DOI: 10.21475/ajcs.21.15.09.sp

Australian Journal of Crop Science
Southern Cross Publishing

CROP ADAPTATION TO CLIMATE CHANGE

High-Temperature Stress in Drought-Prone Areas

IAEA
Joint FAO/IAEA Centre
Nuclear Techniques in Food and Agriculture

DOI: 10.21475/ajcs.21.15.09.sp

Australian Journal of Crop Science
Southern Cross Publishing

Volume 15 Number 8 Year 2021

CROP ADAPTATION TO CLIMATE CHANGE: High-Temperature Stress in Drought Prone Areas

FAO/IAEA Co-ordinated Research Project on Climate Proofing Crops: Genetic Improvement for Adaptation to High Temperature in Drought-Prone Areas and Beyond

Guest Editors: Fatma Sarsu, Brian Forster and Sobhana Sivasankar

DISCLAIMER

Recommended citation

Cover photos credits:
Fatma Sarsu, Ms Sobhana Sivasankar and Brian Forster
Joint FAO/IAEA Division

CONTACT INFORMATION OF THE AUTHORS

Fatma Sarsu
Plant Breeding and Genetics Section, Joint Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture
Vienna, Austria
f.sarsu@iaea.org

Sobhana Sivasankar Plant Breeding
and Genetic Section, Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture, Vienna Austria
S.Sivasankar@iaea.org

B.P. Forster
BioHybrids International Limited
P.O. Box 2411, Early
Reading RG6 5FY, United Kingdom
Email: Brianforster@biohybrids.co.uk; Brianpforster@gmail.com

Ashwani Pareek
Stress Physiology and Molecular Biology Laboratory, School of Life Sciences, Jawaharlal Nehru University, New Delhi 110067, India
Email: ashwanip@mail.jnu.ac.in

Qing-Yao Shu
National Key Laboratory of Rice Biology, Institute of Crop Sciences, Zhejiang University, Hangzhou 310058, China
Email: qyshu@zju.edu.cn

Claudia Díaz-Camino
Departamento de Biología Molecular de Plantas Instituto de Biotecnología Universidad Nacional Autónoma de México Av. Universidad 2001, CP 62210, Cuernavaca, Morelos, México
Email: claudia@ibt.unam.mx

Qun Sun
Key Laboratory of Bio-resource and Bio-environment of the Ministry of Education, College of Life Science, Sichuan University, Chengdu, Sichuan 610064, P.R. China
Email: qunsun@scu.edu.cn

María Caridad González Cepero
National Institute of Agricultural Science, Cuba
Email: mcaridad09@yahoo.es

Ligia Carmenza Muñoz
Universidad Nacional de Colombia- sede Palmira, Carrera 32, Chapinero, Palmira, Colombia
Email: lcmunozf@unal.edu.co

Paul Mbogo Kusolwa
Department of Crop Science and Production, Sokoine University of Agriculture P. O. Box 3005 Morogoro Tanzania
Email: kusolwap@gmail.com

Muhammad Ashraf
Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan
Email: ashraflyp@yahoo.com

Ardashir Kharabian-Masouleh
The University of Queensland, Australia
Queensland Alliance for Agriculture and Food Innovation, St Lucia QLD 4068 Australia

Shiva Yazdanparast Ardabili
Southern Cross Publishing, Brsibane, QLD Australia

CONTENT

DOI: 10.21475/ajcs.21.15.09.sp-i

Australian Journal of Crop Science AJCS

CROP ADAPTATION TO CLIMATE CHANGE: High-Temperature Stress in Drought-Prone Areas

Fatma Sarsu*[1], Brian Forster*[2], Sobhana Sivasankar[1]

[1]Plant Breeding and Genetics Section, Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture, Vienna, Austria
[2]BioHybrids International Ltd, Earley, Reading, RG6 5FY, UK

***Corresponding author: f.sarsu@iaea.org; fatma.sarsu@gmail.com**

Abstract

The papers presented in this special issue are focused on the development of mutant lines and new crop varieties of rice and common bean with improved adaptation to climate change. Also included are the development or adaptation of screening techniques that enable efficient selection of desired phenotypes in plant breeding programmes. The breeding methods used are based on mutation induction and mutation detection, where mutation induction is via physical mutagenesis using gamma and X-ray irradiation. Selection for improved mutant lines is achieved through screening for plant performance in the laboratory, green house and field under hight temperature and/or drought stress. The papers presented are the result of a 5-year coordinated research project (CRP) on Climate Proofing Crops: Genetic Improvement for Adaptation to High Temperatures in Drought Prone Areas and Beyond, funded by the IAEA. The CRP, initiated in 2011, focused on tolerance to increased temperatures in rice and common bean. All participating countries generated new mutant populations in rice and bean and identified heat-tolerant lines with better yields than local standard varieties.

Key Words: Heat temperature stress, tolerance, climate change, crop adaptation, rice, common bean, mutation breeding.

Introduction

The combination of climate change, lack of arable land and limited water resources seriously hamper efforts to produce the estimated 70% increase in food required to feed an ever-increasing population (FAO 2009; Beddington, *et al*., 2011). Climate change involves rising ambient temperatures, which is a serious threat for crop productivity. An Inter-Governmental Panel on Climate Change report predicted that global surface temperatures will increase by 2°C between 2046 and 2065 and up to 3.7°C by 2100 (IPCC, 2012). This is a catastrophic scenario for crop production as current crop varieties lack adaptation to these temperatures. Rice (*Oryza sativa* L) is the most important staple food of more than half of the world's population. Reduction in rice yields have already been reported in many countries such as Australia, Bangladesh, China, India, Japan, Pakistan, the Philippines, Thailand and the USA (Mohapatra *et al*., 2013; *Sarsu et al*., 2018 a). Rice, like all crops, is grown in areas that are suited to its cultivation, and this includes optimal temperatures. Any increase in temperature (especially at sensitive stages such as flowering), will have a direct and negative affect on grain yield. Indeed, a 40% yield reduction in rice has been predicted by the end of the 21st century. (Shah *et al*., 2011 and 2018).

Common bean (*Phaseolus vulgaris* L.), is one of the most important legume crops. It is a valuable source of protein, carbohydrate and other nutrients in diets worldwide. Beans are grown in a wide range of climates, but predominantly in Central and South America, especially along the Andes, and in East and Central Africa. Optimum temperatures for crop production range from 14˚C to 28˚C. Day temperatures above 30˚C and night temperatures above 20˚C significantly reduce grain yield (Sivasankar *et al*., 2018). The common bean is also cultivated intensely in tropical regions and some temperate regions (Silva and Neves, 2011).

