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Co-expression of Genes Regulating Protein Synthesis and Stability Improves Tolerance and Adaptation to Water Limited Conditions

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ABSTRACT

Growing rice under aerobic or semi-irrigated aerobic conditions is one strategy being considered to conserve water since rice requires more than 50% of irrigation water. Although 60% of the water used for irrigation can be conserved with this method, the rice plants still experience moisture stress between irrigations and have a greater vapor pressure deficit (VPD), which has an impact on plant development, spikelet fertility, and other factors. Enhancing cellular level tolerance (CLT) mechanisms is essential in addition to features associated with water relations. Maintaining protein turnover, protein folding, and protein stability are key CLT mechanisms for preserving cell metabolism. With this background, NAC1, bZIP60, and bHLH57 transcription factors were used to generate rice transgenics that demonstrated better tolerance and productivity. These factors promote protein stability and folding by upregulating dehydrins. Additionally, Pg47, HSF4A, and eIF4E transgenics that enhance mRNA processing, protein translation, and chaperoning showed enhanced stress tolerance. By creating breeding stacks with these two transgenic events, the focus of the current study is to combine these two mechanisms. The true F1's were identified and the F2 lines were screened for presence of genes by PCR analysis. The initial studies of multigene breeding stacks improved tolerance to drought stress compared to wild type and multigene expressing transgenics. Therefore, this strategy can be utilized to increase agricultural productivity under rain-fed environments.

KEYWORDS: Multi-gene transgenic; Transcription factors; Cellular tolerance; Gene stacking.

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1. INTRODUCTION

One of the main staple food crops, rice (*Oryza sativa* L.), is grown on about one-third of the world's cereal crop land and is consumed by more than 2.7 billion people. Nearly 80 percent of the world's rice is farmed in lowland habitats that are rain fed (55%) and irrigated (25%) by rice, which uses about 70 percent of the freshwater resources available. The sustainability of the irrigated rice production system is threatened by the growing scarcity of water. The aerobic cultivation technology has been used because it reduces irrigation water use by roughly 50%. Intermittent moisture stress, however, has an impact on plant development and output in these circumstances.

The productivity of rice can be increased by a number of pertinent characteristics.The first and most focus should be placed on features related to preserving water relations, maintaining cellular metabolism under declining cell turgor, and maintaining positive carbon gain among the many traits that have been shown to be relevant. It is also obvious that the benefits of these features can only be fully realized when they are introgressed onto a genetic background of exceptional quality and moderately high cellular tolerance. Enhancing cellular level tolerance (CLT) processes and qualities related to water relationships have enormous value. Maintaining protein turnover, protein folding, and protein stability are key CLT mechanisms for sustaining cell metabolism.

Various stress-responsive genes have demonstrated functional properties that support cellular metabolism under stressful conditions[1,2,3].Cellular tolerance mechanisms are greatly influenced by genes that control DNA unwinding[4], stress-responsive transcription factors, chaperones that remove secondary structures[5], chaperones that process, fold, and protect proteins[6], and genes that control protein degradation[7] play a significant role in cellular tolerance mechanisms. Rice needs a coordinated expression of numerous mechanisms that are controlled by essential proteins in order to increase its tolerance and productivity. Protein synthesis, protein stability, protein degradation, and effective scavenging of oxidative stress-induced cytotoxic compounds are all necessary for CLT maintenance. Processing of RNA, RNA secondary structures, and ribosome loading for amino acid conversion are all components of protein synthesis. In addition to protein turnover, transcription factors are the primary proteins that respond to a variety of stimuli and control the expression of several genes involved in the tolerance of abiotic stress.

Previous research demonstrated that finger millet stress-induced transcription factors enhanced drought tolerance in model system. The findings offered evidence that particular cis-regulatory elements

are bound by overexpressed transcription factors such EcbZIP60, EcNAC1, EcMYC57, and the HSF group of proteins, which promote stress tolerance[8]. TFs EcNAC, EcMYC, and EcbZIP are engaged in up-regulating the genes linked to maintaining protein quality and stability. Similarly AhBTF3, AhNFY7, and EcZF, these fundamental transcription factors help the transcription machinery[9]. PgHSF4 controls heat shock proteins (HSPs), important chaperones for maintaining protein structural stability[10]. RNA secondary structure processing processes, in addition to transcription factors, are crucial for stress adaption. Helicases eliminate secondary stress-related structures from mRNA processing, releasing the mRNA for translation. The overexpression of helicases like eIF4E and Pg47, which remove RNA secondary structures, in a model system led to better drought tolerance.

