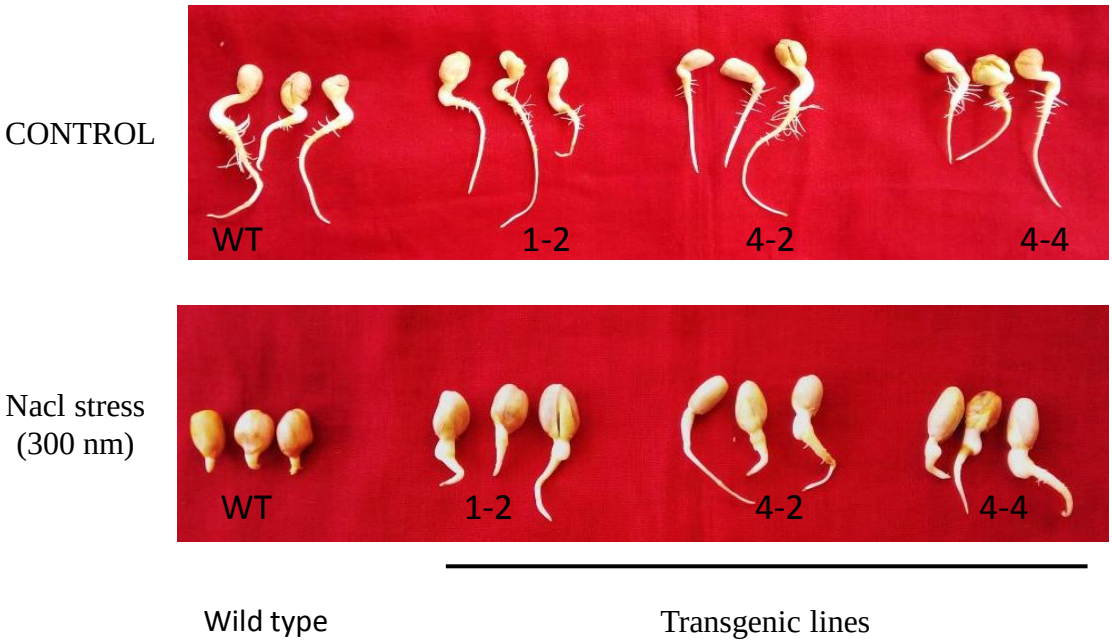



**Plate 33: Salinity induced stress response of Wild type and $T_2$ transgenics seeds co-expressing AKR1 and PIMT2**

# V DISCUSSION

Groundnut (*Arachis hypogaea* L.) also known as peanut, is an annual legume that ranks 3rd among oilseed crop and 13th among the food crops of the world. In addition to providing high quality edible oil (48-50 %) and easily digestible protein (26-28 %), it also provides nearly half of the 13 essential vitamins and 7 of the 20 essential minerals necessary for normal human growth (http://www.icrisat.org/impacts/impact-stories/icrisat-is-groundnut.pdf).

Seed viability is a major constraint in groundnut which lasts only for few months (Sung *et al.,* 1994). The viability of groundnut seeds is considered to be one of the most difficult challenges to maintain (Reusche, 1987). Deterioration of groundnut seeds during storage is a major problem causing reduction of seed nutritional quality and loss of seed viability due to lipid peroxidation. Seeds deteriorate during periods of prolonged storage, but the speed of deterioration varies greatly among species (Priestley 1986, Roberts 1973). Seed viability also controlled by multiple factors such as biotic and abiotic stresses, mechanical damage as well as physiological conditions. Seed moisture content (MC), temperature and oxygen concentration are the major factors which regulate seed viability and germination in the storage environment conditions. Most often, seed viability is linked to seed water content which is regulated by relative humidity of the storage conditions (Walters *et al.,* 1998). Research over the last couple of decades has shown that, in the presence of oxygen, ageing of seeds can occur due to peroxidative changes in polyunsaturated fatty acids. This free radical-induced, non-enzymatic peroxidation has the potential to damage membranes and thus cause deterioration of stored seed (Harrington, 1972; Bewley, 1986; Wilson and McDonald, 1986).

Various environmental stresses or when glucose metabolites non-enzymatically react with protein or when seeds deteriorate during storage lead to the production of reactive oxygen species (ROS) and reactive carbonyl compounds (RCCs) (Foyer *et al.*, 2003). The ROS produced oxidize various biomolecules to inactivate them in vitro. Seeds during storage are like any other dry desiccating tissue and hence expected to produce significant amount of reactive oxygen species and RCCs. Membrane lipids are abundant molecules in the membrane and hence, most probable target of ROS. Carbohydrates and lipids targeted by ROS increases the amount of RCCs that are eventually involved in the formation of advanced glycation end products (AGEs) and advanced lipoxidation end products (ALEs) (Mano *et al.,* 2012) which incidentally have adverse effect on crop establishment.

Deterioration of seeds during storage also seem to be influenced by Amadori and the Miallard reactions which are cytotoxic compounds produced when reactive carbonyl compounds react with protein via glycation resulting in loss of seed viability and seedling vigour. Amadori and millard products often accumulate in dry seeds showed a correlation with the loss of germinability likely through chemical modification of macromolecules during storage (Wettlaufer and Leopold, 1991).

Seed germination has a direct relation with protein synthesis. Deterioration of seeds during storage involves change in seed components with loss in protein integrity. Spontaneous accumulation of damaged proteins in seeds due to ageing /storage /stress

often adversely affects their vigour and viability. One such protein damage is the formation of abnormal non-functional iso-aspartyl residue that results in loss of protein function (Oge *et al.,* 2008). The formation of such iso-aspartyl residue in proteins often leads to the loss of protein function and the consequent loss of cellular function (Johnson *et al.*, 1987; Clarke 2003). Therefore, it is essential to maintain seed proteins in their functional forms (or rapidly repair them upon imbibition) to ensure efficient germination. In mature seeds, metabolism becomes more energetically conservative and hence repair rather than degradation of proteins may become even more important.

In this background, the main aim of the study was to assess the relevance of reactive carbonyl compounds (RCCs) and damage protein through formation of iso-aspartyl residue on seed deterioration and the role of enzymes involved in detoxifying of cytotoxic compounds and protein repair mechanisms.

**5.1 Assessing the genetic variability for seed viability across groundnut genotypes**

Seeds deteriorate during the periods of prolonged storage, but the speed of deterioration varies greatly among species (Priestley 1986, Roberts 1989). Many studies have implicated damage to the membrane as a causative factor (Pearce and Samad, 1980; Bewley, 1986). Therefore, accelerated ageing treatment has been found effective to induce faster deterioration of seeds leading to loss of early seed viability.

In the present study, accelerated ageing technique was initially standardized for groundnut genotypes and standardized protocol was used to impose the accelerated ageing treatment across selected groundnut genotypes. Twenty groundnut genotypes harvested at the same season were subjected to accelerated ageing for 6 days maintaining a temperature of 45 °C and 100 % relative humidity and then assessed for seed viability through TTC test. It was observed that, there is drastic percent reduction in seed viability in all the groundnut genotypes upon 6 days of ageing. However, some of the genotypes such a KCG6 and ICGV9114 showed a very least seed viability whereas, genotypes such as SB3 and SB15 showed high seed viability compared to other genotypes at 6 days of accelerated ageing. Early loss of seed viability seeds upon ageing could be due to lipid peroxidation and loss of membrane phospholipids as they are considered to be the major cause of seed ageing (Priestley 1986; Wilson and McDonald, 1986). Variations in seed longevity been shown to vary across species in relation to number of factors such as taxonomy, seed structure, climate of geographical origin. The storage conditions also reported to alter seed viability and accordingly, at different storage conditions, rice seeds deteriorate faster than wheat and wheat faster than oats (Robertson *et al.,* 1943). Variations in seed longevity across the species have been reported by several other workers (Priestly *et al.,* 1985, 1983; Walters *et al.,* 2005). Although lipid peroxidation seems to be the major cause of seed ageing under accelerated ageing conditions, there are several studies that showed little or no peroxidation from seeds of cucumber (Koostra and Harington, 1992), rice (Matsuda and Hirayama, 1973), peanuts (Pearce and Samad, 1980), Soybean (Priestley and Leoplad, 1983) and wheat (Petruzzelli and Teranto, 1984) indicating something else also could be responsible for seed deterioration.

In the present study, percent seed germination and seedling vigour index also decreased upon ageing treatment. Genotypes such as SB3, SB15 and VB1 even after 6

days of accelerated ageing showed 80 % seed germination whereas, genotypes such as KCG6 and ICGV9114 recorded very less seed germination percentage of approximately 60 % (Table 1). Such variation in seed germination and seedling vigour across the rice genotypes upon ageing treatment was demonstrated by Nisarga (2013). The genotypes with longer viability showed improved seed germination and seedling vigour.

During accelerated ageing, cytotoxic compounds like melondialdehyde, methyl glyoxal, amadori products and Maillard products increased with time via lipid peroxidation and glycation which results in loss in germinability (Wettlauffer and Leopard, 1991). Therefore, reduction of such cytotoxic compounds is necessary for improved seed germination in seeds. In the present study, no significant differences in production of cytotoxic compounds such as Melondialdehyde, Methyl glyoxal and Amadori products under controlled conditions was observed (0 days of ageing). However, upon accelerated ageing, genotypes such KCG6 and ICGV9114 showed significantly high level of cytotoxic compounds compared to genotypes such as SB1, SB3, SB15, VB3 and VB4 which accumulated low level of cytotoxic compounds. The rest of the genotypes however, showed no significant variation in accumulation of cytotoxic compounds (Fig. 9). Therefore, reduced levels of cytotoxic compounds are necessary to maintain seed viability and vigour. The relevance of reduced level of cytotoxic compounds on seed germination was shown through mutant study where, mutation of one of the important detoxifying enzymes, ALDH showed increased accumulation of cytotoxic compounds leading to reduced seed germination (McDonald, 1999).

Based on genetic variability, the contrasting genotypes with high and low seed viability were identified. Accordingly, genotypes such as SB3 and SB15 observed to accumulate low level of cytotoxic compounds and showed high seed viability and germination upon accelerated ageing compared to genotypes like KCG6 and ICGV9114 (Fig. 12 and 13) (Table 2). The differential level of cytotoxic compounds and their involvement in regulating seed viability appears to be due to differential accumulation and expression of the relevant genes in this group of genotypes. Accordingly, it is likely, that, expression of relevant genes would be more in longer viable genotypes. When percent seed germination and seed viability was compared with that of cytotoxic compounds, a strong and significant inverse relationship was observed which indicates that the cytotoxic compounds effect on seed germination (Fig. 10) and seed viability (Fig. 11). Negative relationship between cytotoxic compounds v/s seed germination and seed viability clearly indicates that, if the seeds remain to be viable and protect their germinability, they need to keep cytotoxic compounds low.

Enzymes detoxify specific group of cytotoxic compounds like RCCs and enzymes function in reverting the formation of abnormal iso-aspartyl residue has a greater relevance. Plants possess many defense mechanisms to scavenge RCCs compounds. One such enzyme is Aldo-Keto Reductase (AKR1), which is known to play a pivotal role in detoxifying RCCs in plant. NADPH dependent Aldo-Keto Reductases are known to detoxify the cytotoxic compounds like aldehyde and ketones by reducing them into alcohol thereby reduce polarity and chemical reactivity of carbonyl compounds

(Oberschall *et al.,* 2000). In addition to AKR, over expression of a few other detoxifying enzymes such as aldehyde dehydrogenase or alkenol reductase that catalyse the conversion of reactive aldehyde to carboxylic acid was also found to be effective in reducing the toxicity caused by cytotoxic compounds (Sunkar *et al.,* 2003; Mano *et al.,* 2005). Therefore, it is highly relevant to have efficient detoxification mechanisms in the seeds to have longer life span

In addition, Protein repair appears to play a key role in the long-term survival of seeds in the dry state. PIMT (protein L-isoaspartyl methyltransferase), which limits and repairs age-damaged aspartyl and asparaginyl residues in proteins has been associated with greater seed longevity because, it is highly accumulated in sacred lotus seed (*Nelumbo nucifera*), one of the world's longest living seeds (1,300 years; Shen-Miller, 2002). In plants, PIMT activity is primarily localized in seed tissue during late stages of embryogenesis during and after maturation and desiccation, suggesting the role in rescuing the functionally active conformation of the seeds (Thapar *et al.,* 2001).

With is background, the major emphasis of the study was to co-expressing relevant genes such as AKR1 and PIMT2 in groundnut thereby, enhancing seed viability, vigour and stress tolerance. One of the options of validation is through genetic approach.

### 5.2 Relevance of AKR1 and PIMT2 to improve seed viability through transgenic approach

#### Traditional cloning strategy used to develop gene cassette

Breeding towards dehydration-stress tolerance in plants has met with considerable success in developing cultivars or hybrids that perform better under stress at the field level. However, this approach is time consuming and laborious. It also relies on utilizing existing genetic stocks, whose quantities are limited. An alternative approach is a genetic transformation. Transgenic approaches offer powerful means to gain valuable information towards a better understanding of the mechanism. They also open up new opportunities to improve tolerance to dehydration stress/ storage by incorporating a relevant gene (Bajaj *et al.,* 1999).