A problem frequently encountered in plant breeding is that continuous selection among local genotypes leads to a narrowing of the genetic base. Thus, although breeders may have a lot of germplasm adapted to local conditions and market demands, any change to the environment, such as increased temperature becomes a serious problem as there is little readily available variation for crop improvement. Breeding is dependent on genetic diversity and if the required traits are not present in the primary gene pool the breeder may resort to crossing to more exotic materials including landraces and related wild species. However, this is a time consuming and complicated process as it requires both the introgression of the trait of interest, e.g. high temperature tolerance (targeted breeding), whilst maintaining yield and quality traits (defensive breeding) (see Driedonks *et al.* 2016). An attractive alternative is mutation breeding. Induced mutation is a heritable change in the genetic material of living organisms. Mutation is also a natural phenomenon and a major driver in species diversity and evolution. The use of various mutagens to generate genetic variation in crop plants has a history almost as long as that of conventional breeding (Kharkwal, 2012; Spencer *et al.,* 2018). Additionally, functional genomic studies already initiated for tolerance to heat temperature and drought, the ability of specific genes or members of specific gene families with proven to association can be evaluated by mutation approaches or genetic engineering (Sivasankar et al 2018). Some of these genes are used in patents by the plant biotechnology industry in major crops such as maize (Sivasankar et al 2008).

Induced mutation has been hugely successful in rice and bean breeding. Thus far, 853 mutant varieties have been developed in rice using mostly gamma irradiation, but also Ethyl Methane Sulfonate (EMS) and fast neutron treatments (FAO/IAEA 2021 Mutant Variety Database (MVD); http://mvd.iaea.org/). According to the MVD, 57 common bean *Phaseolus vulgaris* L.) mutant varieties have been released officially. Gamma ray irradiation has been widely used on common bean to increase the genetic diversity for several traits (Arena *et al*., 2013). Some recent mutant traits of interest in common bean include dwarfism, short roots, and various flower colours, seed colour, size and shape (Ellyafa *et al*., 2007; Mahamune and Kothekar, 2011).

The Coordinated Research Project (CRP), Climate Proofing Crops: Genetic Improvement for Adaptation to High Temperatures in Drought-Prone Areas and Beyond, was developed based on the recommendations of a Consultants' Meeting held in Vienna (10-14th May 2010) during which the research concept was formulated based on the needs of IAEA Member States. Considering that many research groups working on crop adaptation to climate change have focused on CO_2 increases in the atmosphere, increased water scarcity, increased salinity and/or spread of pests and diseases, the Consultant group recommended a focus on crop tolerance to high temperatures, specifically on two crops with high impact on food security: cereals (preferentially rice) and legumes (preferentially common bean). The implementation of the CRP involved 17 research teams from 14 countries that worked on developing rice and common bean with tolerance to high temperatures and drought, and on developing and/or adapting protocols for screening mutants with tolerance to high temperatures. Two protocols developed or improved during the CRP were transferred to the scientific community of Member States through the publication of the manual, "Pre-field screening protocols for heat-tolerant mutants in rice" (Sarsu *et al.*, 2018a).

Structure and objectives of the CRP

Seventeen research teams from 14 countries (Australia, China, Colombia, Cuba, India, Japan, Mexico, Pakistan, The Philippines, Senegal, Spain, Tanzania, United Kingdom, and Zimbabwe) participated in the CRP.

The overall objective of the CRP was to: i) develop high yielding food crops that contribute to sustainable food security (with a focus on rice and common bean), ii) improve resource use efficiency (water and nitrogen) and adaptation to high temperatures (increased minima and maxima) as anticipated by climate change predictions for the next 20 to 40 years.

The specific objectives of the CRP included: (i) development of efficient pre-field screening protocols to facilitate the breeding process, especially responses to high temperature stress; (ii) adaptation and application of modern and high throughput biotechnology packages combined with nuclear applications for enhanced crop adaptation and performance; and (iii) development of new high yielding mutant varieties with improved yields under low input cultivation in a range of agro-ecologies, through broadened adaptability. This introductory paper highlights the key findings of the CRP related to the development of heat-tolerant mutants and methods for their detection. In addition to positive developments in heat-tolerant germplasm creation and efficient screening methods, the CRP also identified gaps in current knowledge and areas for future research. Although the papers included in this special issue are confined to two crops, they provide useful information on developing mutation breeding programmes for tolerance to heat temperature stress that can be adapted to other crops.

Specific objectives of the CRP

Explore genetic variability of crops (rice and bean) and identify high yielding genotypes from existing natural and mutated germplasm of rice and nitrogen fixing common bean for adaptation to high temperature

All participating countries generated new mutant populations in rice and bean and identified heat-tolerant lines with better yields than local standard varieties. Some lines were sent to official national variety trials to be evaluated for certification for release as a recommended variety for farmers. Other mutant lines have been incorporated into respective breeding programmes. Some specific national highlights in this special issue United Republic of Tanzania (URT) - Neema *et al.,* 2019, report field performance of heat tolerant upland mutant rice lines generated from both *Oryza sativa* and *Oryza glaberrima*. Seed of four different genotypes from these two-rice species were irradiated with γ rays: *Oryza sativa* at 150, Gy; - Oryza *glaberrima* at 250 Gy. Subsequent M_3 seedling families were screened at 45^{o} C in controlled environment conditions (growth cabinet), and selected mutant families were evaluated in field experiments during the hot, dry season of 2014/2015 in Morogoro, URT. The minimum and maximum temperatures and rainfall during crop growth were observed to be 20^{o}C and 35^{o}C, and 32.7 mm and 155.5 mm, respectively. The data for yield and 12 yield-component parameters such as days to early and 50% flowering, days to physical maturity, plant height, number of tillers, number of panicles, number of spikelet's, filled grains, unfilled grains and 1,000 grain weights were recorded and analysed using ANOVA and Principal Component Analysis. Eight heat-tolerant mutant rice lines with high yields (over 3.5 ton/ha) and low spikelet sterility were selected for incorporation into breeding programmens for further advancement. Cuba - Cepero *et al.*, 2019, evaluated mutant rice lines for tolerance to high- temperature stress and drought in the field. Selected mutant lines were also evaluated for physiological parameters such as cell membrane thermostability, pollen viability, lipid peroxidation, and activities of peroxidase and catalase enzymes under high temperature conditions. Three advanced mutant lines, 8852, 8552 and LP-12 showed tolerance to high temperatures and low water supply in the field. They also showed better pollen viability, cell membrane thermostability, lipid peroxidation and peroxidase activity than the control cv. Amistad-82. These mutant lines are planned to be tested in multi-locational trials for further evaluation and release, subject to superior performance.

Colombia - Muñoz *et al.*, 2019a, explored tepary bean (*Phaseolus acutifolius A. Gray*) and common bean (*Phaseolus vulgaris L.*) and their interspecific lines for heat stress-induced changes in morpho-physiological characteristics of shoot and root, and for yield components under greenhouse conditions and compared to ambient temperatures. High temperatures (HT) significantly affected genotype responses in leaf photosynthetic efficiency (as measured by Fv'/Fm'), total chlorophyll content and stomatal conductance. Genotypes identified with specific response are planned to be evaluated further in field conditions to select the best for varietal development.