The complicated attribute of drought is controlled by the coordinated expression of several genes. Therefore, it stands to reason that combining various cellular tolerance-related systems may increase drought tolerance and productivity.

2. MATERIALS AND METHODS

2.1 Location and plant species used in the study

The experiment was conducted in green house/ containment facility of the Department of Crop Physiology, University of Agricultural Sciences, GKVK campus, Bengaluru, Karnataka. The plant species used for the study was rice (*Oryza sativa* L.) AC39020 genotype.

2.2 Plant material growth conditions

F1 plants were raised in greenhouse and seeds were collected.F2 plants were raised for gravimetry experiment in automated minilysimeter phenomics facility situated in Dept. of Crop Physiology, UAS, Bangalore. One month old seedlings were subjected to moisture stress of 30% FC by a gradual dry down protocol from 100% FC for four days. Growth parameters were collected at three different time intervals. One group were subjected to 30% FC stress for four days, other group was subjecting the stressed plants for recovery for one week and the last group was subjecting the seedlings to prolonged 30% FC stress for one week followed by recovery.

2.3 Extraction of genomic DNA

Genomic DNA was extracted from rice leaf tissue as described by Doyle and Doyle, (1989)[11] with some modifications. The plant material (500 mg) was ground in liquid nitrogen and homogenized in 7.5 mL of pre-warmed (65°C) DNA extraction buffer [2% CTAB; 100 m MTris-HCl, pH 8.0; 20 mM

EDTA, 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 equal 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 6000 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 m MTris-HCl, 1 mM EDTA, pH 8.0) and stored at room temperature. To this mixture 10 µL RNaseA (1 mg/mL) was added and incubated at 37°C for 1 h. An equal volume (500 µL) of cold phenol: chloroform: isoamylalcohol (25:24:1) was added and centrifuged for 5 min at 10000 rpm. The supernatant was mixed with an equal volume of chloroform: isoamylalcohol (24:1) and again centrifuged for 5 min at 10000 rpm. To the resulting supernatant, twice the volume of absolute ethanol was added and incubated at -20°C for 1-2 h. The mixture was centrifuged (10 min, 14000 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.

2.4 Polymerase chain reaction

The DNA fragments were amplified from genomic DNA in a 20 µL reaction volume containing 100 ng of template DNA, 2 µL PCR buffer (10X), 2 µL dNTPs (2 mM), 1 µL forward primer (5 pmol/µL), 1 µL reverse primer (5 pmol/µL), 1 U Taq DNA polymerase and volume made up to 20 µL with sterile water. PCR was performed in Master cycler Gradient (Eppendorf AG, Germany). 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; Step 1: 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 1 min/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 electrophoresis. The presence of the expected PCR products was determined with the help of DNA ladders (GeneRuler DNA ladders, MBI-Fermentas).

2.5 Measurement of growth parameters

The breeding stacks,transgenic and wild type plants were analyzed for the phenotypic characters. Different growth parameters were analyzed by recording the total biomass (g/plant), root

length (cm), temperature (^{o}C) and leaf area (cm^{2}). Leaf area was computed by measuring length and breadth of third fully expanded leaf and was multiplied with correction factor using leaf area meter. Leaf temperature was measured using Infrared (IR) thermometer. Three individual lines from transgenics were taken for measurements.

2.6 Statistical Analysis

The physiological data on leaf area, biomass, root length and temperature were subjected to Tukey's test for ordinary two way ANOVA using statistical analysis software (Graphpad 8.0) to identify bars which are significantly different among treatment combinations.