For development of relevant gene cassette in the present study, NAPIN:PIMT2:TRBCS gene cassette in pCAMBIA2301 binary vector which is under seed specific napin promoter was procured from Dr.Manoj Mazee, NIPGR, New Delhi. Similarly PRBCS:AKR1:TRBCS gene cassette in pi12GW binary vector ,which is codon optimized and custom synthesized by Dept. of Crop Physiology, UAS, GKVK, Bengaluru was also obtained. Here, the aim was to develop a gene cassette co-expressing AKR1 and PIMT2 together in marker free binary vector pmf12GW. The strategy followed in development of double gene cassette co-expressing has been shown in Fig. 19. Construction and validation of a gene co-expression help to identify new genes that may putatively be involved in certain biological processes. The construction of such gene cassettes and transformation to plant system to study their relevance was reported by several workers. Accordingly, the relevance of PIMT in enhancing the seed Longevity and germination, vigour and its predominant activities had already been shown in model system (Oge *et al.,* 2008: Villa *et al.,* 2006). Similarly, overexpression of rice aldo-keto

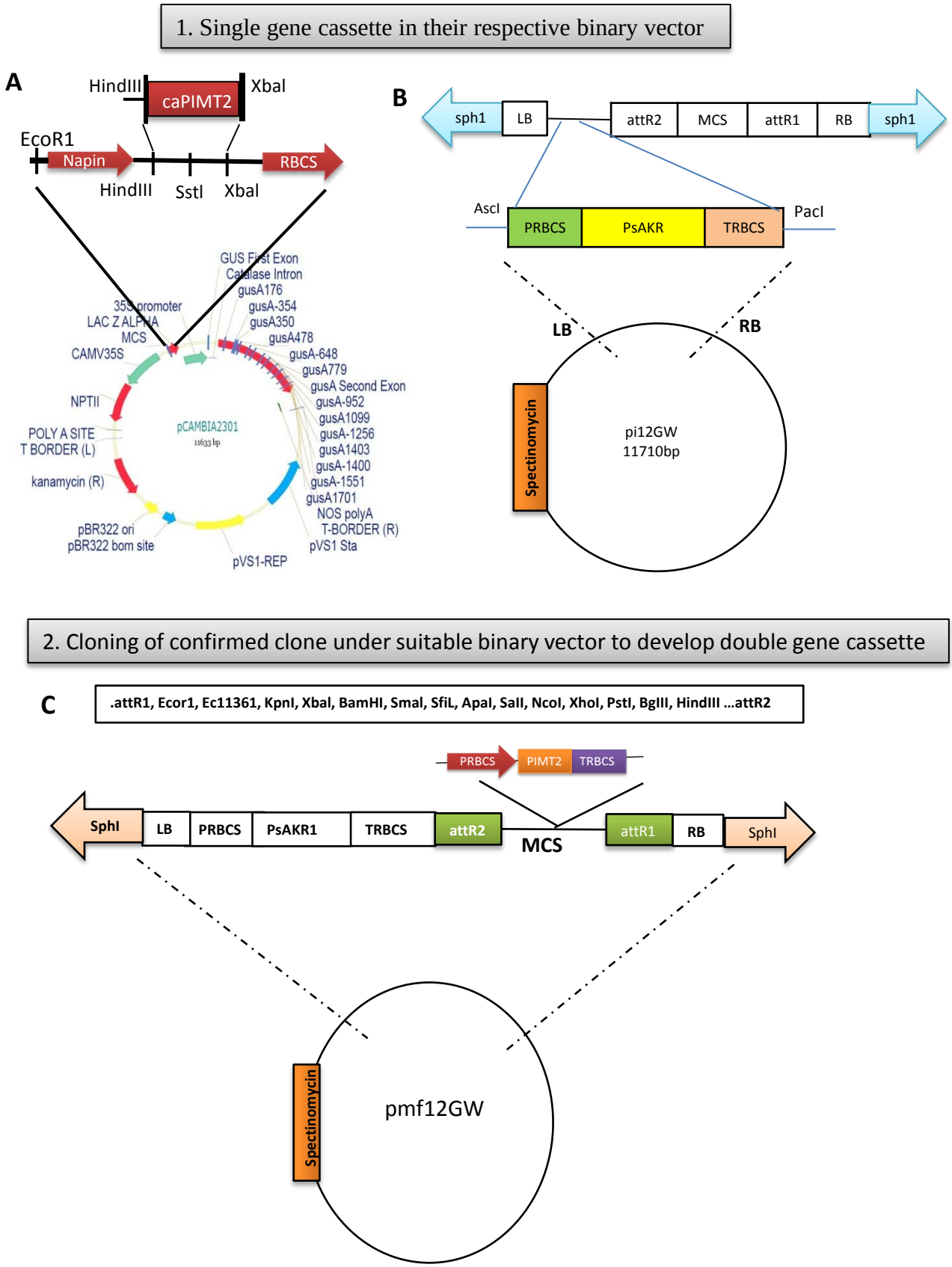



**Fig. 19: Overview of strategy followed in development of double gene cassette co-expressing AKR1 and PIMT2**

reductase increases oxidative and heat stress tolerance by malondialdehyde and methyl glyoxal detoxification and their relevance on seed viability have been demonstrated (Turoczy *et al.,* 2011).

In-silico analysis of caPIMT2 sequence indicates it is high homology at C-terminal region across plant species and variable N-terminal end. Further the amino acid sequence of PIMT2 was analyzed for the presence of five highly conserved domains which were reported earlier in various plant species (Kagan *et al.,* 1997). The five conserved domains are pre-region I (ATISAPHMHA), region I (IALDVGSGTG), region II (APYDHIHVG), region III (QLKPGGRMV) and post region III (VRYVPLTS) which are essential for PIMT function and substrate specificity were detected (Fig. 14). The phylogenetic analysis of CaPIMT2 with other orthologous gene showed high identity with *Medicago trunkatula* and distinctly related to *Nelimbo nucifera* and *Vitis vinifera* (Fig. 15).

### 5.3 Development of groundnut transgenic overexpressing these genes constructs

To achieve improved adaptation under storage conditions, seeds have to evolve two major adaptive mechanisms which include detoxification of cytotoxic compounds and reduce the spontaneous accumulation of abnormal iso-aspartyl residue. From this context the scientific strategy was to overexpress AKR1, PIMT2 and also co-express AKR1+PIMT2 together to improve seed viability, seedling vigour and stress tolerance during seed storage conditions. In this study, to develop transgenic plants, one of the leading groundnut varieties, but having shorter seed viability period namely, KCG6 was used as seed material for transformation.

A genotype independent *in planta* transformation technique was adopted to generate groundnut transgenics. The *in planta* transformation procedure can be applied to all genotypes and cultivars of crops which are susceptible to *Agrobacterium tumefaciens* infection, although the efficiency might vary between crops and genotypes within the species. The success of *in planta* transformation technique has been demonstrated in many species like groundnut (Sundaresha *et al.,* 2009; Manjulatha, 2011), safflower (Rohini *et al.,* 2000), pigeon pea (Rao *et al.,* 2008), bell pepper (Manoj *et al.,* 2008), field bean (Pavani *et al.,* 2006), cotton (Keshamma *et al.,* 2008) and chickpea (Gowri *et al.,* 2008). Tissue culture-independent transformation technique using *Agrobacterium* is targeted to the differentiating cells of the geminating embryos. As a result the success rates can vary greatly depending on the species and genotypes. Fairly good transformation efficiencies have been reported for *in planta* transformation in Arabidopsis. Efficiencies up to 40 % with *Agrobacterium* inoculation of germinating seeds of rice have also been reported (Supartana *et al.,* 2005).

The most important aspects in transformation is selection of putative transformants. Generally, the screening procedure involves the employment of antibiotic or herbicide resistant genes. Since stringent selection can be made by herbicide selectable marker, lethal concentration of glyphosate as a screening strategy was standardized. Glyphosate (N-phosphonomethylglycine) is a non-selective herbicide, capable of inhibiting growth of a broad range of crop and weeds (Amrhein, 1995). Glyphosate interferes in the shikimate acid pathway by inhibiting synthesis of 5-enolpyruvyl-3-

phosphoshikimate (EPSP) by competitively inhibiting EPSPS. Similarly, lethal concentration of kanamycin was also standardized to screen and identify putative transformants. Antibiotics are conventionally used as an effective selectable marker to identify recombinant plants in vitro. It was observed that, at 30 ppm of glyphosate and 400 ppm kanamycin, the wild type groundnut showed complete reduction in root and shoot length and hence, this concentration was considered to be lethal and subsequently, used for screening of transformants. AKR besides as a scavenging enzyme also gives resistant against herbicide. Hence, it was used as selectable marker against glyphosate. Kanamycin was used as selectable marker for PIMT2 transformants as the vector coding for kanamycin resistances (primarily Neomycin phosphotransferase II [NPT II/Neo]).

**Screening of putative transformants for identification of promising lines**

*In planta* transformation essentially involves inoculation of embryo axes of germinating seeds and allows them to grow into seedling *ex-vitro*. The $T_0$ seeds were chimeric with an exception of few cells which are transformed to generate plants with the integration of gene. The reproductive tissue developed from these cells produces transformed $T_1$ seeds, which will be in bulk and difficult to screen and also found to have large number of non-transformed seeds. To reduce all this difficulties, new method of screening the transformants was followed. To improve the efficiency of transformation, $T_0$ generation seeds were subjected to selection pressure with an assumption of creating initial selection pressure, the false transformants can be eradicated and chances of getting true transformants will be high with improved transformation efficiency.

In the present study, *Agrobacterium* infected and 2 days old healthy $T_0$ putative transformants overexpressing AKR1 and co-expressing AKR1 +PIMT2 together were screened on 30 ppm of glyphosate and $T_0$ transformants overexpressing PIMT2 were screened on 400 ppm of kanamycin. To our surprise, $T_0$ transformants showed better performance with good root and shoot compared to wild type (plate 18 & 19) with a percent recovery of 6.2 %, 4.8 % and 5 % respectively (Table 3). These glyphosate and kanamycin tolerant $T_0$ transformants were advanced for next generation.

The $T_1$ seeds were also screened on 30 ppm glyphosate (transformants overexpressing AKR1 & co-expressing AKR1+PIMT2) and 400 ppm kanamycin (transformants overexpressing PIMT2) to identify putative transformants. Totally, 950 seeds co-expressing AKR1 and PIMT2; 720 seeds overexpressing AKR alone along with wild type were screened on 30 ppm glyphosate and found 110 and 60 tolerant plants respectively with good root and shoot compared to wild type. Similarly, 750 seeds from PIMT2 overexpressing plants were screened on 400 ppm of kanamycin and found 70 plants surviving and showed improved tolerance, performed better than wild type induce. The percent recovery in $T_1$ generation was significantly high. Such improved performance of transgenic over wild type upon screening in selection media was shown earlier by number of workers in groundnut (Sundaresha *et al.,* 2009; Manjulatha 2011; Babitha, 2012, Babitha *et al.,* 2013; Pruthvi, 2013; Pruthvi *et al.,* 2013, 2014; Salimat, 2014), pigeon pea (Ramu, 2012), rice (Nisarga, 2013).

**Molecular characterization of $T_1$ transformants and identification of promising lines**

Besides generating large number of transformants identifying the promising lines is crucial. In view of this, the emphasis of the study has been to characterize putative transformants by diverse approaches namely, screening on selectable media which has been demonstrated earlier in our study, followed by molecular confirmation of T-DNA integration and expression of the transgene through PCR and RT-PCR. Out of 110 putative $T_1$ transformants co-expressing AKR1 and PIMT2, 35 putative $T_1$ transformants lines were showed the presences of insert (Plate 23 & Table 3) Similarly, out of 60 putative $T_I$ transformants overexpressing AKR1, 12 putative $T_1$ transformant lines were confirmed the presence of AKR1 gene (Plate 24 & Table 3) and from out of 70 putative $T_1$ transformants overexpressing PIMT2, 26 $T_1$ putative transformant lines showed the presence of PIMT2 (Plate 24 & Table 3). Therefore, the approaches of screening the putative transformants at $T_0$ and $T_1$ generation based on the sensitivity of selection marker and molecular characterization of gene integration clearly demonstrated the relevance of creating initial selection pressure at $T_0$ and subsequently at $T_1$ generation to identify the promising transformants. Through this approach, the probability of identifying the true transformants will be high (Table 3).

**Characterization of stable gene integration and expression in selected superior line**

In an attempt to corroborate between the expression and efficacy in selected transgenic plant, RT-PCR was assessed for transcription accumulation. The total RNA from leaf and seeds were isolated from representative transgenic line and wild type which were reverse transcribed using MMLV to get cDNA. The transgenic lines were confirmed by amplification of transgene and observed a significantly high expression of AKR1 and PIMT2 in transgenic seeds. Since PIMT2 was under seed specific napin promoter the results seems to be valid. Molecular studies clearly indicate stable integration in selected transgenic line.

**5.4 physiological characterizations of $T_2$ transgenic seeds**

Physiological characterization was carried out only in $T_2$ transgenic co-expressing AKR1 and PIMT2. However, the single gene transgenic couldn't able to characterize as the seeds are yet to be harvested.

To identify the promising transformants, transgenic were characterized for five main important parameters:

(a) Seed viability
(b) Germination percent
(c) Seedling vigour index
(d) Cytotoxic compounds accumulation
(e) Stress tolerance.

To test the proof of concept that whether or not co-expression of AKR1 and PIMT2 resulted in reduced seed deterioration process leading to enhanced viability of seeds, the $T_2$ generation groundnut transgenic seeds were exposed to accelerated ageing technique to study the physiological and biochemical changes in seeds during ageing. In

the present study, when the seeds of both wild type and $T_2$ transgenic groundnut were subjected for ageing treatment, there was a drastic reduction in seed viability with the duration of ageing treatment especially in wild type seeds compared to transgenic. This was evidenced by the low extent of TTC staining and lower absorbance value for wild type seeds compared to transgenic. This significant difference may be because the transgenic seeds probably through AKR and PIMT action could detoxify the cytotoxic compounds and reduce the formation of abnormal protein residue and thereby showed less reduction in seed viability upon ageing (Fig. 16). Seed longevity is a quantitative trait controlled by multiple genes (Clerk *et al.,* 2004a) and is strongly affected by environment conditions during seed formation, harvest and storage conditions (Contreras, 2005 and 2007). Similar reduction in cytotoxic compounds to ensure seed viability across rice genotypes upon accelerated ageing was also demonstrated by Nisarga (2013)

When seeds of both wild type and putative $T_2$ transgenic seeds subjected for ageing treatment were tested for seed germination and seedling vigour, it was found that, accelerated ageing reduces percent seed germination. The transgenic lines recorded significantly higher percentage of seed germination up to 70 % compared to wild type (50 %) (Table 4, Plate 31 & 32). Further, transgenic lines performed better in terms of shoot and root growth and showed more fresh weight. Because of higher percentage of seed germination more shoot and root growth, the vigour is more in transgenic lines. The reduced germination and vigour in wild type seeds upon ageing can be attributed to generation of ROS, lipid peroxidation, cytotoxic compounds and protein damage. The relevance of reduced level of cytotoxic compounds on seed germination was showed through a mutant study where, mutation of one of the important detoxifying enzymes, ALDH showed increased accumulation of cytotoxic compounds leading to reduced seed germination (McDonald, 1986). Several AKR's in different organisms have been reported to detoxify effectively a wide range of lipid per-oxidation or glycolysis derived cytotoxic aldehydes (Colrat *et al.,* 1999). It has been also documented that, PIMT targets specific proteins which must be repaired for success for germination of seeds (Vigeswara *et al.,* 2006; Zhu *et al.,* 2006; Dinkins *et al.,* 2007). Overexpression of PIMT1 in *Arabidopsis* enhanced both seed longevity and germination vigour (Oge *et al.,* 2008). All these reports indicate the relevance of protein repair on seed viability.

The major regulatory factors that control seed ageing are oxidative stress, lipid peroxidation and respiration (Sun and Leoplad, 1995; Bailly *et al.,* 1998). Lipid peroxidation and respiration results in the formation of reactive aldehydes such as MDA and acetaldehyde which tend to react with proteins and amino acids (Ponquett, 1992; Almeras *et al.,* 2003). These reactions cause ageing and seed damage (Zhang *et al.,* 1995). In addition to MDA, many other cytotoxic compounds such as HNE, MG, Amadori and Maillard products are also accumulated and in totality, cause cellular damage leading to loss of seed viability. In the present study, the wild type seeds have accumulated significantly high amount of these cytotoxic compounds compared to transgenic lines co-expressing AKR1 and PIMT2 (Fig. 18). This suggest the relevance AKR1 in detoxifying cytotoxic compound and this was evidenced by greater membrane damage and also the lower level of expression of AKR1 in wild type seeds which is responsible for detoxifying these cytotoxic compounds. It is likely that, because of higher expression of detoxifying enzymes in transgenic (Plate 29) the cytotoxic compounds

were detoxified and hence, maintained seed viability, germination and vigour in transgenic.