Colombia - Muñoz *et al.*, 2019b, also explored heat and drought tolerance in tepary bean (*Phaseolus acutifolius* A. Gray) in more detail. EMS induced mutant lines were tested under heat and drought stress in greenhouse conditions. The heat stress treatment was set at 29±5^{o}C during the day and >24^{o}C during the night, with an average relative humidity of 65%. The maximum day/night temperatures of the greenhouse for normal conditions were set at 30°C /20°C. Plants were grown in optimal conditions of soil moisture (80% field capacity) for 10 days and then subjected to drought stress using soil moisture treatments of 80% of field capacity or 40%, which constituted drought. The mutant lines were evaluated for morpho-physiological attributes, seed yield and yield components. The results showed that the mutant lines, CMT38, CMT109 and CMT187, had high seed yield values under heat and drought conditions.

Establishment of protocols for screening mutants for tolerance to high temperatures

Robust pre-field screening protocols were developed that allow plant breeders to screen for enhanced tolerance to heat

stress in rice in a breeding programme using controlled environments (Sarsu *et al*., 2018a). Two critical heat-sensitive stages in the life cycle of the rice crop were targeted, namely, seedling establishment and flowering. The protocols are based on the use of a hydroponics system and/or pot experiments in glasshouse conditions in combination with controlled environment (growth chamber) experiments where heat stress is applied. The protocols are designed to be effective, simple, easy to use and reproducible (Sarsu *et al*., 2018 a). Extensive validation tests were carried out by various CRP partners, including: 1) the FAO/IAEA's Plant Breeding and Genetic Laboratory, Seibersdorf, Austria; 2) School of Life Sciences, Jawaharlal Nehru University New Delhi India; 3) Sokoine University of Agriculture Morogoro, United Republic of Tanzania, and 4) Nuclear Institute for Agriculture and Biology, Faisalabad, Pakistan.

These methods can be used to classify rice genotypes according to their heat- tolerance characteristics. A related book, Pre-Field Screening Protocols for Heat Tolerant Mutants in Rice, was published in open access format, and can be downloaded via the link: https://link.springer.com/book/10.1007%2F978-3-319-77338-4

i) Screening Protocols for Heat Tolerance in Rice at the Seedling and Reproductive Stages

The methods include a new protocol for screening for heat tolerance in rice at the seedling stage (Sarsu *et al*., 2018 a). Young seedlings are exposed to heat stress for 4-5 weeks with visual scoring of symptoms which allows hundreds of seedlings to be evaluated in a short time. The visual screening method was validated through laboratory, glasshouse and field-based experiments. The seedling test can be used to screen M_2 populations, advanced mutant families and lines as well as varieties.

A protocol for screening heat tolerant mutant lines at the flowering stage was also developed (Sarsu *et al*., 2018). Here, plants are subjected to different temperatures from the first day of anthesis (pollen shedding), and subsequent spikelet fertility at maturity is determined.

ii) Physiological Mechanisms Associated with Heat Tolerance

Selected heat-tolerant mutant rice genotypes were tested for physiological and biochemical indicators as part of the pre-field screening protocols (Sarsu *et al*., 2018a). These indicators included electrolyte leakage, malondialdehyde level, total protein content and antioxidant enzyme activity at seedling, vegetative and flowering stages to understand the underlying biological mechanism of heat tolerance, and to explore the potential of pyramiding different traits/alleles for durable heat tolerance. Candidate heat-tolerant mutant lines were tested in hot spot areas in field conditions in China, Cuba, Colombia, India, Pakistan, The Philippines, and United Republic of Tanzania.

The protocols outlined above are designed to enable plant breeders to pre-screen for candidate heat-tolerant lines, effectively reducing the number of plants from a few thousand to less than 100, which can be advanced for field testing.

Observations pertinent to biochemical and physiological responses under heat stress

China - Huang *et al.* (2019), analysed proline accumulation, antioxidant capacity and heat shock protein (HSP) expression in mutant rice lines. Three mutant lines (AG1, AG2 and AG3) were selected and subjected to control temperatures (25°C) and heat stress (45°C) for22 hours at the 5-leaf stage, together with a heat-tolerant cultivar N22 (control) and sensitive wild type. Heat stress produced significantly higher levels of oxidative damage in wild type than in N22 (control) and AG3 mutant line. Proline accumulation was found to be significantly higher in N22 and AG3 compared to wild type. Higher proline accumulation occurred along with elevated T-AOC and SOD activities under heat stress. It is suggested that these could play important roles in thermo-tolerance.

Pakistan - Ashraf *et al*., 2019, identified thermo-tolerant Basmati rice mutants using biochemical and physiological analyses. After vigorous screening of 2,000 mutant lines in the growth chamber/field conditions, 16 were selected. A correlation was established between paddy-field yield under hot field conditions and seedling growth chamber data. Paddy yield was significantly correlated with early seedling-stage traits such as: shoot length (r2 = 0.79), shoot fresh and dry weights (r2 = 0.48 and 0.49), and cell membrane thermo-stability (r2 = 0.60). Additionally, significant higher activities of antioxidant enzymes, SOD and APX, and lower levels of the stress indicators, MDA, esterase and TOS, were observed in heat-tolerant mutants. Six advanced mutant lines were identified with good performance in terms of yield as well as the expression of physiological and biochemical traits at elevated temperatures.

Identification and characterization of genes involved in heat tolerance

In order to identify and characterise genes and mutant alleles involved in heat tolerance, deep sequencing technologies were deployed in both rice and beans for RNA and genomic DNA using appropriate platforms for high throughput analysis. In common bean, studies included: (i) the identification of genes and processes involved in high heat response in symbiotic root nodules of heat tolerant cultivars of common bean, and (ii) the analysis of nitrogen fixation of sensitive and tolerant genotypes under controlled and heat-stress conditions. Some examples from Mexico, India and China are presented below.

Mexico - Camino *et al*., 2019, used next-generation sequencing technology for transcriptome analyses of an unexplored group of peptides encoded by small open reading frames (sORFs)< 150 codons in nitrogen-fixing symbiotic nodules of two heat stress-tolerant genotypes of common bean. A total of 60 differentially expressed sORFs were identified between control and heat stress treatments. The expression profiles of these sORFs suggest that each genotype had adapted molecular signalling pathways to survive heat stress independently. The dataset developed may provide a useful resource for future genetic and genomic studies in these species.

India- Das *et al*., 2019 investigated gain-of-function mutants in rice with tolerance to multiple abiotic stresses. 2000 M_3 Mutant lines were screened at the seedling stage for tolerance to high temperature stress. Three mutant lines were identified with robust seedling development under stress treatments. Screening was also carried out at the flowering stage under heat stress, and these mutants showed higher CO_2 assimilation (10-30%), spikelet fertility (40-45%) and antioxidant activity (15-20% catalase activity) relative to the wildtype control. These 3 mutant lines also had increased CO_2 assimilation, stomatal conductance, transpiration and chlorophyll fluorescence than wildtype plants when exposed to salinity and drought stress. Transcript and protein abundance analyses confirmed higher constitutive levels of HSPs and antioxidant enzymes in the mutant lines compared to wild type. Notably the mutant lines had 25-30% higher grain yield under stress. Based on the above results, these lines are being developed as potential new varieties for dry and saline areas.