3. RESULTS AND DISCUSSION

Two multigene transgenic events (**L-21**=> Transgenic line having Pg47:PgHSF4:eIF4E multigene construct that improves RNA processing mechanisms and **MR7, MR34, MR65**=> Transgenic lines having EcMYC57:EcNAC:bZIP60 multigene construct that improves Protein stability) co-expressing various transcription factors that regulate protein turnover and stability mechanisms were utilized from Department of Crop Physiology, University of Agricultural Sciences, GKVK, Bangalore to stack these diverse mechanisms. In the present study, emphasis is to bring in protein turnover components together in single background. Since both the multigenecoexpressingtransgenics are in background of AC39020 rice genotype, there is an option to combine RNA processing mechanisms and protein stability mechanisms together by specific crosses. Two combinations of crosses were done to bring in different cellular mechanisms in a single elite background.

3.1 Development of Breeding stacks

Transgenic lines were raised in greenhouse and specific cross and reciprocal crosses were done to generate the breeding stacks. The gene integration in transgenic plants was confirmed through PCR using gene specific primers (Fig. 1). Two different combinations of (RNA processing x Protein stability cassettes &Protein stability cassettes x RNA processing) direct and reciprocal crosses were performed. From these breeding stacks minimum of 10 seeds were obtained. Further, the sub set of F1 plants were raised and confirmed for the presence of both the constructs by gene specific primers. As the multigene constructs were cloned in the form of a cassette with all three genes in parent transgenics, pcr confirmation with only one gene primer could be sufficient to confirm the gene integration in breeding

stacks. F2 seed was collected from positive lines and used to check cellular tolerance adopting gravimetry technique.