AKRs catalyze a variety of carbonyl compounds including aldehyde form of glucose which is reduced to corresponding sugars alcohol, sorbitol (Sree *et al.,* 2000). In fact, the reduced level of lipid peroxidation derived aldehydes found in the transgenic lines of tobacco over expressing AKR1 was attributed to the gene action in enhancing detoxification of aldehydes (Turozcy *et al.,* 2011). Further, change in damage and degradation of cellular protein was observed in age-induced seed deterioration and PIMT accumulation and activity in seedling was induced by water and salt stress to indicate the relevance PIMT in stress tolerance (Mudgett and Clarke, 1994; Thapar *et al.,* 2001; Xu *et al.,* 2004). Based on this statement, to study the relevance of AKR1 and PIMT2 on stress response $T_2$ transgenic seeds co-expressing AKR1 and PIMT2 together were subjected to NaCl stress. Both seedling vigour and growth was significantly high in transgenic lines compared to wild type on alleviation of stress (Plate 33) Such improved tolerance of transgenic plants especially under stress conditions has been shown by several earlier workers (Villa *et al.,* 2006; Pruthvi *et al.,* 2014).

AKR has been shown to impart tolerance against variety of oxidative stresses induced by methyl viologen, heavy metal, UV radiation, osmotic and salt stress condition and long periods of drought (Oberschall *et al.,* 2000; Hidege *et al.,* 2003; Hegdues *et al.,* 2004; Mittler *et al.,* 2004, 2011). The stress tolerance and improved growth observed in the present study in transgenic are more likely to be due to the action integrated genes found in the transgenic lines as these genes have shown to impart stress tolerance in a wide range of crop species. Further, several independent research groups have reported the beneficial roles carbonyl detoxifying enzyme such as ALDH (Sunker *et al.,* 2003; Huang *et al.,* 2008), AKR (Oberchell *et al.,* 2000; Turoczy *et al.,* 2011) and AER (Mano *et al.,* 2005) to improve the tolerance of transgenic plants against environmental stress. PIMT also shown to increase germination maize seeds subjected osmotic stress (Thapar *et al.,* 2001). Similarly, PIMT accumulation and activity in seedlings were shown under water and salt stress (Mudgett and Clarke, 1994). More importantly, the role of PIMT in protein repair mechanisms even at 100 °C to give tolerance has been shown in Lotus (Shell *et al.,* 2013).

All the experimental evidences clearly indicate the role of AKR and PIMT in detoxifying cytotoxic compounds and maintaining overall protein integrity respectively and also giving stress tolerances to plants.

## VI SUMMARY

Crop establishment with proper crop stand under rainfed condition is the main constraint in groundnut which affects the potential yield. Early seed germination and seedling vigour substantially improves the crop establishment. Often, groundnut seeds deteriorate rapidly during storage due to accumulation of cytotoxic compounds like Reactive Oxygen Species (ROS) and Reactive Carbonyl Compounds (RCC) leading to generation of Advanced Glycation End-products and Advanced Lipoxidation End-products via non-enzymatic attack of glucose metabolites and lipid degradation respectively. Seed deterioration is also due to protein inactivation due to formation of abnormal non-functional iso-aspartyl residues which is a major source of spontaneous age-related protein damage in cell resulting in loss of seed viability and vigour. Therefore, if cytotoxic compounds are detoxified and also if protein damage is repaired during storage, seed viability may enhance thereby help in early seed germination and improve the crop establishment in the field.

Plants have developed many defense mechanisms to detoxify the cytotoxic compounds. One among them is Aldo-Keto Reductase (AKR) which is a potential scavenger of RCC and thus prevents oxidative stress damage. Similarly, Protein L-isoaspartyl Methyltransferase (PIMT) converts abnormal non-functional isoaspartyl residue into functional asparate residue thereby maintains overall protein integrity.

In this background, the major emphasis of the present study is to validate the relevance of AKR1 and PIMT2 in improving seed viability, seedling vigour in groundnut transgenic co-expressing these two genes.

**The silent findings of the present study are summarized as follows.**

1. Assessing the genotypic variability for seed viability in selected groundnut genotypes.

Initially, to study the genetic variability for seed viability and relevance of cytotoxic compounds on seed viability, 20 different groundnut genotypes harvested at same season were subjected to accelerated ageing technique for 6 days maintaining the temperature of 45 °C and 100 % relative humidity. At the end of ageing treatment, seed viability, seed germination, seedling vigour and cytotoxic compounds namely Melondialdehyde, Methyl glyoxal and Amadori products were quantified. The result of the study indicates, accelerated ageing decreases the seed viability, germination and seedling vigour and increase accumulation of cytotoxic compounds in 20 groundnut genotypes over control. However, a significant genetic variability for seed viability, seed germination and seedling vigour was observed across the groundnut genotypes upon ageing treatment.

Based on the levels of accumulation of cytotoxic compounds and seed viability, the contrasting genotypes were identified. Accordingly, some of the genotypes namely KCG6 and ICGV9114 were found to be susceptible to ageing treatment, as they showed very less seed viability, germination percentage and accumulate higher cytotoxic compounds resulting in early loss of vigour, whereas, genotypes like SB3 and SB15 found to have high seed viability, germination with low level of cytotoxic compounds.

Further, the correlation study suggest, there is inverse relationship between cytotoxic compounds and seed viability. As the cytotoxic compounds level increases, seed viability as well as seed germination decreases.

**Development of transgenic with relevant genes**

A few genes are known to improve the seed viability and vigour by regulating the level of cytotoxic compounds and also by maintaining the protein integrity. Therefore, the strategy here was to develop transgenic over-expressing relevant genes such as AKR1 and PIMT2 and co-expressing them together and validate its relevance. Accordingly, single gene cassette namely RBCS:AKR1:TRBCS and NAPIN:PIMT2:RBCS were confirmed in their respective binary vector. Similarly, double gene cassette co-expressing AKR1 and PIMT2 (RBCS:AKR1:TRBCS::NAPIN:PIMT2:TRBCS) was confirmed in pmf12GW marker free binary vector.

Modified *in planta* transformation technique was adopted to develop transgenic. A short viable and leading groundnut variety KCG6 was used for transformation. The putative $T_0$ transformants were also screened on selectable media (glyphosate/ kanamycin) and relatively tolerant plants were obtained and advanced to $T_0$ plants. Further, $T_1$ seeds were screened on 30 ppm of glyphosate (over-expressing AKR1 and co-expressing AKR1+PIMT2) and 400 ppm of kanamycin (overexpressing PIMT2) to identify the putative transform ant, which were further confirmed by PCR analysis with transformation efficiency of 4 % (AKR+PIMT2), 3 % (AKR1), 3.8 % (PIMT2) of PCR positive and also RT PCR characterization of the amplified products confirmed the expression of integrated genes.

Outcome of this experiment suggests that, *in planta* method is a potential technique to develop transgenic in groundnut. Promising transformants were identified based on physiological screening.

**Characterization of $T_2$ generation transgenic for seed viability, seedling vigour and abiotic stress responses**

With the hypothesis of AKR1 scavenges RCC and PIMT2 performs protein repair mechanisms during storage condition, their relevance on seed viability, seedling vigour and stress tolerances was examined. $T_2$ generation seeds co-expressing AKR1 and PIMT2 were subjected to accelerated ageing treatment for 8 days (45 °C, 100 % RH) and relevant observations are made. Upon ageing treatment, seed viability, percent seed germination as well as seedling vigour reduced significantly in both wild type and transgenic lines. However, the extent of reduction was found to be high in wild type compared to transgenic lines. Further, AKR1+PIMT2 transgenic have accumulated less cytotoxic compounds namely, MDA, MG, Amadori products compared to wild type and also these transgenic lines put on more fresh weight, shoot and root growth compared to wild type.

Similarly, to study the relevance of AKR1 and PIMT2 on stress response, the transgenic lines co-expressing AKR1 and PIMT2 together were exposed to salinity

induction response and found that, the transgenic lines showed higher percentage of seedling survival and seedling vigour upon alleviation of stress compared to wild type.

All this experimental results signify the relevance of AKR1 and PIMT2 in regulating seed viability, seedling vigour, growth and stress tolerance.

**In a nut shell the outcome of the study can be summarized as follows:**

- Genetic variability for seed viability, seedling vigour and accumulation of cytotoxic compounds was observed in selected groundnut genotypes upon ageing treatment.
- There is inverse relationship between cytotoxic compounds and seed viability and germination in groundnut genotypes upon accelerated ageing.
- Modified *in planta* method is a potential technique to develop transgenics in groundnut. Promising transformants were identified based on physiological screen.
- T-DNA integration and semi-quantitative RT-PCR expression of AKR1 and PIMT2 in groundnut transgenic confirms stable integration.
- The transgenic lines showed higher seed viability, germination percent, seedling vigour with low level of cytotoxic compounds compared wild type upon accelerated ageing. And these transgenic lines significantly performed better with higher seedling vigour than wild type under salinity induction response study.

**Future line of work:**

- Characterization of transgenic for iso-aspartyl residue and PIMT activity
- Molecular confirmation of these transgenics through southern hybridization and dot blot analysis.

## VII REFERENCES


ALMERAS, E., STOLZ, S., VOLLENWEIDER, S., REYMOND, P. AND FARMER, E. E., 2003, Production and scavenging of reactive oxygen species in chloroplasts and their functions. *Plant J.*, **34:** 205-216.

AMRHEIN, N., DEUS, B., GEHRKE, P. AND STEINRUCKEN, H. C., 1980, The site of the inhibition of the shikimate pathway by glyphosate. II. Interference of glyphosate with chorismate formation *in vivo* and *in vitro*. *Plant Physiol*., **66**: 830-834.

ANDERSON. J. J. B. AND MILLER, C. P., 1998, Lower lifetime estrogen exposure among vegetarians as a possible risk factor for osteoporosis: a hypothesis. *Vegetarian Nutrition,* **2:** 4-12.

ASADA, K., 2006, Production and scavenging of reactive oxygen species in chloroplasts and their functions. *Plant Physiology, Soc Plant Biol.,* **215:** 1023- 1035.

BABITHA, K. C., 2012, Development of multiple gene construct with regulatory genes and their functional validation. PhD thesis. University of Agricultural Sciences, Bengaluru, India.

BABITHA, K. C., RAMU, S. V., PRUTHVI, V., MAHESH, P., NATARAJA.K. N. AND UDAYAKUMAR, M., 2013, Co-expression of AtbHLH17 and AtWRKY28 confers resistance to abiotic stress in Arabidopsis. *Transgenic Research*, **22**: 327-341.

BADAWI, G. H., YAMAUCHI, Y., SHIMADA, E., SASAKI, R., KAWANO, N. AND TANAKA, K., 2004a, Enhanced tolerance to salt stress and water deficit by overexpressing superoxide dismutase in tobacco *(Nicotiana tabacum*) chloroplasts. *Plant Sci.,* **66**: 919–928.

BADAWI, G. H., KAWANO1, N., YAMAUCHI , Y., SHIMADA, E., SASAKI, R., KUBO, A. AND TANAKA, K., 2004b, Over-expression of ascorbate peroxidase in tobacco chloroplasts enhances the tolerance to salt stress and water deficit. *International journal for plant physiology*, **121**: 231–238.

BAILLY, C., BENAMAR, A., CORBINEAU, F. AND COME, D., 1998, Free radical scavenging as affected by accelerated ageing and subsequent priming in sunflower seeds. *Physiol. Plant*, **104**: 646–652.

BAILLY, C. AND KRANNER, I., 2011, Analyses of reactive oxygen species and antioxidants in relation to seed longevity and germination. *Methods Mol. Biol.*, **773**: 343-367.

BAJAJ, S., TARGOLLI, J., LIU, L. AND DAVID, T., 1999, Transgenic approaches to increase dehydration-stress tolerance in plants. *Molecular Breeding,* **5**: 493–503.

BARTELS, D., ENGELHARDT, K., RONCARATI, R., SCHNEIDER, K., ROTTER, M. AND SALAMINI, F., 1991, An ABA and GA modulated gene expressed in the barley embryo encodes an aldose reductase related protein. *EM BOJ.*, **10**: 1037–1043.

BATTY, D. P. AND WOOD, R. D., 2000, DNA damage recognition and nucleotide excision repair in mammalian cells. *Cold Spring Harb. Symp. Quant. Biol.*, **65:** 173-182.

BAZIN, J., LANGLADE, N., VINCOURT, P., ARRIBAT, S., BALZERGUE, S., EL-MAAROUF-BOUTEAU, H. AND BAILLY, C., 2011, Targeted mRNA oxidation regulates sunflower seed dormancy alleviation during dry after-ripening. *The Plant Cell*, **23**: 2196-2208.

BERJAK, P. AND PARMENTER, N. W., 1997, Progress in understanding and manipulation of desiccation-sensitive (recalcitrant) seeds. *Basic and Applied Aspects of Seed Biology,* **22**: 689-703.

BEWLEY, J. D., LARSEN, K. M., JOANNE, E. T. AND PAPP, J. E. T., 1983, Water stress induced changes in the pattern of protein synthesis in maize seedling mesocotyls: A comparison with the effects of heat shock. *J. Expt. Bot.,* **34** (146): 1126-1133.

BEWLEY, J, D., 1986, Membrane changes in seeds as related to germination and the perturbations resulting from deterioration in storage. *Crop Science Society of America, Inc. Madis.,* **23**: 1678-1723.

BEWLEYL, J. AND BALCK, 1994, Seed Germination and Dormancy. *The Plant Cell,* **9**: 1055-1066.

BRITT, A. B., 1996, DNA damage and repair in plants. *Ann. Rev. Plant Physiol. Plant Mol. Biol.*, **47**: 75–100.

CHAKRABORTY, M. K., CHAUDHARI, A. N., SHUKLA, Y. M. AND PATEL, K.V., 1991, Biochemical characterization of groundnut (*Arachis hypogaea* L.) seed in relation to its viability. Indian J, Agri. Sci., 61: 332-334.

CHEN, T., NAYAK, N., MAJEE, S. M., LOWENSON, J., SCHAFERMER, K., ELIPOPOULOS, A. C., LIOYD, T. D., DINKINS, R. AND PERRY, S. E., 2010, Substrate of the Arabidopsis thaliana Protein Isoaspartyl Methytransferase1 Identified Using Phage Display and Biopanning. *Journal of Biological Chemistry,* **285**: 37281-37292.

CLARKE, S., 2003, Ageing as war between chemical and biochemical processes: Protein methylation and the recognition of age-damaged proteins for repair. *Ageing Res. Rev*., **2**: 263-285.

CLERKE, E. J. M., BLANKESTIJN, H., RUYS, G. J., GROOT, S. AND KOORNNEEF, M., 2004a, Genetic differences in seed longevity of various Arabidopsis mutants. *Physiologia Plantarum*, **121:** 448-461.