Other highlights include:

China - Wang *et al*., 2019, studied heat shock protein (HSP) and antioxidant enzyme genes as part of the mechanism of heat tolerance in hybrid rice II YOU 838. Gene expression was studied in the mutant plants exposed to heat stress during flowering (anthesis) using quantitative real-time PCR. Gene

expression of the heat shock protein 70 (HSP70), heat shock protein 90 (HSP90, small heat shock protein (smHSP), superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) were investigated. Quantitative RT-PCR showed increased expression of smhsp, hsp90, hsp70, CAT, SOD and POD in flag leaves under heat stress. The maximum expression values were observed on Day 2 or Day 3 after the beginning of the heat stress. These were the critical periods for response of heat stress in II YOU 838.

Identification and characterization of temperature-sensitive genic male sterility genes and application in rice breeding; development of DNA markers of heat tolerance for marker assisted selection

Temperature-sensitive genic male sterility (TGMS) as implied is a genetic/environment interaction producing male sterility. Male sterility is a key component in the production of high yielding F_1 hybrids and is therefore of interest. In studies reported by Huang 2014, Zhang H-L 2014 a and b, Zhang H-L 2015, a temperature –sensitive gene associated with male sterility in the rice line Zhu1S was found. Pedigree analysis revealed that the mutant allele of a rice RNase Z orthologue, RNZm or OsaTRZ1(m) controlled TGMS in most Chinese commercial two-line rice hybrids (Zhang et al. 2014a, 2015). DNA markers have been developed for marker assisted selection (MAS) of TGMS rice using sequence information from this allele.

Facilitation of technology transfer through peer reviewed publications, workshops, training courses, field days and networks of participating research groups and potential end users.

The integration of mutation breeding and genetics (including functional genomics) in the CRP was achieved through bi- and multi-lateral collaborations based either on the target crop, screening methodologies or common research aims and facilities. Fruitful collaborations led to the development of new techniques/technologies which were disseminated through peer-reviewed publications, national training courses/workshops and human capacity building (BSc, MSc and PhD programmes). Participating countries have published their work in various forms; collectively there are 25 peer-reviewed publications (15 published and 10 accepted), 10 conference proceedings, 1 book chapter,1 book accepted to be published and a manual. Details are given in reference 3, 7, 8, 9, 10, 14, 17, 18, 19, 20, 21, 22, 23, 27, 28, 29, 30, 31, 32, 33 and 34. In addition, knowledge transfer was achieved through training courses/workshops organised in Cuba and Colombia focused on mutation breeding and CRP results. Human capacity building supported 8 BSc, 7 MSc and 9 PhD programmes. Bi- and multi-lateral collaborations have been strengthened among participants for germplasm exchange, testing of advanced lines in different locations and molecular analysis of heat-tolerant mutant lines.
An aim of this special issue is to transfer the knowledge, outcomes and outputs of the CRP to a wider audience.

Conclusions

Advancing breeding techniques in developing crops tolerant to high-temperature stress is a fundamental goal in achieving food security under climate change. The CRP coordinated and supported the generation of new mutants in rice and common bean and the development of protocols/technologies for screening for the trait using physiological, biochemical and molecular parameters. The project succeeded in bringing together an international group of scientists with similar aims and objectives, who, through networking, shared specialized technical know-how and developed high-throughput pre-screens and field evaluations for high-temperature stress tolerance.
Major outcomes can be summarized as:
(i) New mutant cultivars/ lines adapted to climate change (see relevant papers: Neema *et al.,* 2019, Cepero *et al*., 2019, Muñoz *et al*., 2019a, b, and Ashraf *et al*., 2019).
(ii) Hands-on protocols and robust screening techniques for heat tolerance (Sarsu *et al*., 2018a).
(iii) Biological mechanisms under-pinning heat tolerance (see relevant papers Ashraf *et al*., 2019, Cepero *et al*., 2019, Das *et al*., 2019, Das *et al*., 2019, Huang *et al*., 2019, Muñoz *et al*., 2019a Wang *et al*., 2019).
(iv) Development of DNA markers for marker-assisted selection (see relevant papers Camino *et al*., 2019, Das *et al*., 2019, Zhang *et al*., 2014a, 2015).
(v) Genetic variability for heat tolerance explored and new germplasm identified (see relevant papers Ashraf *et al*., 2019. Camino *et al*., 2019 and Das *et al*., 2019.
(vi) Increased knowledge and experience in using nuclear technologies to develop crops resilient to high temperatures.
(vii) Human capacity building in mutation breeding and enabling technologies for heat tolerance in rice and common bean.
(viii) Research results disseminated via proceedings, presentations at conferences, workshops, peer-reviewed papers and about 40 scientific publications (papers, book, book chapters and a manual) and through outreach activities including training, farmers' day, workshops, public exhibitions.
(ix) Integration between mutation breeding and functional genomics through bi- and multi-lateral collaborations.
(x) Enhanced public awareness about the potential of plant mutation breeding in meeting the challenges of food security with increasing climate change.

In summary, this project of the Joint FAO/IAEA Division has developed tools and adapted methodologies and procedures to allow plant breeders to develop improved crop varieties with higher and wider adaptability to temperature stress. Abiotic stresses (heat, drought, salinity, flooding, soil degradation and low fertility) and biotic stresses (new pests and diseases) will continue to be major constraints to global agricultural production. The Joint Division continues to support improved crop production through nuclear technologies including mutation breeding. At the present time, there are 11 national and two regional Technical Cooperation projects in this research area, aside from this ongoing Coordinated Research Project.

Acknowledgements

The Authors thank all their colleagues who were enthusiastic participants of this CRP, the group of consultants who helped formulate the CRP, the ex-scientific secretary of the CRP, Ms Maria Madelina Spencer who initiated the work, and all associated colleagues who contributed in reviewing the presented papers in this special issue.

References

Arena C, Micco VD, Maio AD (2013) Growth alteration and leaf biochemical responses in Phaseolus vulgaris exposed to different doses of ionising radiation. Plant Biology 16 (1), 194-202.

Beddington J (2011) Achieving food security in the face of climate change, https://cgspace.cgiar.org/bitstream/handle/10568/10701/Climate_food_commission-SPM-Nov2011.pdf

Das P, Mishra M, Lakra N, Singla-Pareek SL, Pareek A (2013) Mutation Breeding: A Powerful Approach for Obtaining Abiotic Stress Tolerant Crops and Upgrading Food Security for Human Nutrition. Editors: Tomlekova N, Kozgar I and Wani R. Publisher: Wageningen Academic Publishers, Pp 15-37.