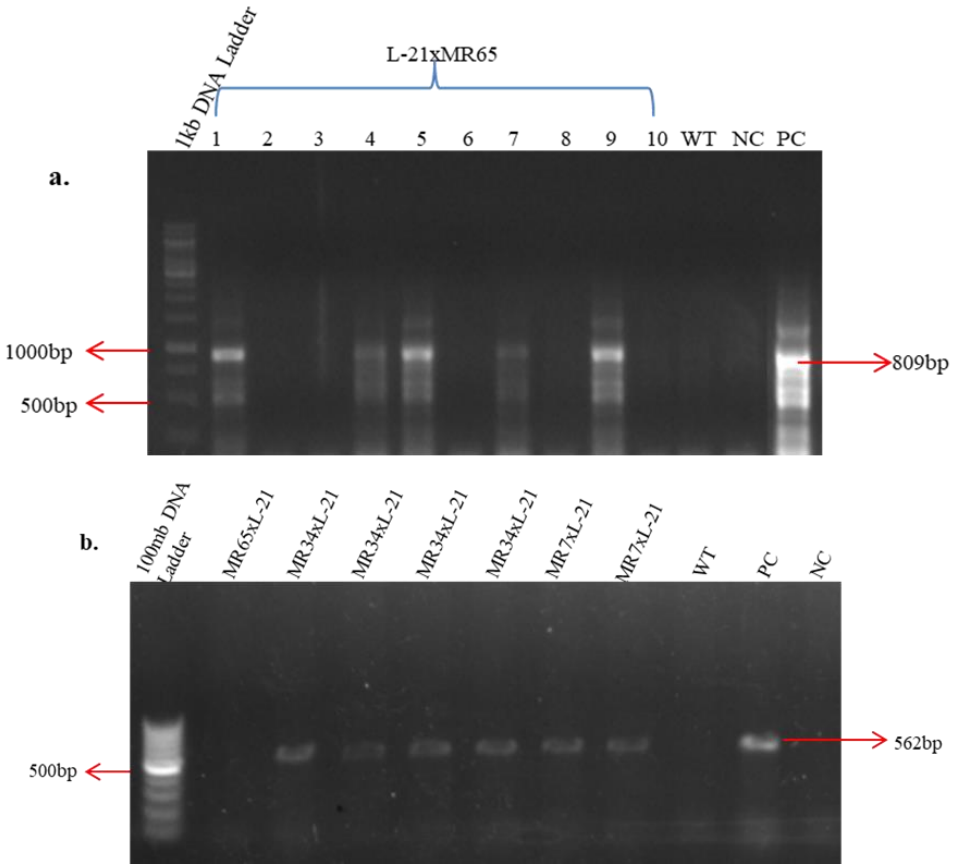

Fig.1. a) Molecular Confirmation of gene integration of EcMYC57:EcNAC:bZIP60 construct with EcMYC57 CDS primers. For confirmation of transgene integration 10 crossed F1 plants were selected and genomic DNA was isolated. PCR amplification of MYC CDS primer. PC- Positive control, NC- Negative control, WT- Wild type, **b) Molecular Confirmation of gene integration of Pg47:PgHSF4:eIF4E construct with Pg47 CDS primers.**For confirmation of transgene integration 7 crossed F1 plants were selected and genomic DNA was isolated. PCR amplification of Pg47 CDS primer. PC- Positive control, NC- Negative control, WT- Wild type.(L-21- Transgenics having Pg47:PgHSF4:eIF4E construct and MR7,MR34&MR65- Transgenic lines having bHLH57:EcNAC:bZIP60 construct).

3.2 Imposing moisture stress by dry downgravimetryprotocol

To assess the cellular level tolerance of crossed material in comparison with their respective independent transgenic lines and WT, a dry down gravimetry experiment in 30 day old seedlings was planned and conducted in Phenomics facility at UAS(B). The platform was protected by a semi-automated rain out shelter which was indigenously designed and built. A novel minilysimeter was designed utilising 20 L capacity buckets, with a manifold for uniform distribution of water and tap to regulate drainage. Weighing scales consisting load cell sensors were fabricated provided by 'Orbifold Solutions', a private company. The software for continuous data acquisition and controlling the

irrigation system was developed by Orbifold solutions in collaboration with the Department of Crop Physiology, UASB.

Rice seedlingswere gradually subjected to 30% FC moisture stress from 100% FC by a dry down protocol whereas control plants were maintained at 100% FC. DNA was extracted from these F2 plants and confirmed the gene integration from both the cassettes by PCR. Physiological parameters like biomass was recorded to study the stress response of these stacked transgenic plants in comparison with their respective multigene transgenics and WT. Growth parameters like total leaf area, root length and leaf temperature parameters was recorded at different growth intervals. For one set of replication,data was collected at the end of stress (30%FC for 4 days) and second set was collected after recovery (for one week) and the data for third set was collected after imposing 30%FC stress for one week followed by recovery. The data was evident that leaf area was better in breeding stacks compared to multigene parents and wild type (Fig. 2) among all the treatments.