CLERKE, E. J. M., LITHY, M. E., VIERLING, E., RUYS, G. J. AND KOORNNEEF, M., 2004b, Analysis of natural allelic variation of Arabidopsis seed germination and seed longevity traits between the accessions Landsberg *erecta* and Shakdara using a new recombinant inbred line population. *Plant Physiology*, **135:** 432-443.

COLRAT, S., LATCHE, A., GUIS, M., PECH, J. C., BOUZAYEN, M., FALLOT, J. AND ROUSTAN, J. P., 1999, Purification and characterization of a NADPH-dependent aldehyde reductase from mung bean that detoxifies eutypine, a toxin from Eutypa lata. *Plant Physiol.*, **119**: 621-626.

COME, D. AND CORBINEAU, F., 1989, Some aspects of metabolic regulation of seed germination and dormancy. *Plenum Press*, **66**: 165-80

CONTRERAS, S., 2007, Effects of maternal plant environment on lettuce (*Lactuca sativa L*.) seed dormancy, germinability, and storability. Ohio State Univ., Columbus, PhD Diss.

DEBEAUJON, L., LEPINIEC, L., POURCEL, J. M. AND ROUTABOUL, M., 2007, Seed coat development and dormancy. *Plenum press*, **44:** 25-49.

DINKINS, R. D., MAJEE, S. M., NAYAK, N. R., MARTIN,D., XU, Q., BELCASTRO, M. P., HOUTZ, R. L., BEACH, C. M. AND DOWNIE, A. B., 2008, Changing transcriptional initiation sites and alternative 5'-3' splice site selection of the first intron deploys Arabidopsis PROTEIN ISOASPARTYL METHYLTRANSFERASE2 variants to different sub-cellular compartments. *Plant J.*, **55**: 1-13.

DONG, J. Z. AND DUNSTAN, D. I., 1996, Reliable method for extraction of RNA from various conifer tissues. *Plant Cell Rep.*, **15:** 516-521.

DONOHUE, K., DORN, L., GRIFFITH, C., KIM, K., AGUILERA, A., POLISETTY, C. R. AND SCHMITT, J., 2005, The evolutionary ecology of seed germination of Arabidopsis thaliana: variable natural selection on germination timing. *Evolution,* **59**: 758–770.

DOYLE, J. J. AND DOYLE, J. L., 1987, A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem Bull.*, **19**: 11-15.

ELLIS, R. H. AND FILHO, P. C., 1992, Seed development and cereal seed longevity. *Seed Sci. Res*., **2**: 9-15.

ELLIS, R. H., HONG, T. D. AND ROBERTS E. H., 1992, Low moisture content limit to the negative logarithmic relation between seed longevity and moisture content in three sub species of rice. *Ann. Bot*., **69**: 53-58.

FANG, P., LI, X., WANG, J. AND XING, L., 2010, Crystal structure of the Protein L-isoaspartyl Methyltransferase from *Escherichia coli. Planta,* **58**: 163-167.

FEENEY, R. E. AND WHITAKER, J. R., 1982, The Maillard reaction and its prevention. *Food Protein Deterioration Academic Press*, **22**: 201-229.

FENG, WANG, X., ZHANG, X., PHAT, M., DANG, C., HOLBROOK, O., ALBERT, K., CULBREATH, WU, Y. AND GUO1, B., 2012, Peanut (*Arachis hypogaea L.*) Expressed Sequence Tag Project: Progress and Application.

FOURIE, P. C. AND BASSON, D. S., 1990, Sugar content of almond, pecnand macadamia nuts. *J Agric Food Chem.,* **38**:101-104.

FOYER, G. AND NOCTOR, 2003, Redox sensing and signaling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria. *Physiol. Plant*, **119**: 355-364.

GALLARDO, K., JOB, C., GROOT, S. P. C., PUYPE, M., DEMOL, H. AND VANDEKERCKHOVE, J., 2002, Importance of methionine biosynthesis for Arabidopsis seed germination and seedling growth. *Physiol. Plant.*, **116**: 238–247.

GALLETTI, P., INGROSS, D., MANNA, C., CLEMENTE, G. AND ZAPPIA, V., 1995, Protein damage and methylation-mediated repair in the erythrocyte. *Biochemical Journal*, **306:** 313-25.

GARG, N. A., AND MANCHANDA, G., 2009, Plant Biosystems, ROS generation in plants: Boon or bane? *An International Journal Dealing with all Aspects of Plant Biology: Official Journal of the Societa Botanica Italiana,* **143**: 81-96, DOI: 10.1080/11263500802633626

GOEL, A., GOEL, A. K. AND SHEORAN, I. S., 2003, Change in oxidative stress enzymes during artificial ageing in cotton (*Gossypium hirsutum* L.) seeds. *Journal of Plant Physiology*, **160**: 1093-1100.

GOWRI N. M., RAMU, S. V., SREEVATHSA, R., ASHA RANI, B. M., KUMAR, A. R. V. AND GAYATHRI, M. C., 2008, *In planta* transformation strategy to generate transgenic plants in chick pea: proof of concept with a cry gene. *J. Plant Biol.,* **35 (**3): 201-206.

HARINGTON, J.F., 1972, seed storage and longevity, *Academic Press*, *New York*, **45**: 145-245.

HEGEDUS, A., ERDEI, T., JANDA, E., TÓTH, G., HORVÁTH, E., DUDITS, 2004, Transgenic tobacco plants overproducing alfalfa aldose/aldehyde reductase show higher tolerance to low temperature and cadmium stress. *Plant Sci*., **166**: 1329-1333.

HIDEG, E., NAGY T., OBERSCHALL, A. AND DUDITS, 2003, Detoxification function of aldose/aldehyde reductase during drought and ultraviolet. *Plant Cell Environ*., **26**: 513-522.

HODGE, J. E., 1993, Dehydrated foods, chemistry of browning reaction in model system. *J.Agric Food Chem.,* **1**: 928-943.

JOHNSON, L. B. AND CUNNINGHAM, B. A., 1987, Peroxidase activity in healthy and leaf-rust-infected wheat leaves. *Phytochemistry*, **11**: 547-551.

JUN, J., FANG L., PETER P.,WAYNE, G., WAMER, B., PAUL, C., WANG, F., YANG, Q., SUN, B., XING, B. AND DONG, J., 2009, The scavenging of reactive oxygen species and the potential for cell protection by functionalized fullerene materials. *Biomaterials,* **30**: 611–621.

KAGAN, R. M., MCFADDEN, H. J., MCFADDEN, P. N., O'CONNOR, C. AND CLARKE, S., 1997A, Molecular phylogenetic of a protein repair methyltransferase. *Comp Biochem Physiol.*, **117**: 379–385.

KESHAMMA, E., ROHINI, S., RAO, K. S., MADHUSUDHAN, B. AND UDAYAKUMAR, M., 2008, Tissue culture-independent *in planta* transformation strategy: an *Agrobacterium tumefaciens* mediated gene transfer method to overcome recalcitrance in cotton (*Gossypium hirsutum* L.). *J. Cotton Sci.,* **12:** 264–272.

KESTER, S. T., GENEVE, R. L. AND HOUTZ, R. L., 1997, Priming and accelerated ageing affect L-isoaspartyl methyltransferase activity in tomato (*Lycopersicon esculentum* Mill.). *Seed. J. Exp. Bot.,* **48**: 943–949.

KIM, E., LOWENSON, J. D., MACLAREN, D. C., CLARKE, S. AND YOUNG, S. G. 1997, Deficiency of a protein-repair enzyme results in the accumulation of altered proteins, retardation of growth, and fatal seizures in mice. *Proc. Natl. Acad. Sci.*, **94**: 6132–6137.

KOOSTRA, P. T. AND HARRINGTON, J. F., 1992, Biochemical effect of age on membrane lipids of *Cucumis sativus* L. seeds. *Proceedings of the International Seed Testing Association,* **34**: 329-340.

LEE, A. T. AND CERAMI, A., 1989, Non-enzymatic glycosylation of DNA by reducing sugars. In: Baynes JW, Monnier VM, eds. The Maillard reaction in ageing, diabetes and nutrition. *Alan R Liss, Inc.,* **66**: 291-299.

LU, X., FENG, S., SHAO, Y., JIANG, L. AND HOU, K., 2007a, Effects of arsenic on seed germination and physiological activities of wheat seedlings. *Journal of environmental sciences*, **19**: 725–732.

LU, Z., HALL, J. D. AND MOUNT, D. W., 2007b, Arabidopsis UVH3 gene is a homolog of the *Saccharomyces cereviseae* RAD2 and human XPG DNA repair genes. *Plant J.,* **26:** 329–338.

MANJULATHA, 2011, Cloning and characterization of helicase and validation of their relevance's in stress response. PhD Thesis. Sri Krishnadevaraya University Anantapur-515055, INDIA.

MANO, J., 2012, Reactive carbonyl species: Their production from lipid peroxides, action in environmental stress, and the detoxification mechanism. *Science Research Center*, **45**: 1-8.

MANO, J., BELLES-BOIX, E., BABIYCHUK, D., TORII, E., HIRAOKA, K. AND TAKIMOTO, K., 2005, Protection against photo oxidative injury of tobacco leaves by 2-alkenal reductase and detoxification of lipid peroxide-derived reactive carbonyls. *Plant Physiol*., **139:** 1773-1783.

MANOJ K. A., REDDY, K. N., ROHINI, S., GIRIJA, G. AND UDAYAKUMAR, M., 2009, Towards crop improvement in capsicum (*Capsicum annum L*.): transgenics (uidA::hpt II) by a tissue-culture independent Agrobacterium-mediated *in planta* approach. *Sci. Hortic.,* **119**: 362– 370.

MATSUDA, H. AND HIRAYAMA, O., 1973, Changes of lipid components and lipolytic acyl hydrolase activities in rice grains during their storage. *Journal of Agricultural Chemistry Society of Japan*, **47**: 279-384.

MC DONALD, M. B., 1999, Seed deterioration: Physiology, repair and assessment. *Seed Science and Technology*, **27**: 177-237.

MITTLER, R., VANDERAUWERA, S., GOLLERY, M. AND VAN BREUSEGEM, F., 2004, Reactive oxygen gene network of plants. *Trends Plant Sci.,* **9:** 490–498.

MITTLER, R., VANDERAUWERA, S., SUZUKI, N., MILLER, G., TOGNETTI, V. B., VANDEPOELE, K., GOLLERY, M., SHULAEV, V. AND VAN-BREUSEGEM F., 2011, ROS signaling: the new wave? *Trends Plant Sci*., **16**: 300-309.

MIURA, K. Y., LIN, M., YANO, T. AND NAGAMINE, 2002, Mapping quantitative trait loci controlling seed longevity in rice (*Oryza sativa* L.). *Theor. Appl. Genet.*, **104** 981–986.

MIYATA, T., KUROKAWA, K. AND STRIHOU, V., 2001, Molecular and Cellular Nephrology, Institute of Medical Advanced Glycation and Lipoxidation End Products: Role of Reactive Carbonyl Compounds Generated during Carbohydrate and Lipid Metabolism. *J Am Soc Nephrol*., **11**: 1744–1752.

MUDGETT, M. B. AND CLARKE, S., 1994, Hormonal and environmental responsiveness of a developmentally regulated protein repair L-isoaspartyl methyltransferase in wheat. *J. Biol. Chem.,* **269:** 25605– 25612.

MUDGETT, M. B. AND CLARKE, S., 1996, A distinctly regulated protein repair L-isoaspartyl methyltransferase from *Arabidopsis thaliana*. *Plant Mol. Biol.,* **30**: 723–737.

MUDGETT, M. B., LOWENSON, J. D. AND CLARKE, S., 1997, Protein repair L isoaspartyl methyltransferase in plants. Phylogenetic distribution and the accumulation of substrate proteins in aged barley seeds. *Plant Physiol.,* **115**: 1481–1489.

MUDGETT, M. B., LOWENSON, J. D. AND CLARKE, S., 1997, Protein repair L-isoaspartyl methyltransferase in plants: phylogenetic distribution and the accumulation of substrate proteins in aged barley seeds. *Plant Physiol.,* **115**: 1481–1489.

MURTHY, N., PRAKASH, U, M., KUMAR, P. AND WENDELL, Q., 2003, Mechanisms of seed ageing under different storage conditions for *Vigna radiate* L. Wilczek: lipid peroxidation, sugar hydrolysis, Maillard reactions and their relationship to glass state transition. *Journal of Experimental Botany*, **54**: 1057-1067.

NAGAMIYA, K., MOTOHASHI, T., NAKAO, K., PRODHAN, S. H., HATTORI, E., HIROSE, S., OZAWA, K., OHKAWA, Y., TAKABE, T., TAKABE, T. AND KOMAMINE, A., 2007, Enhancement of salt tolerance in transgenic rice expressing an *Escherichia coli* catalase gene, katE. *Plant Biotechnol. Rep.,* **1**: 49-55.

NISARGA, K. N., 2013, Role of aldo-keto reductases in regulating seed viability and seedling vigour in rice. MSc thesis, University of Agriculture Sciences, Bengaluru, India.

NOCTOR, G. AND FOYER, C. H., 1998, Re-evaluation of the ATP: NADPH budget during C3 photosynthesis. A contribution from nitrate assimilation and its associated respiratory activity?. *J. Exp. Bot.*, **49:** 1895-1908.

OBERSCHALL, A., DEÁK, M., TÖRÖK, K., SASS, L., VASS, I., KOVÁCS, I., FEHÉR, A., DUDITS, D. AND HORVÁTH, G. V., 2000, A novel aldose/aldehyde reductase protects transgenic plants against lipid peroxidation under chemical and drought stresses. *Plant J.*, **24**: 437-446.

OGE, L., BOURDAIS, G., BOVE, J., COLLET, B., GODIN, B., GRANIER, F., BOUTIN, J. P., JOB, D., JULLIEN, M. AND GRAPPINA, P., 2008, Protein repair L-Isoaspartyl methyltransferase1 is involved in both seed longevity and germination vigour in Arabidopsis. *The Plant Cell,* **20**: 3022–3037.

OSBRONE, D. J., 1980, Senescence in seeds. In: Thimann KV, ed. Senescence in plants. Boca Raton: *CRC Press*, **45:** 13-37.

PAVANI, C., 2006, Development and characterization of transgenics over expressing cry genes in field bean against *Helicoverpa armigera* (Hubner). MSc Thesis, University of Agricultural Sciences, Bangalore, India.

PEARCE, R. S. AND SAMAD, I. M., 1980, Changes in fatty acid content of polar lipids during ageing of seeds of peanut (*Arachis hypogea L.). Journal of Experimental Botany*, **31**: 1283-1290.

PETRUZZELLI, L. AND TARANTO, G., 1984, Phospholipid changes in wheat embryos aged under different storage conditions. *Journal of Experimental Botany*, **35**: 517-520.

PILLAY, D. T., 1997, Protein synthesis in ageing soybean cotyledons, loss in translational capacity. *Biochem. Biophys. Res. Commun.,* **79**: 796–804.