Driedonks N, Rieu I, Vrizen WH (2016) Breeding for plant heat tolerance at vegetative and reproductive stages. Plant Reprod 29:67. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4909801/

Ellyafa K, Ahmed OH, Shaharudin S, Rahman DA (2007) Gamma Radiosensitivity Study on Snap Bean (*Phaseolus vulgaris*). International Journal of Agricultural Research 2(9), 844-848.

FAO (2009) How to Feed the World in 2050 http://www.fao.org/fileadmin/templates/wsfs/docs/expert_paper/How_to_Feed_the_World_in_2050.pdf

Fahad S, Huang J, Cui K, (2011). Impact of high-temperature stress on rice plant and its traits related to tolerance 2011 The Journal of Agricultural Science 149(05):545-556 DOI: 10.1017/S0021859611000360

Fahad S, Ihsan MZ, Khalig A, Daur I, Saud S, Alzemenan S, Nasim W, Abdullah M, Muhammad A, Imtiaz AK, Wu C, Wang D, Huang J (2018) Consequences of high temperature under changing climate optima for rice pollen characteristics-concepts and perspectives Taylor and Francis Online published 06 March 2018 https://www.tandfonline.com/doi/abs/10.1080/03650340.2018.1443213?journalCode=gags20

González MCC, Marinez A (2016) Selección de genotipos de arroz de buen comportamiento agronómico en condiciones de bajo suministro de agua. vol. 37, no. 1 (enero-marzo) https://ediciones.inca.edu.cu

Huang J-Z, E Z-G, Zhang H-L, Shu Q-Y (2014) Workable male sterility systems for hybrid rice: Genetics, biochemistry, molecular biology, and utilization. Rice. 7: 13.

IAEA Mutant variety database, https://mvd.iaea.org/ (accessed 29 April 2021).

IPCC (2012). Summary for Policy makers, in: Managing the Risks of Extreme Events and Disasters to Advance Climate Change Adaptation. A Special Report of Working Groups I and II of the Intergovernmental Panel on Climate Change, eds Field CB, Barros V, Stocker TK, Qin D, Dokken DJ, Ebi KL, *et al.* (Cambridge University Press),1–19.

Kharkwal MC (2012) A Brief History of Plant Mutagenesis. In: Plant Mutation Breeding and Biotechnology, eds Shu QY, Forster BP and Nakagawa H. CAB Interanational and FAO, C02, 21-30.

León LP, González MCC, Castro R, (2016) Evaluación de cultivares de arroz en diferentes zonas de producción arrocera de "Los Palacios", Pinar del Río, para su utilización en programas de mejoramiento. Cultivos Tropicales, 2016, vol. 37, no. 1 (enero-marzo) https://ediciones.inca.edu.cu

Mahamune SE, Kothekar VS (2011) Gamma Ray Induced Flower Color and Seed Mutants in French Bean (*Phaseolus vulgaris* L.). Recent Research in Science and Technology 3(5), 33-35.

Mohapatra T, Robin S, Sarla N, Sheshashayee M, Singh AK, Singh K, *et al.* (2014) EMS induced mutants of upland rice variety Nagina22: generation and characterization. Proc. Indian Natl. Sci. Acad. 80 163–172. 10.16943/ptinsa/2014/v80i1/55094

Peláez P, Trejo MS, Iñiguez LP, Estrada-Navarrete G, Covarrubias AA, Reyes JL. and Sanchez F (2012) Identification and characterization of microRNAs in *Phaseolus vulgaris* by high-throughput sequencing. BMC Genomics, 13(1), 1–18. http://doi.org/10.1186/1471-2164-13-83

Sarsu F, Spencer MM, Hao C, Pareek A, Das A, Padolina A (2014) Climate proofing of food crops: Genetic improvement for adaptation to high temperatures in drought prone areas and beyond. 4th International Rice Congress, 27 October- 01 November 2014, Bangkok, Thailand. Book of Abstract, p 85.

Sarsu F, Caridad M, Muñoz C (2015a) Development of Heat Tolerance Screening Techniques of Common and Tepary Beans Mutants for Adaptation to High Temperature in Drought Prone Areas. Plant Abiotic Stress III. 29 June- 1 July 2015, Vienna, Austria. Book of Abstract page 48.

Sarsu F, Ashraf M, Caridad M, Kusolwa P, Kihupi L, Rajab M, Yona N (2015b) Rapid Screening Methods for Heat Stress Tolerance of Mutant Rice in the face of Climate Change. Plant Abiotic Stress III. 29 June- 1 July 2015, Vienna, Austria. Book of Abstract page 48.

Sarsu F, Ghanim AMA, Das P, Bahuguna R, Mbogo KM, Ashraf M, Pareek SLS, Pareek A, Forster B, Ingelbrecht I, (2018) Pre-Field Screening Protocols for Heat Tolerant Mutants in Rice, Springer Book Open Access. ISBN 978-3-319-77337-7, https://link.springer.com/book/10.1007%2F978-3-319-77338-4

Sarsu F, Ghanim AMA, Das P, Bahuguna R, Mbogo KP, Ashraf M, Singla-Pareek SL, Pareek A, Forster BF, Ingelbrecht I (2017) Pre-Field Screening for Heat Tolerant Mutants in Rice, Inter Drought V,21-25 February 2017, Book of Abstracts, p156, abstract number 444.

Sarsu F, Ghanim AMA, Das P, Bahuguna R, Mbogo KM, Ashraf M, Pareek SLS, Pareek A, Forster BP, Ingelbrecht I (2018b) Screening Protocols for Heat Tolerant Mutants In: Rice. Plant Mutation Breeding and Biotechnology Symposium, 26-31 August 2018, Vienna Austria.

Sarsu F, Ghanim AMA, Das P, Bahuguna R, Mbogo KM, Ashraf M, Pareek SLS, Pareek A, Forster B, Ingelbrecht I (2018a) Pre-Field Screening Protocols for Heat Tolerant Mutants in Rice, Springer Book Open Access. ISBN 978-3-319-77337-7, https://rd.springer.com/book/10.1007%2F978-3-319-77338-4

Sivasankar S, Bergvinson D, Gaur P, Kumar S, Beebe S, Tamo M (2018) Achieving sustainable cultivation of grain legumes. Burleigh Dodds Series in Agricultural Sciences, ISBN-10: 9781786761361

Sivasankar S, Helentjaris TG, Xu D, Lyznik A (2008) Transcriptional activators involved in abiotic stress tolerance. Patent US7317141

Silva JAL, Neves JA (2011) Componentes de produção e suas correlações em genótipos de feijão-caupi em cultivo de sequeiro e irrigado. Revista Ciência Agronômica 42, 702-713.

Spencer LMM, Forster BP, Jankuloski L (2018). Manual on Mutation Breeding, ISBN 978-92-5-130526-3. http://www.fao.org/3/I9285EN/i9285en.pdf

Su P, Feng H, Chen H, Yang CM, Wu XB, Huang Q (2013) Influences of Natural High-temperature Stress on Heat Resistance of Rice. Southwest China Journal of Agricultural Sciences 26:1387-1391.