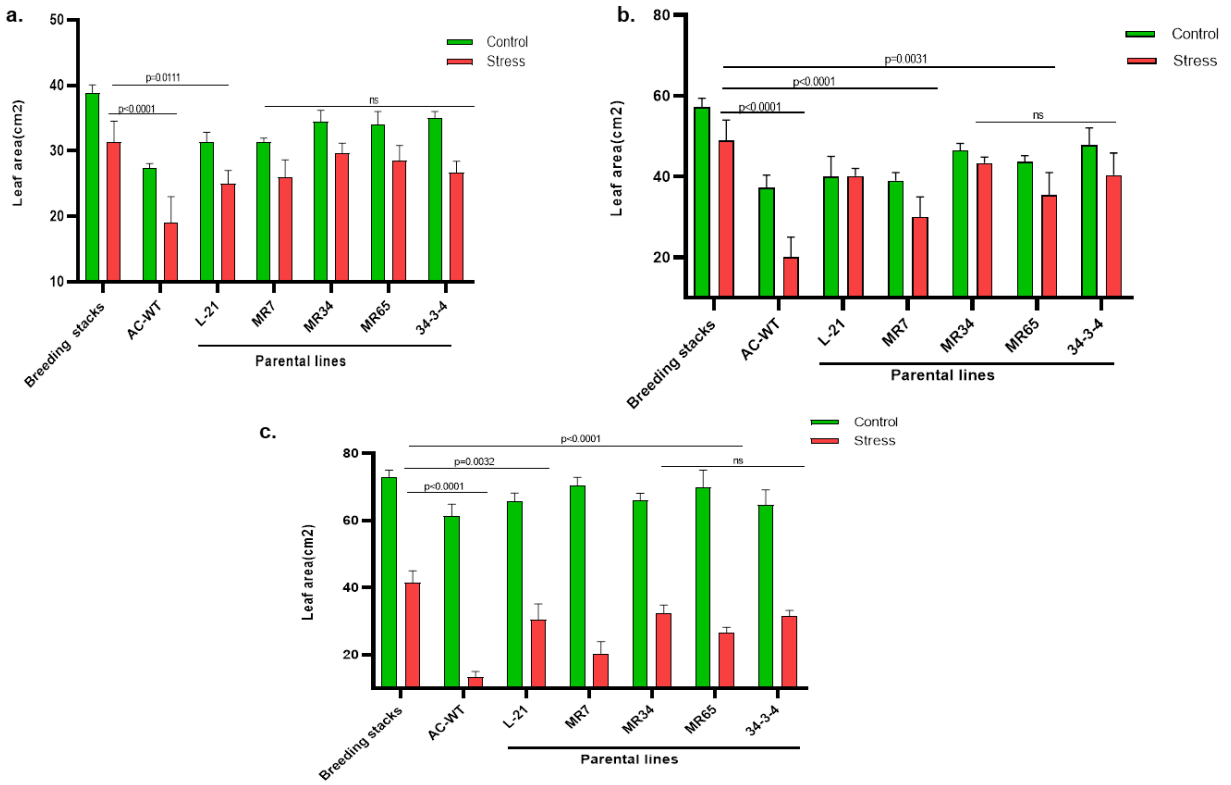

Fig.2.Graphical representation of growth parameters (Leaf area (cm^2)) in breeding stacks, transgenic and wild type plants under control and stress conditions. The leaf area of third fully expanded leaf was recorded using Leaf area meter. **a**, Leaf area was collected at the end of stress, **b**, after recovery , and **c**, after prolonged stress of one week followed by recovery. Three biological replicates were used for each sample. AC-Wildtype, L-21,MR7,MR34,MR65& 34-3-4 - Parental

transgenic lines carrying various multigene constructs. Significant difference by Tukey's test for ordinary two way Anova of treatments ($p < 0.0001$) using Graphpad Prism 8 software.

Total biomass also showed same trend that breeding stacks were better compared to parents and wildtype because of improved physiological processes due to co-expression of regulatory genes (Fig. 3).