PONQUETT, R. T., SMITH, M. T. AND ROSS, G., 1992, Lipid autoxidation and seed ageing: putative relationships between seed longevity and lipid stability. *Seed Sci. Res.,* **2**: 51-54.

PRAKASH, S., JOHNSON, R. E. AND PRAKASH, L., 2005, Eukaryotic translation synthesis DNA polymerases: specificity of structure and function. *Annu. Rev. Biochem.,* **74**: 317–353.

PRIESTLEY, D. A., AND LEOPOLD, A. C., 1983, Lipid changes during natural ageing of soybean seeds. *Physiologia Plantarum,* **59**: 467-470.

PRIESTLEY, D. A., CULLINAN, V. I. AND WOLFE, J. 1985, Differences in seed longevity at the species level. *Plant, Cell and Environment,* **8**: 557–562.

PRIESTLEY, D. A., 1986, Seed ageing. Publishing Associates Itheca New York, pp: 137-55.

PRIETO-DAPENA, P., CASTANO, R., ALMOGUERA, C. AND JORDANA, J. 2006, improved resistances to controlled deterioration in transgenic seeds. *Plant physiol,* **142**: 1102-1112.

PRUTHVI, V., 2013, Validation of abiotic stress responsive transcription factors by over expression in crop plants. PhD thesis, University of Agricultural Sciences, Bengaluru, India.

PRUTHVI, V., RAMA, N., GEETHA, G. AND KARABA N. N., 2013, Expression analysis of drought stresses specific genes in Peanut (*Arachis hypogaea* L.). *Physiol Mol Biol Plants*., **19**(2): 277–281.

PRUTHVI, V., NARASIMHAN, R. AND KARABA, N. K., 2014, Simultaneous Expression of Abiotic Stress Responsive Transcription Factors, AtDREB2A, AtHB7 and AtABF3 Improves Salinity and Drought Tolerance in Peanut (*Arachis hypogaea* L.). PLOS ONE | DOI:10.1371/journal.pone.0111152

RAJJOU, L. AND DEBEAUJON, I. 2008, Seed longevity: Survival and maintenance of high germination ability in dry seeds. *C.R. Biol.*, **331**: 796-805.

RAJJOU, L., LOVIGNY, Y., GROOT, S. P. C., BELGHAZI, M., JOB, C. AND JOB, D., 2008, Proteome wide characterization of seed ageing in Arabidopsis. A comparison between artificial and natural ageing protocols. *Plant Physiol.*, **148**: 620–641.

RAMU, S. V., 2012, Development and evaluation of transgenic Pigeon Pea (*Cajanas cajan* L.) lines tolerant to *Helicovorpa armigera*. PhD thesis, Department of microbiology and biotechnology, Jnanbharathi campus, Bangalore University, India.

RAO, K. S., SREEVATHSA, R., SHARMA, P. D., KESHAMMA, E. AND UDAYAKUMAR, M., 2008, *In planta* transformation of pigeon pea: a method to overcome recalcitrance of the crop to regeneration in vitro. *Physiol. Mol. Biol. Plant.*, **14:** 321–328.

RAO, M. S., BHAGYASRI AND MOHAMED, A.I., 1998, Yield protein, and oil quality of soybean genotypes selected for tofu production. *Plant Foods Hum. Nutr*, **52**: 241-251.

RAVANEL, S., GAKIÈRE, B., JOB, D. AND DOUCE, R., 1998, The specific features of methionine biosynthesis and metabolism in plants. *Proc. Natl. Acad. Sci. USA.*, **95**: 7805–7812.

REUSCHE, G. A., 1987, Peanut seed production. - *J, Seed Techno.*, **11:** 88-96.

ROBERT, R. J, DAWS, M. I. AND HAY, F. R., 2009, Ecological correlates of ex situ seed longevity: a comparative study on 195 species. *Annals of Botany*, **104**: 57-69.

ROBERTS, E. H. AND ELLIS, R. H., 1989, Water and seed survival. *Ann.Bot.*, **63**: 39–52.

ROBERTS, E. H., 1973, predicting the storage life of seeds. *Seed Sci. Technol.*, **1**: 499–514.

ROBERTSON, D. W., LUTE, A. M. AND KROEGER, H. 1943, Germination of 20-year old wheat, oats, barley, corn, rye, sorghum, and soybean. *Agronomy Journal***, 35**: 786-795.

ROHINI, V. K. AND RAO, K. S., 2000, Embryo transformation, a practical approach for realizing transgenic plants of safflower (*Carthamus tinctorius* L.). *Ann. Bot.*, **86:** 1043-1049.

ROLDA, N. T. AND ARIZA, R. R., 2009, Repair and tolerance of oxidative DNA damage in plants. *Mutation Research*, **681**: 169–179.

SALIMAT, M., 2014, Role of helicase in stress tolerance: an analysis through transgenic approach in crops. PhD thesis, University of Agriculture Sciences, Bengaluru, India.

SANKARA, R. AND MAHESHWARI, R., 2010, Branch-branch connections in trees analogous to hyphal fusions in fungal colonies. *Curr. Sci.,* **98**: 240-242.

SATTLER, S. E., GILLILAND, L. U., MAGALLANES, L. M., POLLARD, M. AND DELLAPENNA, D., 2004, Vitamin E is essential for seed longevity and for preventing lipid peroxidation during germination. *Plant Cell*, **16:** 1419–1432.

SCOTT, H., WETTLAUFER, L. AND CARL, A., 1991, Relevance of Amadori and Maillard Products to Seed Deterioration, *Plant Physio.,* **97**: 165-169.

SEN, S. AND OSBORAE, D. J., 1977, Decline in ribonucleic acid and protein synthesis with loss of viability during the early h of imbibition of rye (*Secale cereale* L.) embryos. *Biochemistry Journal,* **22**: 156-166.

SHEN-MILLER, J., MUDGETT, M. B., SCHOPF, J. W., CLARKE, S. AND BERGER, R., 1995, Exceptional seed longevity and robust growth: ancient sacred lotus from China. *Am J Bot.,* **82**: 1367–1380.

SHEN-MILLER, J., 2002, Sacred lotus, the long-living fruits of China Antique. *Seed Sci. Res.,* **12**: 131–43.

SHEN-MILLER, J., LINDNER,P., XIE, Y., VILLA, S., WOODING, K., CLARKE, S. G., RACHEL R. O. L. AND JOSEPH A. L., 2013, Thermal-Stable Proteins of Fruit of Long-Living Sacred Lotus *Nelumbo nucifera* Gaertn var. China Antique. *Tropical Plant Biol.,* **6**:69–84.

SIMPSON, P. J., TANTITADAPITA, C., REED, A. M., MATHER, O. C., BUNCE, C. M., WHITE, S. A. AND RIDE, J. P., 2009, Characterization of two novel aldo-keto reductases from arabidopsis: expression patterns, broad substrate specificity, and an open active-site structure suggest a role in toxicant metabolism following stress. *Journal of Molecular Biology*, **392**: 465–480.

SREE, B. K., RAJENDRAKUMAR, S. V. AND REDDY, A. R., 2000, Aldose reductase in rice (*Oryza sativa L.*): stress response and developmental specificity. *Plant Sci.,* **160**: 149–157.

SUN, W. Q. AND LEOPOLD, A. C., 1995, The Maillard reaction and oxidative stress during ageing of soybean seeds. *Physiologia Plantarum,* **94**: 94-105.

SUNDARESHA, S., MOHAJ K, A., ROHINI, S., MATH, S. A., KESHAMMA, E., CHANDRASHEKAR, S. C. AND UDAYAKUMAR, M., 2009, Enhanced protection against two major fungal pathogens of groundnut, *cercospora arachidicola* and *Aspergillus flavus* in transgenic groundnut overexpressing a tobacco β 1-3 glucanase. *Eur.J.Plant Pathol*., **126**: 497-508.

SUNG, J. M. AND JENG, T. L., 1994, Lipid peroxidation and peroxide-scavenging enzymes associated with accelerated ageing of peanut seed. *physiologia plantarum*, **91**: 51-55.

SUNKAR, R., BARTELS, D. AND KIRCH, H., 2003, Overexpression of a stress-inducible aldehyde dehydrogenase gene from Arabidopsis thaliana in transgenic plants improves stress tolerance. *Plant J.,* **35**: 452–464.

SUPARTANA, P., SHIMIZU, T., NOGAWA, M., SHIOIRA, H., NAKAJIMA, T., HARAMOTO, N., NOUZE, M. AND KOIJMA, M., 2006, Development of simple and efficient *in planta* transformation method for wheat (*Triticum aestivum L.*) using *Agrobacterium tumefaciens. J. Biosci. Bioeng.,* **102**: 162-170.

TAKAGI, D., INOU, H., ODAWARA, M., SHIMAKAWA, G. AND CHIKAHIRO MIYAKE, 2014, the Calvin Cycle Inevitably Produces Sugar-Derived Reactive Carbonyl Methylglyoxal During Photosynthesis: A Potential Cause of Plant Diabetes. *Plant Cell Physiol.,* **55**(2): 333–340.

THAPAR, N., KIM, A. K. AND CLARKE, S., 2001, Distinct patterns of expression but similar biochemical properties of protein L-isoaspartyl methyltransferase in higher plants. *Plant Physiol.,* **125:** 1023–1035.

TRAWATHA, S. E., TEKRONY, D. M. AND HILDEBRAND, D. F., 1995, Relationship of soybean quality to fatty acid and C6-aldehyde levels during storage. *Crop science,* **35:** 1415-1422.

TUROCZY, Z., KIS, P., TOROK, M., LENDVAI, D., DUDITS, D. AND HORVÁTH, 2011, Overproduction of a rice aldo-keto reductase increases oxidative and heat stress tolerance by malondialdehyde and methylglyoxal detoxification. *Plant Mol. Biol*., **75**: 399-412.

VERMA, P., SINGH, A. AND KAUR, H., 2009, Protein L-isoaspartyl methyltransferase1 (CaPIMT1) from chickpea mitigates oxidative stress-induced growth inhibition of *Escherichia coli. planta*, **231**: 329-336.

VERMA, P., KAUR, H., PETLA, B. P., RAO, V., SAXENA, S. C. AND MAJEE, M., 2013, Protein L-Isoaspartyl Methyltransferase2 is differentially expressed in chickpea and enhances seed vigour and longevity by reducing abnormal isoaspartyl accumulation predominantly in seed nuclear proteins. *Plant Physiol*., **161**: 1141–1157.

VIGNESWARA, V., LOWENSON, J. D., POWELL, C. D., THAKUR, M., BAILEY, K., CLARKE, S., RAY, D. E., AND CARTER, W. G., 2006, Proteomic identification of novel substrates of a protein isoaspartyl methyltransferase repair enzyme. *J. Biol. Chem.*, **281**: 32619–32629.

VILLA, S. T., XU, Q., DOWNIE, A. B. AND CLARKE, S. G., 2006, Arabidopsis protein repair L-isoaspartyl methyltransferases: Predominant activities at lethal temperatures. *Physiol. Plant.*, **128**: 581–592.

VISICK, J. E., CAI, H. AND CLARKE, S. 1998, The L-isoaspartyl protein repair methyltransferase enhances survival of aging *Escherichia coli* subjected to secondary environmental stresses. *J. Bacteriol*., **180**: 2623–2629.

WALTERS, C. AND KOSTER, K. L., 2007, Structural dynamics and desiccation damage in plant reproductive organs. *Bio chem.*, **11**: 251–280.

WALTERS, C., 1998, Understanding the mechanisms and kinetics of seed ageing. *Seed Science Research,* **8:** 223–244.

WALTERS, C., HILL, L. M., AND WHEELER, L. J., 2005, Dying while dry: kinetics and mechanisms of deterioration in desiccated organisms. *Integr Comp Bio.,* **45:** 751-758.

WONG, J. H., CAI, N., BALMER, Y., TANAKA, C. K., VENSEL, W. H., HURKMAN, W. J. AND BUCHANAN, B. B., 2004, Thioredoxin targets of developing wheat seeds identified by complementary proteomic approaches. *Phytochemistry,* **65**: 1629–1640.

WETTLAUFER, S. H. AND LEOPOLD, A. C., 1991, Relevance of Amadori and Maillard products to seed deterioration. *Plant Physiology*, **97:** 165-169.

WILSON, D. O. & MCDONALD, M. B., 1986, The lipid peroxidation model of seed ageing. - *Seed Sci, Technol.,* **14**: 269-300.

XU, Q., BELCASTRO, M. P., VILLA, S. T., DINKINS, R. D., CLARKE, S. G. AND DOWNIE, A. B., 2004, A second protein L-isoaspartyl methyltransferase gene in Arabidopsis produces two transcripts whose products are sequestered in the nucleus. *Plant Physiol.,* **136**: 2652–2664.

XU, R., FAZIO, G. C. AND MATSUDA, S. P. T., 2004, On the origins of triterpenoid skeletal diversity. *Phytochemistry,* **65:** 261-291.

ZHANG, M., NAKAMURA, Y., TSUDA, S., NAGASHIMA, T. AND ESASHI, Y., 1995, Enzymatic conversion of volatile metabolites in dry seeds during storage. *Plant Cell Physio.,* **36**: 157–164.

ZHU, J. X., DOYLE, H. A., MAMULA, M. J. AND ASWAD, D. W., 2006, Protein repair in the brain, proteomic analysis of endogenous substrates for protein L-isoaspartyl methyltransferase in mouse brain. *J. Biol. Chem*., **281**: 33802–33813.

ZHU, J., DONG, C. H. AND ZHU, J. K., 2007, Interplay between cold- responsive gene regulation, metabolism and RNA processing during plant cold acclimation. *Curr.Opin.Plant Biol.,* **10:** 290–295.

ZIMNIAK, P. AND SINGH, S. P., 2011, Families of glutathione transferases. In Toxicology of Glutathione Transferases (Awasthi YC ed.). *Taylor & Francis CRC Press, Boca Raton*., **11**: 11–26.