Wang Y, Gao P, Huang M, Chen H, Yang ZR, Sun Q (2015) Effects of High Temperature on the Activity and Expression of Antioxidative Enzymes in Rice Flag Leaves during Flowering Stage. Plant Science Journal. 33(3):213-219.

Wang Y, Gao P, Fu Y, He J, Sun Q, Huang M (2016) Identification of Heat Resistance in Radiation Breed Hybrid Rice II YOU 838. Hubei Agricultural Sciences,2016, Vol. 55(23):6061-6066.

Zafar SA, Hameed A, Ashraf M, Khan AS (2014) Screening for heat tolerance in rice (*Oryza sativa* L.) through morpho-physiological profiling (Paper presented in the International Conference on Stress Biology and Biotechnology:

Challenges & Management. Institute of Agricultural Sciences, University of the Punjab, Quaid-e-Azam Campus, Lahore, Pakistan. pp15.

Zafar SA, Hameed A, Khan AS, Ashraf M (2017) Heat shock induced morpho- physiological response in indica rice (*Oryza sativa* L.) at early seedling stage. Pak J Botany Pak J Bot. 49(2): 453-463, 2017.

Zhang H-L, Huang J-Z, Liu Q-L, Nawaz Z, Lu H-P, Gong J-Y, Zhu Y-J, Yan W, Shu Q-Y (2014a) Characterization of an RNase Z nonsense mutation identified exclusively in environment-conditioned genic male sterile rice. Molecular Breeding. 34: 481-489.

Zhang H-L, Huang J-Z, Chen X-Y, Tan Y-Y, Shu Q-Y (2014b) Competitive amplification of differentially melting amplicons facilitates efficient genotyping of photoperiod- and temperature- sensitive genic male sterility in rice. Molecular Breeding. 34:1765-1776.

Zhang H-L, Chen X-Y, Huang J-Z, E Z-G, Gong J-Y, Shu Q-Y (2015) Identification and transition analysis of photo-/thermo-sensitive genic male sterile genes in two-line hybrid rice in China (in Chinese with English abstract). Scientia Agricultura Sinica. 48(1): 1-9 (doi: 10.3864/j.issn.0578-1752.2015.01.001).

DOI: 10.21475/ajcs.21.15.09.sp-1

Australian Journal of Crop Science AJCS

Rice mutants with tolerance to multiple abiotic stresses show high constitutive abundance of stress-related transcripts and proteins

Priyanka Das[1], Rajeev N Bahuguna[1], Ray Singh Rathore[1], Sheenu Abbat[1], Ramsong C. Nongpiur[1], Fatma Sarsu[2], Sneh L. Singla-Pareek[3] and Ashwani Pareek[1*]

[1]Stress Physiology and Molecular Biology Laboratory, School of Life Sciences, Jawaharlal Nehru University, New Delhi 110067, India
[2]Plant Breeding and Genetics Section, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, Vienna, Austria
[3]Plant Stress Biology, International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi 110067, India

***Corresponding author: ashwanip@mail.jnu.ac.in**

Abstract

Mutation breeding has a long track record in the development of crop cultivars with improved tolerance to abiotic stresses such as heat, salinity and drought. *Oryza sativa* L. cv IR64 is a very popular high yielding rice, but susceptible to major abiotic stresses, such as low and high temperatures, salinity and drought. We subjected IR64 to gamma irradiation and generated a mutant population (M_3) with ~2,000 families. These were screened at the seedling stage for tolerance to high-temperature stress using hydroponics and controlled-environment chambers, resulting in the identification of three mutant lines showing a robust seedling phenotype. Under heat stress, higher CO_2 assimilation (10-30%), higher spikelet fertility (40-45%) and higher antioxidant activity (15-20% catalase activity) confirmed superiority of the selected mutant lines over wild type plants at seedling and flowering stages. Upon exposure to salinity and drought stress, the three selected lines also exhibited better tolerance than wild type in terms of higher CO_2 assimilation, stomatal conductance, transpiration and chlorophyll fluorescence. Transcript and protein abundance analyses confirmed higher constitutive levels of heat shock proteins and antioxidant enzymes in the mutant lines relative to wild type. Tolerance to multiple abiotic stresses was reflected in higher (25-30%) grain yield than wild type. It is anticipated that the mutant lines identified will be useful for developing new improved cultivars for dry and saline areas and may be exploited to dissect the molecular basis of multiple stress tolerance in crop plants.

Key Words: Rice, Mutant, Gamma irradiation, heat, salinity, drought.
Abbreviations: CAT_Catalase, APX_Ascorbate Peroxidase, SOD_Superoxide Dismutase, GLY_Glyoxalase, HSP_Heat Shock Protein.

Introduction

Rice is a staple food crop especially in Asia and Africa. Despite growing demands to feed an ever-increasing population, rice production has been decreasing at a rate of 2.7% per year in 1970–1990 to 1.2% per year in 1990–2007 (Wassman et al., 2009). Sensitivity of rice to abiotic stresses such as heat, drought and salinity pose major constraints to yield (Grover and Minhas, 2000). 'Abiotic stress tolerance' and 'stability in yield' are complex genetic traits which involve highly regulated expression of a plethora of genes or the indirect effect of a single key regulatory gene (Yokoi et al., 2002). Hence, it is a daunting task for rice researchers to adopt a suitable method/technique to tailor abiotic-stress tolerance with improved yield and yield stability under unfavourable environmental conditions.

Mutation breeding has made notable contributions towards the production of high-yielding and stress-tolerant crops (Cassell and Doyel, 2003; Parry et al., 2009; Bado et al., 2015). There are now simple and effective protocols available to screen for salt-tolerant rice mutants (Badoet al., 2016a; Joshi et al., 2016). Rice seeds irradiated with C or Ne ions have successfully generated mutant genotypes with tolerance to salinity (Hayashi et al., 2007). Moreover, the mutant rice genotype, Zhefu 802, induced by gamma radiation in the parental cultivar Simei no. 2, showed cold tolerance (Ahloowalia et al., 2003). Many other rice genotypes such as, A-20, Atomita 2, Changwei19, Emai No. 9, Fuxuan No. 1, Liaoyan 2, Mohan-CSR 4, Jiaxuan No. 1, Shua 92, Nipponbare, Basmati 370, IR 58614, Zhefu 802, Kasmir basmati and mutated IR8 have been generated through mutation breeding for improving tolerance against various abiotic stresses (Singh, 2000; Baloch et al., 2003; Ahloowalia et al., 2003; Chhun et al., 2003; Saleem et al., 2005; Hayashi et al., 2008; Jain and Suprasanna, 2011). Cultivars have also been developed from these lines and released all over the world.