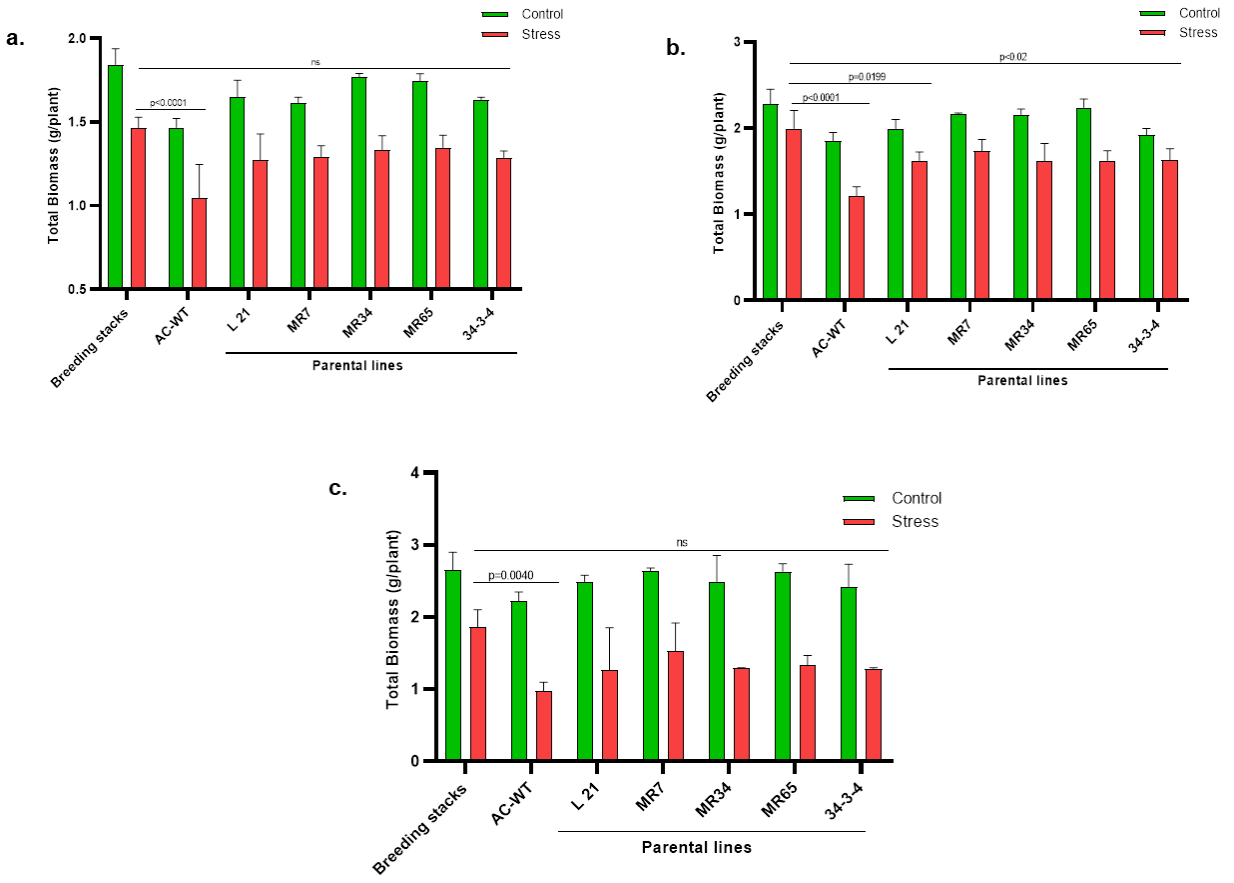

Fig.3.Graphical representation of growth parameters (Total biomass (g/plant)) in breeding stacks, transgenic and wild type plants under control and stress conditions. a. Biomass collected at the end of stress, **b**, after recovery ,**c**, after prolonged stress for one week followed by recovery. Three biological replicates were used for each sample. Significant difference by Tukey's test for ordinary two way Anova of treatments ($p < 0.0001$) using Graphpad Prism 8 software.

Root length was slightly enhanced compared to the rest due to improved water mining capacities in breeding stacks (Fig.4). Leaf temperature(^{o}C) was also recorded using Infrared (IR) thermometer across the treatments and found that breeding stacks were able to withstand to higher temperatures and

remained cool by maintaining the gas exchange and stomatal conductance compared to parents and wildtype (Fig.5).