**APPENDEX I**
**Details of the primers used in the study**

| SL. NO | Primers | | Nucleotide sequence |
|---|---|---|---|
| 1 | PsAKR | Forward | GATGCACAGAGAGGACGATAGC |
| | | Reverse | ATCAAAGCTGCGCCTTGTAGC |
| 2 | caPIMT2 | forward | CACATCAAGCAAGGTGGCTGAA |
| | | Reverse | CAGAGGTCTCAGTCCGGATACTG |
| 3 | Napin | Forward F1 | GCCATGCCAGAAGATTAGCT |
| | | Forward F2 | CATAGCATGCAGCCGCGGAG |
| 4 | CaPIMT2 _GSP | Reverse 1 | CAGCACCTTGCTTGATGTG |
| | | Reverse 2 | GCTGCAACCTCTCCACCATTGC |
| 5 | RBCS nest | Forward | CCACGTGTCACATCGTCATGGTG |
| | | Reverse | ATGCGGTTGTAGCATTCC |
| 6 | igraRT | Forward | CCGGCAACCCTCACATCAAAAGATTGGC |
| | | Reverse | GGAGGCATTGGAGGCAGGTCACAGATTC |
| 7 | PIMT2 RT | Forward | GGTGGGAGAATGGTGATTCCTG |
| | | Reverse | GACTAGTGAGGGGCACATAAGC |
| 8 | Tubulin | Forward | GAGCTCTACTGCCTCGAACATGG |
| | | Reverse | CCGGGTGGAAGAGCTGGCGGTAGG |

# APPENDIX II

## A. Media compositions

**LB medium:**

Tryptone-10g

Yeast extract-5g

NaCl- 10g

pH-7

Distilled water- 1L

**YEM medium:**

Mannitol- 10g

$KH_2PO_4$- 200mg

$K_2HPO_4$- 200mg

Yeast extract- 1g

$MgSO_4$(1M)- 800

µl $CaCl_2$(1M)- 400

µl Agar - 18g

Distilled water – 1L

**2% Cetyl triamonium bromide (CTAB) buffer**:

2% CTAB

1.4M NaCl

100mM Tris-HCl

20mM EDTA

**AB MEDIA:**

- **AB salts:**

NH4CL-20g

MGSO4- 6g

KCL- 3g

CaCL2- 3g

FeSO4.7H2O- 0.05g

- **AB buffer:**

KH2PO4- 6g

NaH2PO4- 20g

pH- 7

- **AB glucose:**

Glucose – 5.5g

Distilled water- 1L

**PHOSPHATE BUFFERED SALINE (PBS)**

10x PBS (sodium-potassium combo), 1 L:

• 2 g KCl

• 2.4 g KH2PO4

• 80 g NaCl

• 11.45 g Na2HPO4

Dissolve everything in approximately 800 ml of $H_2O$ then fill up to 1000 ml. Autoclave and store at room temperature.

**10x PBS, pH 7.4 (sodium), 100 mL:**

- 7.6 g NaCl (Mw 58.4425 g/mol, M= 1.3 mol/L)
- 0.99g Na2HPO4 (Mw 141.959 g/mol, M= 0.07 mol/L)
- 0.41g Na2HPO4 x H2O (Mw 137.992 g/mol, M= 0.03 mol/L)

Adjust the pH with 1M NaOH or 1 M HCl. Fill up to 100 ml. Autoclave and store in room temperature.

# APPENDIX III

## Nucleotide sequence of PIMT2- $5^1$ - $3^1$ – 1180bp

GAAAATTGTAGAAAGAAAGTGTGAGAAGGGTAGAAATGAATGTTCGGTACTATTTCCCTGTATCGGTGGCG
TATGATTCCCGCGCGTGTTACGAAGCAGCAACAACAAAACCAACTAATCACTCTCTTTCTCTAACCCTCTT
ACACCACCGTTCTCTTTCTCTTCCCTTCTTCCAAAACCCTAATTTCCTCACGGGAAACACTATGCAGCGAT
TCTGCTCCGTGAATGGCATAAGTAAGAACAGAGCAATGGTGGAGAGGTTGCAGCATTTTGGAGTAATCACA
TCAAGCAAGGTGGCTGAAGTAATGGAGACTGTTGATAGAGCTTTGTTTGTACCTAGTGGATCAGCTCCTTA
TGATGACAGCCCAATGGCCATAGGCTACAATGCCACTATATCTGCACCTCATATGCATGCTACCTGCCTTC
AGTTGCTGGAGGAGAATTTGCAACCTGGGATGCGCGCTCTCGACGTTGGCTCTGGAACAGGGTATCTGACT
GCGTGTTTTGCCTTGATGGTTGGACCCCGAGGCCGTGCTGTTGGTGTAGAGCATATTCCTGAATTGGTTTC
TTTTGCAATAGAAAATATTCAGAAAAGTGCTGCAGCTACACTATTGAAAGATGGTTCCCTTTTTGTTCATG
TTGGTGATGGAAGGGAAGGTTGGCCAGAATTTGCTCCTTATGATGCCATTCACGTTGGAGCAGCAGCACCA
GAAATTCCCCAACCACTTATTGACCAGTTGAAGCCTGGTGGGAGAATGGTGATTCCTGTTGGTAACATGTT
TCAAGATTTAAAGGTGGTGGACAAGAATTCTGATGGTTCTATCAGTATCCGGACTGAGACCTCTGTTCGTT
ATGTGCCCCTCACTAGTCGAGAAGCTCAACTGCGCGGCTACTAAGATGGTGCCAAAATCTTGAATTGTGGA
AGGTTAATGCTTCAGGTGTCAGTGTTTGGAACTTGAATGTACACACATGACGTTAGAATTTGTACAGTTAA
TGTTTGCTGAATTTTGCAAGCTTATACATATATCTATCAGTGGTTGGAACTTGGATGTAAGCACAAGCTGT
TAGAATTTGTAAAGGTAAAGGTTGGTGCTCTTCTAACAGGATAGATTCATGTAAATCCCTTGCAGCAATAA
TAATTTTGATAC

## Nucleotide sequences AKR-$5^1$ -$3^1$ length-1589bp.

TCTAGACTCGAGGAATTCCAAGCAACGAACTGCGAGTGATTCAAGAAAAAAGAAAACCTGAGCTTTCGATC
TCTACGGAGTGGTTTCTTGTTCTTTGAAAAAGAGGGGGATTAAAAAAAAAAAGGGCCCATGGTACCCGGGA
TGCACAGAGAGGACGATAGCACATCAACTGGGAGGAGGGAGGAGAGACTGAGCACAGGGAAGGAGACAGCC
TACAGCCTGGACCTGATCAAAGAATTTTTCAGACAGCACTCTACAGACCTCAAAGGCATGGACTTATCCTC
TTCCCACAGAGGCTTTATGGTAACTGCCTCGCTCCTCTGGCCTACAGTAATTATGTGGCTCCAAGAGAACT
TCATTCCTCTATCCACGCCGAGGGAAGGACCCTCTCGGTCAGATTGATTATGTTGTTTGTGAACACCAGTA
AGAGATCGAGGACTTTGCCTCCAGTGGGAAGTACTGTTATGAGGATACTTGTGAATCTCTGTCAGTTGCTT
TCAGCAGAGTTTCAGCTCCTGCAAGGTAGCCAGAGGTTGATTTTCAAACTTTGCTACAGAACATGTCCTGA
TCAAAGAAGAGGAGATACCAGAGTTGCTCAGTGCCCAGGCGATTCCCACCTCTCTCAATGTCTGACTGCAA
CTTTTAGAAACGGGGTTCAGAGACCTGACGCTCCACAGCAACTGTTCGTCCTATGCACTGGGCTACAAGGC
GCAGCTTTGATAAGACCTAGGCCATTTGGAGATTCCGTGCAGATTGCCGGAGGTCAACAATCACTGAGCCA
AAGAAGGAAGGATAATGCCGCAGGATCTGACCTAGCTAAAGGTATTGAACAGGCAATCTTCGATCCTGCTA
TAGAGCATGTGGTTATTGGATTGATGAACGAAAAGAGGAATCCACTGTTTCTACAAGACAGGGGGTCACTA
TTGAGACAGTTCAGAAGGATTGCCGGCAACCCTCACATCAAAAGATTGGCACTTACTGTTCAGATGAGAGA
AAGGAGCCATAGGCTTTTCCAAAGAAGAGGAGGAGTGCACACAGTGAGAATCGAAGATGTTGACATAGTCG
AGCCTCATCCACTCCAAAGACCAGGAAGGGGTTGGAGATCAGGAATCTGTGACCTGCCTCCAATGCCTCCA
TATGGACCAGGGCATATGTCGCAACCAGCCTTTGAGGAAATGATTATATCGTCGAGGTAATAACCCGGGCG
GCCGCGGATCCAGATCTGAATTCAGAGATGATGATGATCTATCTCTCTCTCTCTCTCTCTGCAGTTTCAGT
GCATACAAGAAACCAAAAATGAAAGCAAAAAATGGATGAGAAGTTTGAGGTTTTCCATTTAAGTTTAATAA
TGGTCGTGAGACAGGGCTTGTGTGGAGTCAGCTCTGTTTACTCCCTCCTTTCATGGCTTGATCTTGTTTAT
TGTCAGCATTGTGGCTTAGCAGATTTTCACCAGTTTTACCCTGTAAATTAGTACCATTCTGATCAATCGTA
ATAAACTACTAGCAGTGTGAGGTACTATTGCTGGAACTGAATTGATTGTGCAGTAAAGAGGTGAATAGCAG
CCAGTATGAAGGTTTTGAGCTCTAGA

## Nucleotide sequence of pi12GW binary vector- 10810bp.

AGCTTAGCTTGAGCTTGGATCAGATTGTCGTTTCCCGCCTTCAGTTTAAACTATCAGTGTTTGACAGGATA
TATTGGCGGGTAAACCTAAGAGAAAAGAGCGTTTATTAGAATAACGGATATTTAAAAGGGCGTGAAAAGGT
TTATCCGTTCGTCCATTTGTATGTGCATGCCAACCACAGGGTTCCCCTCGGGATCAAAGTACTTTGATCCA
ACCCCTCCGCTGCTATAGTGCAGTCGGCTTCTGACGTTCAGTGCAGCCGTCTTCTGAAAACGACATGTCGC
ACAAGTCCTAAGTTACGCGACAGGCTGCCGCCCTGCCCTTTTCCTGGCGTTTTCTTGTCGCGTGTTTTAGT
CGCATAAAGTAGAATACTTGCGACTAGAACCGGAGACATTACGCCATGAACAAGAGCGCCGCCGCTGGCCT
GCTGGGCTATGCCCGCGTCAGCACCGACGACCAGGACTTGACCAACCAACGGGCCGAACTGCACGCGGCCG
GCTGCACCAAGCTGTTTTCCGAGAAGATCACCGGCACCAGGCGCGACCGCCCGGAGCTGGCCAGGATGCTT
GACCACCTACGCCCTGGCGACGTTGTGACAGTGACCAGGCTAGACCGCCTGGCCCGCAGCACCCGCGACCT
ACTGGACATTGCCGAGCGCATCCAGGAGGCCGGCGCGGGCCTGCGTAGCCTGGCAGAGCCGTGGGCCGACA
CCACCACGCCGGCCGGCCGCATGGTGTTGACCGTGTTCGCCGGCATTGCCGAGTTCGAGCGTTCCCTAATC
ATCGACCGCACCCGGAGCGGGCGCGAGGCCGCCAAGGCCCGAGGCGTGAAGTTTGGCCCCCGCCCTACCCT
CACCCCGGCACAGATCGCGCACGCCCGCGAGCTGATCGACCAGGAAGGCCGCACCGTGAAAGAGGCGGCTG
CACTGCTTGGCGTGCATCGCTCGACCCTGTACCGCGCACTTGAGCGCAGCGAGGAAGTGACGCCCACCGAG
GCCAGGCGGCGCGGTGCCTTCCGTGAGGACGCATTGACCGAGGCCGACGCCCTGGCGGCCGCCGAGAATGA
ACGCCAAGAGGAACAAGCATGAAACCGCACCAGGACGGCCAGGACGAACCGTTTTTCATTACCGAAGAGAT
CGAGGCGGAGATGATCGCGGCCGGGTACGTGTTCGAGCCGCCCGCGCACGTCTCAACCGTGCGGCTGCATG
AAATCCTGGCCGGTTTGTCTGATGCCAAGCTGGCGGCCTGGCCGGCCAGCTTGGCCGCTGAAGAAACCGAG
CGCCGCCGTCTAAAAAGGTGATGTGTATTTGAGTAAAACAGCTTGCGTCATGCGGTCGCTGCGTATATGAT
GCGATGAGTAAATAAACAAATACGCAAGGGGAACGCATGAAGGTTATCGCTGTACTTAACCAGAAAGGCGG
GTCAGGCAAGACGACCATCGCAACCCATCTAGCCCGCGCCCTGCAACTCGCCGGGGCCGATGTTCTGTTAG
TCGATTCCGATCCCCAGGGCAGTGCCCGCGATTGGGCGGCCGTGCGGGAAGATCAACCGCTAACCGTTGTC
GGCATCGACCGCCCGACGATTGACCGCGACGTGAAGGCCATCGGCCGGCGCGACTTCGTAGTGATCGACGG
AGCGCCCCAGGCGGCGGACTTGGCTGTGTCCGCGATCAAGGCAGCCGACTTCGTGCTGATTCCGGTGCAGC
CAAGCCCTTACGACATATGGGCCACCGCCGACCTGGTGGAGCTGGTTAAGCAGCGCATTGAGGTCACGGAT
GGAAGGCTACAAGCGGCCTTTGTCGTGTCGCGGGCGATCAAAGGCACGCGCATCGGCGGTGAGGTTGCCGA
GGCGCTGGCCGGGTACGAGCTGCCCATTCTTGAGTCCCGTATCACGCAGCGCGTGAGCTACCCAGGCACTG
CCGCCGCCGGCACAACCGTTCTTGAATCAGAACCCGAGGGCGACGCTGCCCGCGAGGTCCAGGCGCTGGCC
GCTGAAATTAAATCAAAACTCATTTGAGTTAATGAGGTAAAGAGAAAATGAGCAAAAGCACAAACACGCTA
AGTGCCGGCCGTCCGAGCGCACGCAGCAGCAAGGCTGCAACGTTGGCCAGCCTGGCAGACACGCCAGCCAT
GAAGCGGGTCAACTTTCAGTTGCCGGCGGAGGATCACACCAAGCTGAAGATGTACGCGGTACGCCAAGGCA
AGACCATTACCGAGCTGCTATCTGAATACATCGCGCAGCTACCAGAGTAAATGAGCAAATGAATAAATGAG
TAGATGAATTTTAGCGGCTAAAGGAGGCGGCATGGAAAATCAAGAACAACCAGGCACCGACGCCGTGGAAT
GCCCCATGTGTGGAGGAACGGGCGGTTGGCCAGGCGTAAGCGGCTGGGTTGTCTGCCGGCCCTGCAATGGC
ACTGGAACCCCCAAGCCCGAGGAATCGGCGTGACGGTCGCAAACCATCCGGCCCGGTACAAATCGGCGCGG
CGCTGGGTGATGACCTGGTGGAGAAGTTGAAGGCCGCGCAGGCCGCCCAGCGGCAACGCATCGAGGCAGAA
GCACGCCCCGGTGAATCGTGGCAAGCGGCCGCTGATCGAATCCGCAAAGAATCCCGGCAACCGCCGGCAGC
CGGTGCGCCGTCGATTAGGAAGCCGCCCAAGGGCGACGAGCAACCAGATTTTTTCGTTCCGATGCTCTATG
ACGTGGGCACCCGCGATAGTCGCAGCATCATGGACGTGGCCGTTTTCCGTCTGTCGAAGCGTGACCGACGA
GCTGGCGAGGTGATCCGCTACGAGCTTCCAGACGGGCACGTAGAGGTTTCCGCAGGGCCGGCCGGCATGGC
CAGTGTGTGGGATTACGACCTGGTACTGATGGCGGTTTCCCATCTAACCGAATCCATGAACCGATACCGGG
AAGGGAAGGGAGACAAGCCCGGCCGCGTGTTCCGTCCACACGTTGCGGACGTACTCAAGTTCTGCCGGCGA
GCCGATGGCGGAAAGCAGAAAGACGACCTGGTAGAAACCTGCATTCGGTTAAACACCACGCACGTTGCCAT
GCAGCGTACGAAGAAGGCCAAGAACGGCCGCCTGGTGACGGTATCCGAGGGTGAAGCCTTGATTAGCCGCT
ACAAGATCGTAAAGAGCGAAACCGGGCGGCCGGAGTACATCGAGATCGAGCTAGCTGATTGGATGTACCGC
GAGATCACAGAAGGCAAGAACCCGGACGTGCTGACGGTTCACCCCGATTACTTTTTGATCGATCCCGGCAT
CGGCCGTTTTCTCTACCGCCTGGCACGCCGCGCCGCAGGCAAGGCAGAAGCCAGATGGTTGTTCAAGACGA