Heat stress has a dynamic impact on productivity of crop plants where sensitivity varies with the development stage and tissue type (Barnabas et al., 2008; Sakata and Higashitani, 2008). Heat stress at the seedling stage or flowering stage or grain filling stage can have a significant impact on plant survival and grain yield (Bahuguna et al., 2015). Most reports on crop varieties tolerant to abiotic stress have centred on single stresses, and experiments have been usually conducted under controlled environmental conditions. However, under field conditions, crops are seldom exposed to a single stress; rather they are exposed to more than one type of stress either sequentially or simultaneously. Therefore, improving crops for tolerance towards multiple stresses is an imperative. Although,

efforts need to focus on integrating tolerance to multiple abiotic stresses, reports in rice are scant. Most successful breeding programmes have been directed towards improving tolerance to a distinct stress, such as drought, salinity, flooding or high temperature. In this study we generate and screened ~2,000 M_3 families derived from gamma-irradiated rice (cv IR64) for heat tolerance at the seedling stage (by evaluating phenotypic, physiological and biochemical parameters). Subsequently, three selected putative heat-tolerant mutant lines were screened for their tolerance at flowering and grain filling stages to multiple abiotic stresses such as heat, salinity and drought.

Results

Heat stress at the seedling stage

Exposure of rice wild type (WT) seedlings to heat stress affected the growth of shoot and roots relative to control seedlings without stress (Fig1a). Under control conditions with no heat stress, no difference in seedling shoot length was observed between WT and the selected mutant families (D100/79, D100/96 and D100/11). This was not the case for high temperature stress (Fig1b). Under heat stress conditions, mutant lines exhibited a higher shoot length ranging from a 25% increase in D100/79 to 39% increase in D100/96 as compared to WT (Fig1b). Comparisons of root length revealed that even under control conditions, mutant seedlings had longer roots as compared to the WT (Fig1c). In the mutant families D100/79, D100/111 and D100/96, roots were 17%, 59% and 22% longer than WT under control conditions. A similar phenotype was observed under heat stress conditions, where roots of D100/79, D100/111 and D100/96 were 30%, 48% and 30% longer compared to WT, respectively (Fig1c). For fresh weight, a similar observation was made, where the mutant families had higher weights than WT seedlings under both control and heat stress conditions (Fig1d). Under control conditions, the fresh weight of D100/79, D100/111 and D100/96 seedlings were 9%, 25% and 13% higher than WT, respectively. Further, under heat stress conditions, the fresh weight of D100/79, D100/111 and D100/96 seedlings were 22%, 25% and 23% higher relative to WT, respectively (Fig1d). To understand the basis of improved seedling phenotypes, we carried out heat stress associated physiological and biochemical analyses such as membrane stability, activity of antioxidant enzymes and chlorophyll content in the seedlings (Lakra et al., 2017). Under control condition without stress, significant differences in electrolyte leakage were observed between WT and the selected mutant families where the mutants showed decreased electrolyte leakage compared to WT (Fig2a). The electrolyte leakage in D100/79, D100/111 and D100/96 seedlings were 4%, 6% and 9% lower than WT, respectively. Heat stress treatment significantly increased electrolyte leakage across the WT and mutant plants, but the mutant families recorded significantly lower electrolyte leakage relative to WT, with D100/111 recording the lowest electrolyte leakage (26% less than WT; Fig2a). Membrane stability was checked using lipid peroxidation (MDA content) analysis. Under control conditions, no significant difference in lipid peroxidation was observed among WT and mutant seedlings. However, under heat stress, lipid peroxidation was highest for WT plants (~400nmol g^{-1} fresh weight). Mutant families recorded 0.5% to 2% lower MDA content as compared to WT under similar conditions, although the only significant change as compared to WT was for D100/79 (Fig2b). Interestingly, significant differences in catalase activity of two mutants (D100/79 and D100/96) were observed under control conditions as compared to WT. D100/79 and D100/96 showed 10.8% and 4% higher catalase activity than WT controls, respectively. However, D100/111 seedlings did not show any significant increase in catalase activity in comparison to WT. Interestingly, heat stress led to a significant increase in catalase activity in D100/111 seedlings and similar increase was also observed in D100/96 seedlings, when compared to WT (Fig2c). Quite contrary to the catalase activity, under control conditions, no significant differences were observed between APX activity of WT and the three mutant families (Fig2d). But after heat stress treatment, D100/79 and D100/111 mutant seedlings showed significantly higher APX activity (8.2% and 5.2% increase) compared to WT. No significant differences were observed in the APX activity of WT and D100/96 heat-stressed seedlings. The total chlorophyll content of WT and mutant seedlings was analysed under heat stress. While no differences were observed for chlorophyll content of WT and mutant seedlings under control conditions, under heat stress two of the mutants viz. D100/79 and D100/111, maintained a chlorophyll content which was respectively 50% and 16% higher than WT (Fig2e).

Heat tolerance at reproductive stages

Since heat stress at reproductive stages has severe effects on yield of rice, the effects of heat stress at the flowering stage on the reproductive development of WT and mutant plants were analysed (Fig3a). Figure 3b showed panicles of plants under control conditions and after being subjected to heat stress at flowering. Under control conditions, there was no marked difference between spikelet fertility of WT and mutant plants which ranged from 85.7% to 89.9% (Fig3c). Heat stress exposure (40^{o}C) during the flowering stage led to significant reductions in spikelet fertility of WT and the D100/79 mutant line, recording only 47.7% and 47.3% spikelet fertility, respectively (Fig3c). Interestingly, mutant families D100/111 and D100/96 recorded ~80% spikelet fertility under heat stress which is comparable to that of unstressed, control plants (Fig3c). All the three mutant families recorded a significantly higher number of grains per panicle as compared to WT (Fig3d). Under heat stress, the difference between the number of grains per panicle of WT and mutants was more pronounced, numbers for D100/79, D100/111 and D100/96 were 35.5%, 50.0% and 44.0% higher than that WT controls (Fig3d). The weight of 1,000 grains from each mutant family was then compared with that of WT. The 1,000-grain weight of each mutant family was significantly higher than WT under control and stress conditions. 1,000 grain weight differences were 44.6% and 47.5% for D100/79, 42.8% and 43% for D100/111, 41% and 36% for D100/96, as compared to WT under control and heat stress respectively (Fig3e). These analyses showed better performances of the mutant families, not only under heat stress, but also under control conditions.