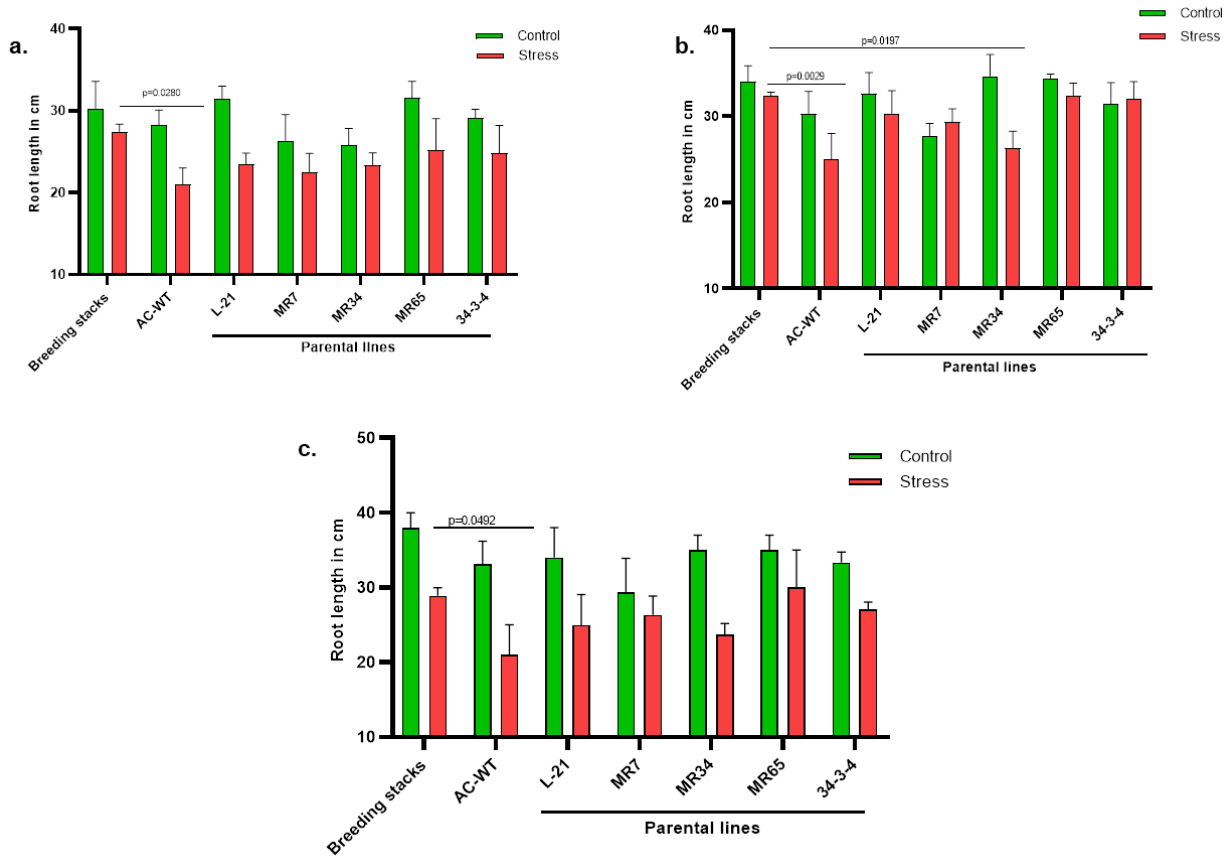

Fig.4.Graphical representation of growth parameters (Root length in cm) in breeding stacks, transgenic and wild type plants under control and stress conditions. a. Root length collected at the end of stress, **b**, after recovery, and **c,** after prolonged stress for one week followed by recovery. Three biological replicates were used for each sample. Significant difference by Tukey's test for ordinary two way Anova of treatments ($p < 0.0001$) using Graphpad Prism 8 software.

Out of curiosity, we checked the gene integration of two cassettes in some of the selected high biomass and low biomass yielding plants. The data was evident that especially under stress the plants where gene integration from both cassettes taken place has yielded higher biomass compared to the ones in which gene integration was not there.The data clearly indicates that the stacked transgenics were showing better tolerance compared to their respective single gene cassettes and wildtype due to co-expression of various regulatory genes involved in imparting cellular tolerance.