TCTACGAACGCAGTGGCAGCGCCGGAGAGTTCAAGAAGTTCTGTTTCACCGTGCGCAAGCTGATCGGGTCA
AATGACCTGCCGGAGTACGATTTGAAGGAGGAGGCGGGGCAGGCTGGCCCGATCCTAGTCATGCGCTACCG
CAACCTGATCGAGGGCGAAGCATCCGCCGGTTCCTAATGTACGGAGCAGATGCTAGGGCAAATTGCCCTAG
CAGGGGAAAAAGGTCGAAAAGGTCTCTTTCCTGTGGATAGCACGTACATTGGGAACCCAAAGCCGTACATT
GGGAACCGGAACCCGTACATTGGGAACCCAAAGCCGTACATTGGGAACCGGTCACACATGTAAGTGACTGA
TATAAAAGAGAAAAAAGGCGATTTTTCCGCCTAAAACTCTTTAAAACTTATTAAAACTCTTAAAACCCGCC
TGGCCTGTGCATAACTGTCTGGCCAGCGCACAGCCGAAGAGCTGCAAAAAGCGCCTACCCTTCGGTCGCTG
CGCTCCCTACGCCCCGCCGCTTCGCGTCGGCCTATCGCGGCCGCTGGCCGCTCAAAAATGGCTGGCCTACG
GCCAGGCAATCTACCAGGGCGCGGACAAGCCGCGCCGTCGCCACTCGACCGCCGGCGCCCACATCAAGGCA
CCCTGCCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGC
TTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGG
GCGCAGCCATGACCCAGTCACGTAGCGATAGCGGAGTGTATACTGGCTTAACTATGCGGCATCAGAGCAGA
TTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGG
CGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCA
CTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCC
AGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAG
CATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCC
CCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCC
CTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCC
AAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGA
GTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGT
ATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGT
ATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCAC
CGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATC
CTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGCAT
GATATATCTCCCAATTTGTGTAGGGCTTATTATGCACGCTTAAAAATAATAAAAGCAGACTTGACCTGATA
GTTTGGCTGTGAGCAATTATGTGCTTAGTGCATCTAATCGCTTGAGTTAACGCCGGCGAAGCGGCGTCGGC
TTGAACGAATTTCTAGCTAGACATTATTTGCCGACTACCTTGGTGATCTCGCCTTTCACGTAGTGGACAAA
TTCTTCCAACTGATCTGCGCGCGAGGCCAAGCGATCTTCTTCTTGTCCAAGATAAGCCTGTCTAGCTTCAA
GTATGACGGGCTGATACTGGGCCGGCAGGCGCTCCATTGCCCAGTCGGCAGCGACATCCTTCGGCGCGATT
TTGCCGGTTACTGCGCTGTACCAAATGCGGGACAACGTAAGCACTACATTTCGCTCATCGCCAGCCCAGTC
GGGCGGCGAGTTCCATAGCGTTAAGGTTTCATTTAGCGCCTCAAATAGATCCTGTTCAGGAACCGGATCAA
AGAGTTCCTCCGCCGCTGGACCTACCAAGGCAACGCTATGTTCTCTTGCTTTTGTCAGCAAGATAGCCAGA
TCAATGTCGATCGTGGCTGGCTCGAAGATACCTGCAAGAATGTCATTGCGCTGCCATTCTCCAAATTGCAG
TTCGCGCTTAGCTGGATAACGCCACGGAATGATGTCGTCGTGCACAACAATGGTGACTTCTACAGCGCGGA
GAATCTCGCTCTCTCCAGGGGAAGCCGAAGTTTCCAAAAGGTCGTTGATCAAAGCTCGCCGCGTTGTTTCA
TCAAGCCTTACGGTCACCGTAACCAGCAAATCAATATCACTGTGTGGCTTCAGGCCGCCATCCACTGCGGA
GCCGTACAAATGTACGGCCAGCAACGTCGGTTCGAGATGGCGCTCGATGACGCCAACTACCTCTGATAGTT
GAGTCGATACTTCGGCGATCACCGCTTCCCCCATGATGTTTAACTTTGTTTTAGGGCGACTGCCCTGCTGC
GTAACATCGTTGCTGCTCCATAACATCAAACATCGACCCACGGCGTAACGCGCTTGCTGCTTGGATGCCCG
AGGCATAGACTGTACCCCAAAAAAACATGTCATAACAAGAAGCCATGAAAACCGCCACTGCGCCGTTACCA
CCGCTGCGTTCGGTCAAGGTTCTGGACCAGTTGCGTGACGGCAGTTACGCTACTTGCATTACAGCTTACGA
ACCGAACGAGGCTTATGTCCACTGGGTTCGTGCCCGAATTGATCACAGGCAGCAACGCTCTGTCATCGTTA
CAATCAACATGCTACCCTCCGCGAGATCATCCGTGTTTCAAACCCGGCAGCTTAGTTGCCGTTCTTCCGAA
TAGCATCGGTAACATGAGCAAAGTCTGCCGCCTTACAACGGCTCTCCCGCTGACGCCGTCCCGGACTGATG
GGCTGCCTGTATCGAGTGGTGATTTTGTGCCGAGCTGCCGGTCGGGGAGCTGTTGGCTGGCTGGTGGCAGG
ATATATTGTGGTGTAAACAAATTGACGCTTAGACAACTTAATAACACATTGCGGACGTTTTTAATGTACTG
AATTAACGCCGAATTGAATTATCAGCTTGCATGCCGGTCGATCTAGTAACATAGATGACACCGCGCGCGAT
AATTTATCCTAGTTTGCGCGCTATATTTTGTTTTCTATCGCGTATTAAATGTATAATTGCGGGACTCTAAT

CATAAAAACCCATCTCATAAATAACGTCATGCATTACATGTTAATTATTACATGCTTAACGTAATTCAACA
GAAATTATATGATAATCATCGCAAGACCGGCAACAGGATTCAATCTTAAGAAACTTTATTGCCAAATGTTT
GAACGATCTGCTTGACTCTAGCTAGAGTCCGAACCCCAGAGTCCCGCTCAGAAGAACTCGTCAAGAAGGCG
ATAGAAGGCGATGCGCTGCGAATCGGGAGCGGCGATACCGTAAAGCACGAGGAAGCGGTCAGCCCATTCGC
CGCCAAGCTCTTCAGCAATATCACGGGTAGCCAACGCTATGTCCTGATAGCGGTCCGCCACACCCAGCCGG
CCACAGTCGATGAATCCAGAAAAGCGGCCATTTTCCACCATGATATTCGGCAAGCAGGCATCGCCGTGGGT
CACGACGAGATCCTCGCCGTCGGGCATCCGCGCCTTGAGCCTGGCGAACAGTTCGGCTGGCGCGAGCCCCT
GATGCTCTTCGTCCAGATCATCCTGATCGACAAGACCGGCTTCCATCCGAGTACGTGCTCGCTCGATGCGA
TGTTTCGCTTGGTGGTCGAATGGGCAGGTAGCCGGATCAAGCGTATGCAGCCGCCGCATTGCATCAGCCAT
GATGGATACTTTCTCGGCAGGAGCAAGGTGAGATGACAGGAGATCCTGCCCCGGCACTTCGCCCAATAGCA
GCCAGTCCCTTCCCGCTTCAGTGACAACGTCGAGCACAGCTGCGCAAGGAACGCCCGTCGTGGCCAGCCAC
GATAGCCGCGCTGCCTCGTCTTGGAGTTCATTCAGGGCACCGGACAGGTCGGTCTTGACAAAAAGAACCGG
GCGCCCCTGCGCTGACAGCCGGAACACGGCGGCATCAGAGCAGCCGATTGTCTGTTGTGCCCAGTCATAGC
CGAATAGCCTCTCCACCCAAGCGGCCGGAGAACCTGCGTGCAATCCATCTTGTTCAATCATGCCTCGATCG
AGTTGAGAGTGAATATGAGACTCTAATTGGATACCGAGGGGAATTTATGGAACGTCAGTGGAGCATTTTTG
ACAAGAAATATTTGCTAGCTGATAGTGACCTTAGGCGACTTTTGAACGCGCAATAATGGTTTCTGACGTAT
GTGCTTAGCTCATTAAACTCCAGAAACCCGCGGCTGAGTGGCTCCTTCAACGTTGCGGTTCTGTCAGTTCC
AAACGTAAAACGGCTTGTCCCGCGTCATCGGCGGGGGTCATAACGTGACTCCCTTAATTCTCATGTATGAT
AATTCGAGGGTACCCCCACCGCGGTGGCGGCCGCTCTAGAACTAGTTAATTAAGGAATTATCGAACCACTT
TGTACAAGAAAGCTGAACGAGAAACGTAAAATGATATAAATATCAATATATTAAATTAGATTTTGCATAAA
AAACAGACTACATAATACTGTAAAACACAACATATCCAGTCACTATGGTCGACCTGCAGACTGGCTGTGTA
TAAGGGAGCCTGACATTTATATTCCCCAGAACATCAGGTTAATGGCGTTTTTGATGTCATTTTCGCGGTGG
CTGAGATCAGCCACTTCTTCCCCGATAACGGAGACCGGCACACTGGCCATATCGGTGGTCATCATGCGCCA
GCTTTCATCCCCGATATGCACCACCGGGTAAAGTTCACGGGAGACTTTATCTGACAGCAGACGTGCACTGG
CCAGGGGGATCACCATCCGTCGCCCGGGCGTGTCAATAATATCACTCTGTACATCCACAAACAGACGATAA
CGGCTCTCTCTTTTATAGGTGTAAACCTTAAACTGCATTTCACCAGTCCCTGTTCTCGTCAGCAAAAGAGC
CGTTCATTTCAATAAACCGGGCGACCTCAGCCATCCCTTCCTGATTTTCCGCTTTCCAGCGTTCGGCACGC
AGACGACGGGCTTCATTCTGCATGGTTGTGCTTACCAGACCGGAGATATTGACATCATATATGCCTTGAGC
AACTGATAGCTGTCGCTGTCAACTGTCACTGTAATACGCTGCTTCATAGCACACCTCTTTTTGACATACTT
CGGGTATACATATCAGTATATATTCTTATACCGCAAAAATCAGCGCGCAAATACGCATACTGTTATCTGGC
TTTTAGTAAGCCGGATCCTCTAGATTACGCCCCGCCCTGCCACTCATCGCAGTACTGTTGTAATTCATTAA
GCATTCTGCCGACATGGAAGCCATCACAGACGGCATGATGAACCTGAATCGCCAGCGGCATCAGCACCTTG
TCGCCTTGCGTATAATATTTGCCCATGGTGAAAACGGGGGCGAAGAAGTTGTCCATATTGGCCACGTTTAA
ATCAAAACTGGTGAAACTCACCCAGGGATTGGCTGAGACGAAAAACATATTCTCAATAAACCCTTTAGGGA
AATAGGCCAGGTTTTCACCGTAACACGCCACATCTTGCGAATATATGTGTAGAAACTGCCGGAAATCGTCG
TGGTATTCACTCCAGAGCGATGAAAACGTTTCAGTTTGCTCATGGAAAACGGTGTAACAAGGGTGAACACT
ATCCCATATCACCAGCTCACCGTCTTTCATTGCCATACGGAATTCCGGATGAGCATTCATCAGGCGGGCAA
GAATGTGAATAAAGGCCGGATAAAACTTGTGCTTATTTTTCTTTACGGTCTTTAAAAAGGCCGTAATATCC
AGCTGAACGGTCTGGTTATAGGTACATTGAGCAACTGACTGAAATGCCTCAAAATGTTCTTTACGATGCCA
TTGGGATATATCAACGGTGGTATATCCAGTGATTTTTTTCTCCATTTTAGCTTCCTTAGCTCCTGAAAATC
TCGCCGGATCCTAACTCAAAATCCACACATTATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCT
AATGCGGCCGCCATAGTGACTGGATATGTTGTGTTTTACAGTATTATGTAGTCTGTTTTTTATGCAAAATC
TAATTTAATATATTGATATTTATATCATTTTACGTTTCTCGTTCAGCTTTTTTGTACAAACTTGTTTGATA
GCTTGGCGCGCCTCGAGGGGGGGCCCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAGGGCA
TGCGGCGCGGCCTTAATTAGATATCACCACTTTGTACAAGAAAGCTGAACGAGAAACGTAAAATGATATAA
ATATCAATATATTAAATTAGATTTTGCATAAAAAACAGACTACATAATACTGTAAAACACAACATATCCAG
TCACTATGGAATTCGAGCTCGGTACCTCTAGGATCCCGGGCCNNNNNGGCCGGGCCCGTCGACCATGGCTC
GAGCTGCAGAGATCTAAGCTTCATAGTGACTGGATATGTTGTGTTTTACAGTATTATGTAGTCTGTTTTTT
ATGCAAAATCTAATTTAATATATTGATATTTATATCATTTTACGTTTCTCGTTCAGCTTTTTTGTACAAAC

TTGTTTGATAGCTTAGCTTGAGCTTGGATCAGATTGTCGTTTCCCGCCTTCAGTTTAAACTATCAGTGTTT
GACAGGATATATTGGCGGGTAAACCTAAGAGAAAAGAGCGTTTATTAGAATAACGGATATTTAAAAGGGCG
TGAAAAGGTTTATCCGTTCGTCCATTTGTATGTGCATGCCC

## Nucleotide sequences of napin promotor 5'-3' 1137 bp
## >gi|167375698|gb|EU416279.1| Brassica napus (napin) promoter region and 5' UTR

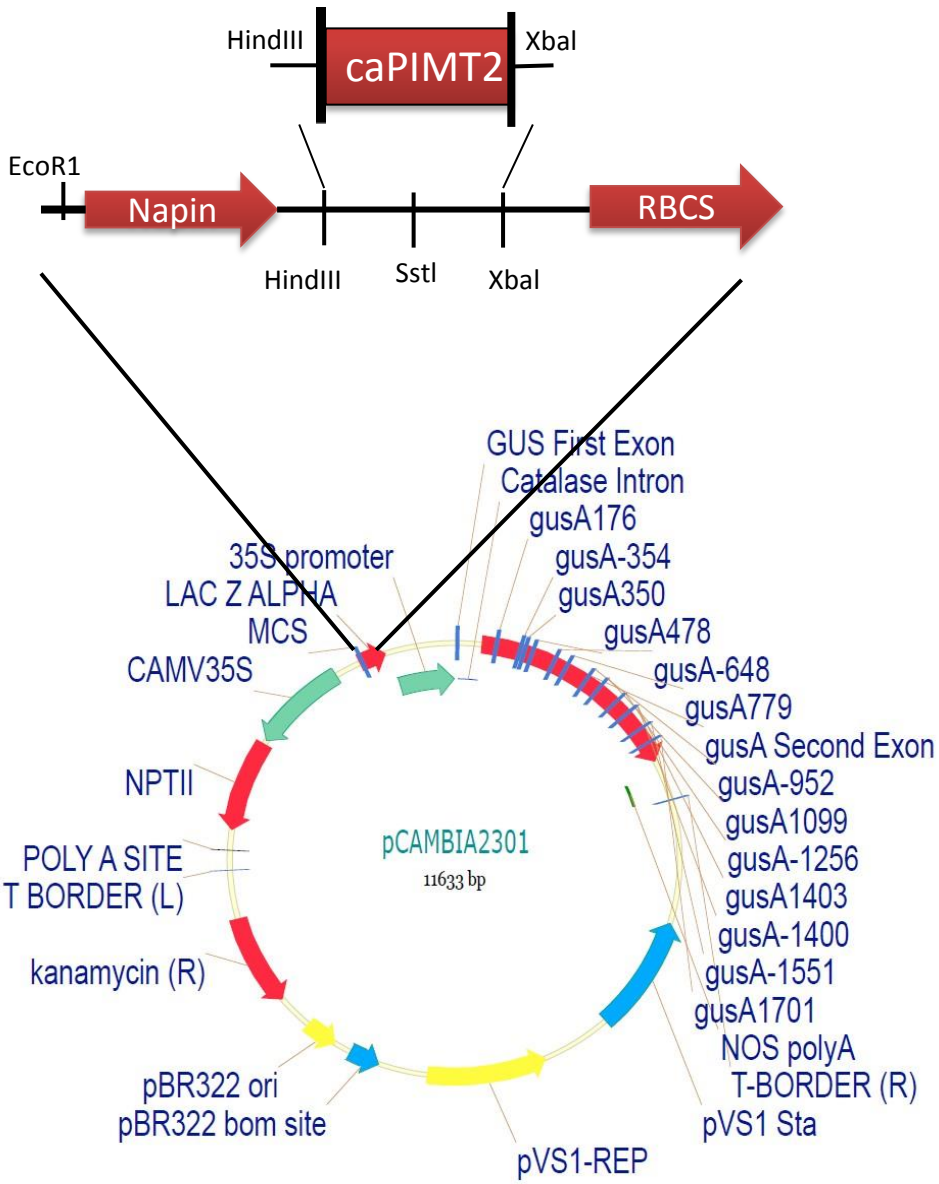
CTTCATCGGTGATTGATTCCTTTAAAGACTTATGTTTCTTATCTTGCTTCTGAGGCAAGTATTCATTTCCA
AGTTACCAGTTACCACTTATATTCTGGACTTTCTGACTGCATCCTCATTTTTCCAACATTTTAAATTTCAC
TATTGGCTGAATGCTTCTTCTTTGAGGAAGAAACAATTCAGATGGCAGAAATGTATCAACCAATGCATATA
TACAAATGTACCTCTTGTTCTCAAAACATCTATCGGATGGTTCCATTTGCTTTGTCATCCAATTAGTGACT
ACTTTATATTATTCACTCCTCTTTATTACTATTTTCATGCGAGGTTGCCATGTACATTATATTTGTAAGGA
TTGACGCTATTGAGCGTTTTTCTTCAATTTTCTTTATTTTAGACATGGGTATGAAATGTGTGTTAGAGTTG
GGTTGAATGAGATATACGTTCAAGTGAATGGCATACCGTTCTCGAGTAAGGATGACCTACCCATTCTTGAG
ACAAATGTTACATTTTAGTATCAGAGTAAAATGTGTACCTATAACTCAAATTCGATTGACATGTATCCATT
CAACATAAAATTAAACCAGCCTGCACCTGCATCCACATTTCAAGTATTTTCAAACCGTTCGGCTCCTATCC
ACCGGGTGTAACAAGACGGATTCCGAATTTGGAAGATTTTGACTCAAATTCCCAATTTATATTGACCGTGA
CTAAATCAACTTTAACTTCTATAATTCTGATTAAGCTCCCAATTTATATTCCCAACGGCACTACCTCCAAA
ATTTATAGACTCTCATCCCCTTTTAAACCAACTTAGTAAACGTTTTTTTTTTTAATTTTATGAAGTTAAGT
TTTTACCTTGTTTTTAAAAAGAATCGTTCATAAGATGCCATGCCAGAACATTAGCTACACGTTACACATAG
CATGCAGCCGCGGAGAATTGTTTTTCTTCGCCACTTGTCACTCCCTTCAAACACCTAAGAGCTTCTCTCTC
ACAGCACACACATACAATCACATGCGTGCATGCATTATTACACGTGATCGCCATGCAAATCTCCTTTATAG
CCTATAAATTAACTCATCCGCTTCACTCTTTACTCAAACCAAAACTCATCAATACAAACAAGATTAAAAAC
ATACACGA

## Nucleotide sequence of RBCS promoter 5'-3' – 1003 bp

TTAGACAAACACCCCTTGTTATACAAAGAATTTCGCTTTACAAAATCAAATTCGAGAAAATAATATATGCA
CTAAATAAGATCATTCGGATCTAATCTAACCAATTACGATACGCTTTGGGTACACTTGATTTTTGTTTCAG
TGGTTACATATATCTTGTTTTATATGCTATCTTTAAGGATCTGCACAAAGATTATTTGTTGATGTTCTTGA
TGGGGCTCAGAAGATTTGATATGATACACTCTAATCTTTAGGAGATACCAGCCAGGATTATATTCAGTAAG
ACAATCAAATTTTACGTGTTCAAACTCGTTATCTTTTCATTCAAAGGATGAGCCAGAATCTTTATAGAATG
ATTGCAATCGAGAATATGTTCGGCCGATATGCCTTTGTTGGCTTCAATATTCTACATATCACACAAGAATC
GACCGTATTGTACCCTCTTTCCATAAAGGAAAACACAATATGCAGATGCTTTTTTCCCACATGCAGTAACA
TATAGGTATTCAAAAATGGCTAAAAGAAGTTGGATAACAAATTGACAACTATTTCCATTTCTGTTATATAA
ATTTCACAACACACAAAAGCCCGTAATCAAGAGTCTGCCCATGTACGAAATAACTTCTATTATTTGGTATT
GGGCCTAAGCCCAGCTCAGAGTACGTGGGGGTACCACATATAGGAAGGTAACAAAATACTGCAAGATAGCC
CCATAACGTACCAGCCTCTCCTTACCACGAAGAGATAAGATATAAGACCCACCCTGCCACGTGTCACATCG
TCATGGTGGTTAATGATAAGGGATTACATCCTTCTATGTTTGTGGACATGATGCATGTAATGTCATGAGCC
ACAAGATCCAATGGCCACAGGAACGTAAGAATGTAGATAGATTTGATTTTGTCCGTTAGATAGCAAACAAC
ATTATAAAAGGTGTGTATCAATAGGAACTAATTCACTCATTGGATTCATAGAAGTCCATTCCTCCTAAGTA
TCTAGAAAC

## APPENDIX IV

A

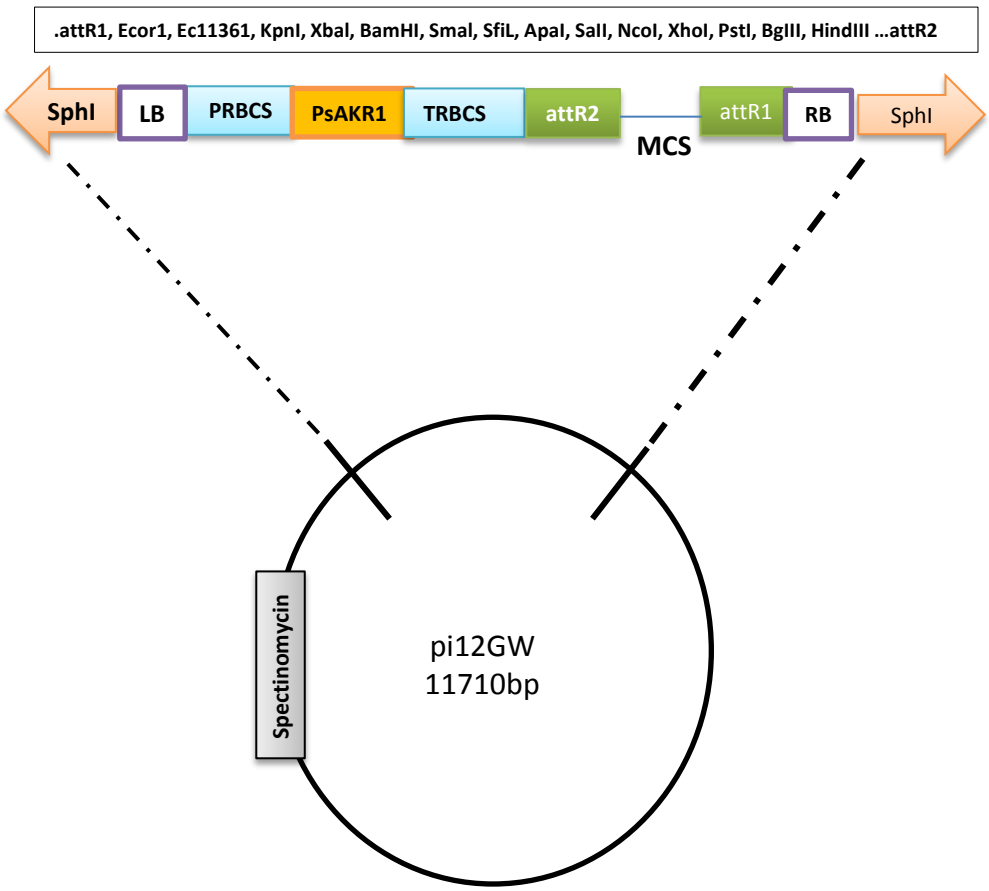



**A. circular map of pCAMBIA2301 vector**

Silent feature of these binary vector-

- high copy number in E.coli for high DNA yields
- pVS1 replicon for high stability in Agrobacterium
- bacterial selection with chloramphenicol or kanamycin
- plant selection with kanamycin
- simple means to construct translational fusions to gusA reporter genes.

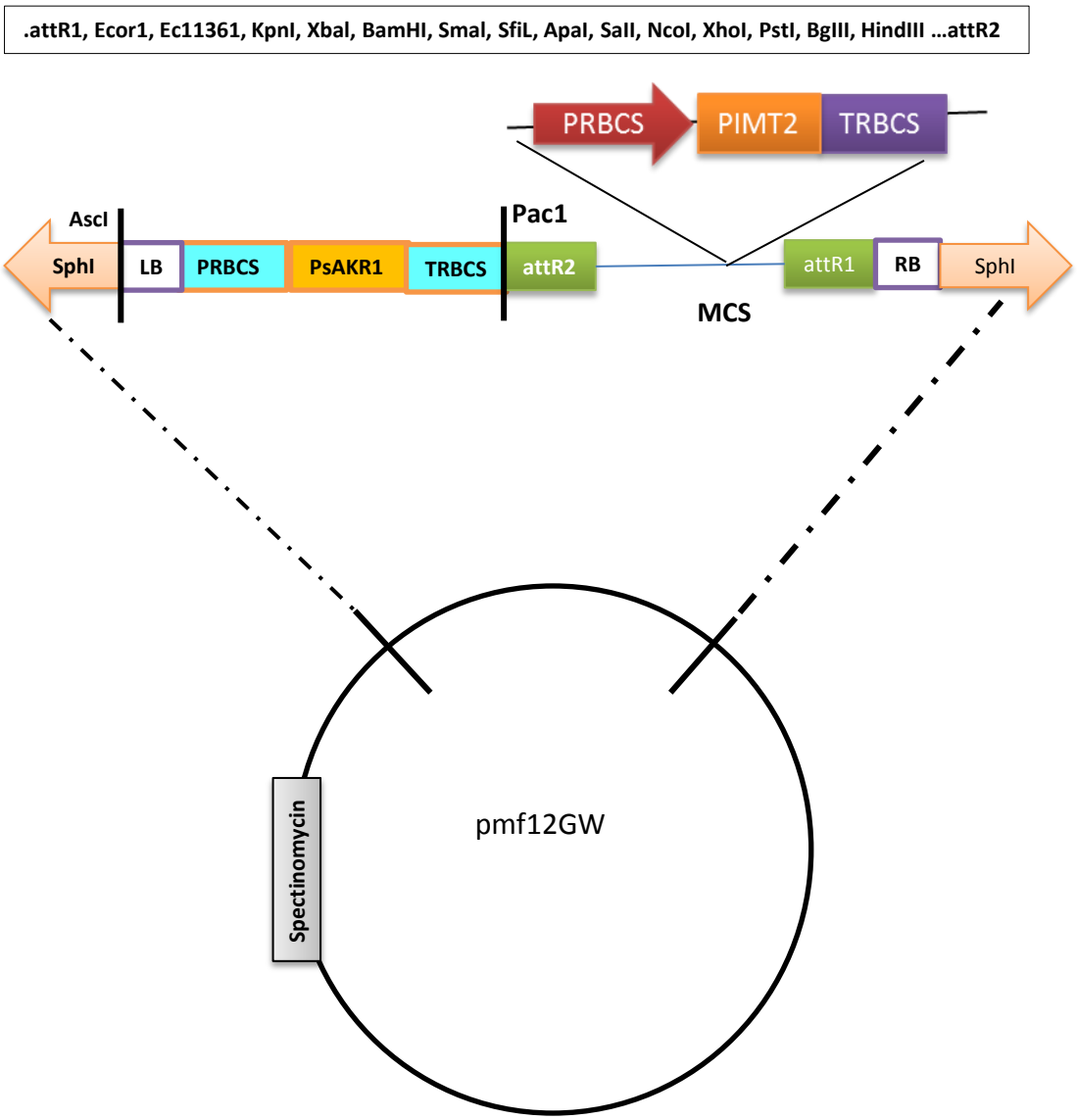
B




**B. Circular map of pi12GW binary vector**

The binary vector pi12GW with attR2 and attR1 sites and colony selection with spectinomycin.Plasmid carrying AKR1 as selection marker with attR2 and attR1 recombibation sites without CCDB gene. Can be used for both single gene as well as multisite stacking via gateway strategy.

C




**C. Circular map of pmf12GW binary vector**

- Bacterial selection with spectinomycin.
- Plants selection with AKR1 (igrA) .