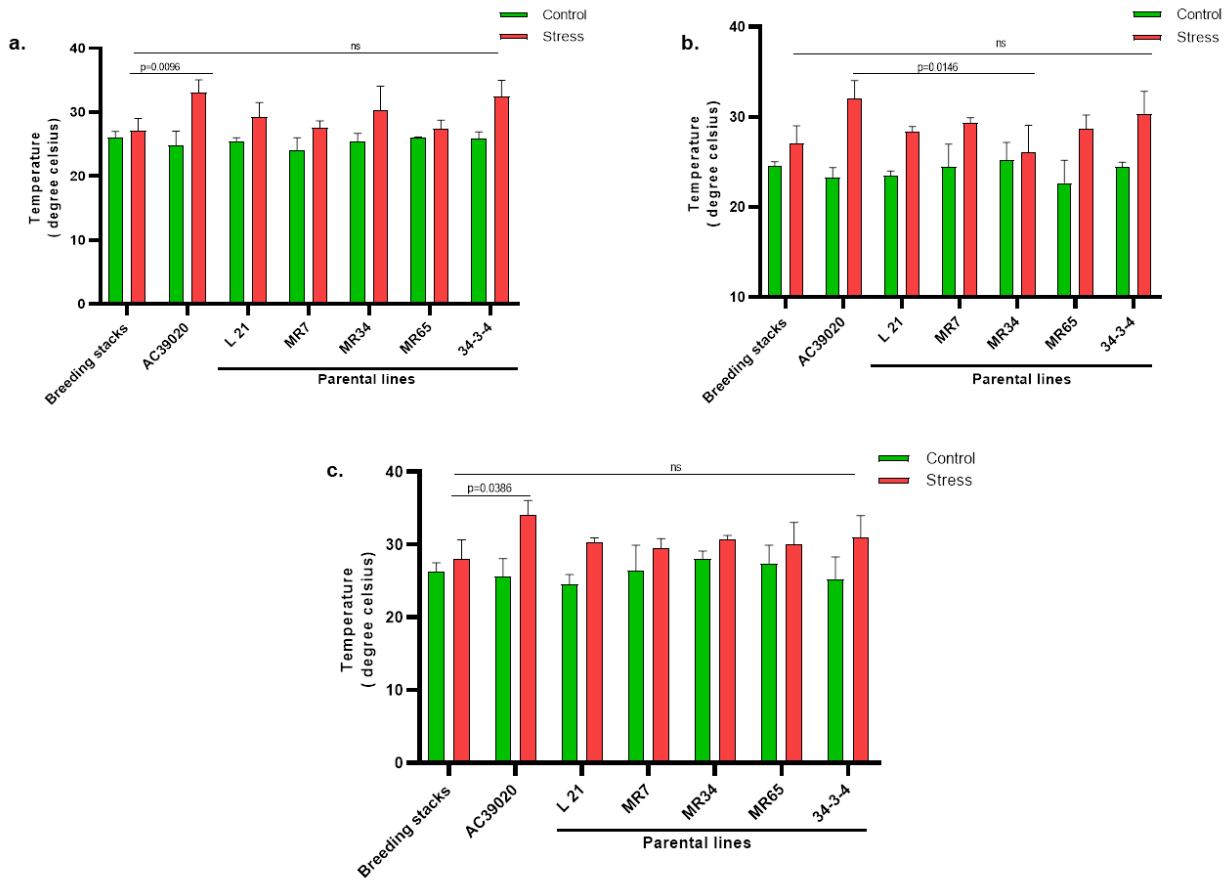

Fig. 5.Graphical representation of temperature(^{o}C) variations in breeding stacks, transgenic and wild type plants under control and stress conditions. a. Leaf temperature was recorded using Infrared (IR) thermometer at the end of stress, **b**, after recovery, and **c**, after prolonged stress for one week followed by recovery. Three biological replicates were used for each sample. Significant difference by Tukey's test for ordinary two way anova of treatments ($p < 0.0001$) using Graphpad Prism 8 software.

4. CONCLUSION

Rice is one of the most common crops grown and consumed in rainfed regions, which makes up roughly 25% of all rice production worldwide. Rainfed rice farming is susceptible to variations in temperature and rainfall because of its dependence on the climate. Therefore there is a need to avoid moisture stress by maintaining superior water relations to sustain the growth under dehydration conditions. In this context, plants have developed a variety of adaptive mechanisms, and among them, cellular level tolerance (CLT) and improving water relations are thought to be crucial for enhancing adaption to circumstances of moisture stress. Protein synthesis, maintenance, and turnover are important CLT mechanisms that are typically impacted by stressful conditions[12]. As multiple adaptive traits/processes can be combined to improve a crop's stress tolerance, the strategy used in this study is to

combine genes that enhance protein synthesis and stability as well as cellular tolerance mechanisms. Multigene cassettes were developed because the simultaneous expression of pertinent genes in a single locus is required to enhance tolerance. This concept has been proved in other species also like Arabidopsis,groundnut, tobacco, finger millet etc where a combination of set of transcription factors improve tolerance under stress conditions. By doing so, the simultaneous expression of the transcription factors AtWRKY28 and AtbHLH17 significantly increased the stress tolerance of Arabidopsis under various abiotic stress conditions[13]. By using this approach, OsAlfin1, which controls root growth, PDH45, and PgHSF4, which are involved in protein turnover and protection, were stacked and expressed in peanut[14].

In summary, breeding stacks developed by crossing multigene transgenics by putting different genes together showed improved growth over control and parents. The breeding stacks of these multigene transgenics showed superior stress tolerance over single gene cassettes. The transgenes might be enhancing the expression of specific target genes including oxidative stress responsive genes and hence improve the tolerance**.**

5. ACKNOWLEDGEMENT

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6. DECLARATION OF COMPETING INTEREST

Authors declare no conflict of interest.

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