Gas exchange and chlorophyll fluorescence analyses

Gas exchange traits and chlorophyll fluorescence data revealed significantly better performance of mutant families as compared to WT under drought (70 to 80kPa), salinity (200mM) and heat stress(40^{o}C) at flowering (Fig4a-d) and early grain filling stages (Fig4e-h). Heat and osmotic stress (salinity and drought) treatments significantly reduced CO_2 assimilation in the three mutant genotypes as compared to WT controls. WT plants recorded lower CO_2 assimilation rates under various stresses as compared to the mutants, being lowest under drought stress viz. 6.9 and 7.7μmol m^{-2} s^{-1} at flowering and grain filling stages, respectively (Fig4a and e). However, under heat stress treatment at grain filling, no significant difference was observed in the CO_2 assimilation rate of D100/79 plants as compared to WT (Fig4e). All three mutant lines showed significantly higher CO_2 assimilation than WT across the osmotic stress treatments, recording 9.5 to 16.1μmol m^{-2} s^{-1}photosynthetic activity at flowering and grain filling (Fig4a and e). Further, stomatal conductance reduced significantly across the treatments with WT plants recording the lowest

measurements (0.07 and 0.1mol H_2O m^{-2} s^{-1}, at flowering and grain filling, respectively) under drought stress (Fig4b and f). On the other hand, mutant families recorded relatively higher stomatal conductance under various treatments. D100/96 recorded the highest stomatal conductance after stress treatments at flowering and grain filling stages (Fig4b and f). Further, both drought and salinity stress reduced transpiration rate, and WT plants recorded the lowest rates under drought (2.2 to 3.3mol H_2O m^{-2} s^{-1}) and salinity stress (3.2 to 4.5mol H_2O m^{-2} s^{-1}) at both growth stages (Fig4c and g). Conversely, the three mutant families showed higher transpiration rates under drought (3.3 to 7mol H_2O m^{-2} s^{-1}) and salinity (5.3 to 7.9mol H_2O m^{-2} s^{-1}), at flowering and grain filling stages (Fig4c and g). Mutant families recorded higher transpiration rates even under heat stress, with D100/96 showing the highest transpiration rate, 12.4 and 9.1mol H_2O m^{-2} s^{-1} at flowering and grain filling, respectively (Fig4c and 4g). Furthermore, chlorophyll fluorescence reduced significantly across the stress treatments with highest reductions recorded in WT plants at flowering (0.49 to 0.54) and grain filling (0.55 to 0.62) (Fig4dand h). Conversely, mutant lines maintained significantly higher chlorophyll fluorescence (0.57 to 0.77) over WT plants across the stress treatments and growth stages.

Grain yield under various osmotic stresses

Grain yield was reduced significantly in WT plants and mutants exposed to salinity, drought or heat stress at flowering and grain filling stages (Fig 5). However, the reduction in grain yield was significantly lower in the mutant families compared to WT across stress treatments. Highest reductions in grain yield were recorded for WT plants under drought stress at both flowering (61%) and grain filling (64%) stages, compared to control conditions (Fig 5). Yield loss under drought was lower in the mutant lines showing only 52 to 60% and 48 to 49% reduction at flowering and grain filling stages, respectively (Fig 5). Similarly, both heat and salinity stress reduced yield in WT plants by 27 to 60%, and heat-induced loss was greater (39%) at flowering. However, the mutant families showed significantly lesser reductions in grain yield under both heat and salinity stresses except the line D100/79. Mutant lines D100/111 and D100/96 recorded significantly higher yields under heat and salinity stresses, with D100/111 recording the highest yield under salinity (30, and 48g $plant^{-1}$) and heat (48 and 56g $plant^{-1}$) stress at flowering and grain filling, respectively (Fig 5).

Transcript and protein abundance of stress related genes

Transcript abundance of various key stress-responsive genes have been reported to provide information on regulating osmotic and heat stress tolerance in rice (Kumari et al., 2009; Lakraet al*.,* 2015; Soda et al., 2016). qRT-PCR analyses of histone-binding protein, antioxidant enzymes (APX and SOD) and heat shock protein-70 under heat stress treatment revealed significant differences all genotypes tested (Fig6a). WT plants recorded lowest expression levels of histone binding protein-1b (*HBP1b*), *APX*, *SOD* and *HSP70*. In contrast, all three mutant families displayed significantly higher expression levels of these genes compared to WT, with D100/111 and D100/96 showing considerably higher levels of *HBP1b, APX, SOD* and *HSP-70* activity (Fig6a). Conversely, *CAT* expression levels in the mutant lines was similar to that observed in WT under heat stress, except in D100/96 which recorded significantly higher level of *CAT* expression (Fig6a). Thus, the tolerance traits observed in the three mutant families could be attributed to the expression of these positive regulators of stress.

Discussion

Rice cultivars with multiple abiotic stress tolerance in field conditions are of great elevance (Nguyen and Ferrero, 2006, Das et al., 2014). Mutation breeding provides a promising approach for tailoring rice genotypes for resilience towards environmental stresses (Das et al., 2014; Bado et al., 2015). Although sensitive to several abiotic stresses, IR64 is one of the most popular rice cultivars due to its high-quality grain, high yield and excellent cooking qualities; it is grown in over 200 countries (Laborte et al., 2015, Das et al., 2015b). With an objective of generating new variation in IR64 for resilience to abiotic stresses, we raised a mutant population from gamma-ray mutagenesis and performed screening for high-temperature tolerance. A simple but efficient protocol was developed where screening was done at the seedling stage and further evaluation of selected lines was carried out at flowering and grain filling stages with major emphasis on the phenotypic, physiological, biochemical and molecular parameters.

Rice seeds (M_o) were exposed to 100 Gy of gamma irradiation followed by repeated selfing (M_1 and M_2) to obtain M_3families from which stable lines may be developed. The heat stress treatment and recovery were done based on screening protocols for rice seedlings (Bado et al., 2016a; Joshi et al., 2016). As seedling and reproductive stages are the two most sensitive stages in rice, a strategy was devised to screen the whole population for high temperature tolerance of seedling stages followed by testing selected lines at reproductive stages (flowering and grain filling). We tested three putative mutant lines for tolerance to salinity and drought, in addition to high temperature stress. This strategy allowed us to develop a robust and reproducible pre-field screening method through hydroponics in a short time where the temperature, light and humidity could be controlled very precisely in a plant growth chamber. Plant morphology plays an important role in identifying and selecting mutant lines tolerant to heat or any other stress from among a large population (Ayeneh et al. 2002). Therefore, the first round of screening was based mainly on phenotype, such as leaf tip burn, root length, shoot length, greenness and seedling establishment (Fig1). Previous studies exploring the effects of high temperature on rice seedlings have documented damage of plant cellular architecture, which brings about major changes in chloroplast and mitochondria resulting in reduced metabolism and hence, reduced growth of seedlings under such conditions (Pareek et al., 1997).

Table 1. List of primers used for qRT-PCR and their sequences (5'- 3').

Gene	Experiment	Forward Primer sequence (F)	Reverse Primer sequence (R)
HBP	qRT-PCR	GGATAGCCAACCTTCAGCAG	ATTTGGCCCATGTAGTTTGC
SOD	qRT-PCR	CCAGAAGCACCACGCCACCT	GATTGACATGGCCTCCGC
CAT	qRT-PCR	ATGGATCCCTACAAGCATCGG	AGATGATAGTCCTCAAGGAGG
APX	qRT-PCR	CGAGCCCATCAAGGAGGAGA	AGGTGGGGGTGCAGGTTTGTC
HSP70	qRT-PCR	CCAGCTAAGAAGCTCCAAG	CTGAGGGTCTTCCTCGAG
Actin	qRT-PCR	CAGCCACACTGTCCCCATCTA	AGCAAGGTCGAGACGAAGGA

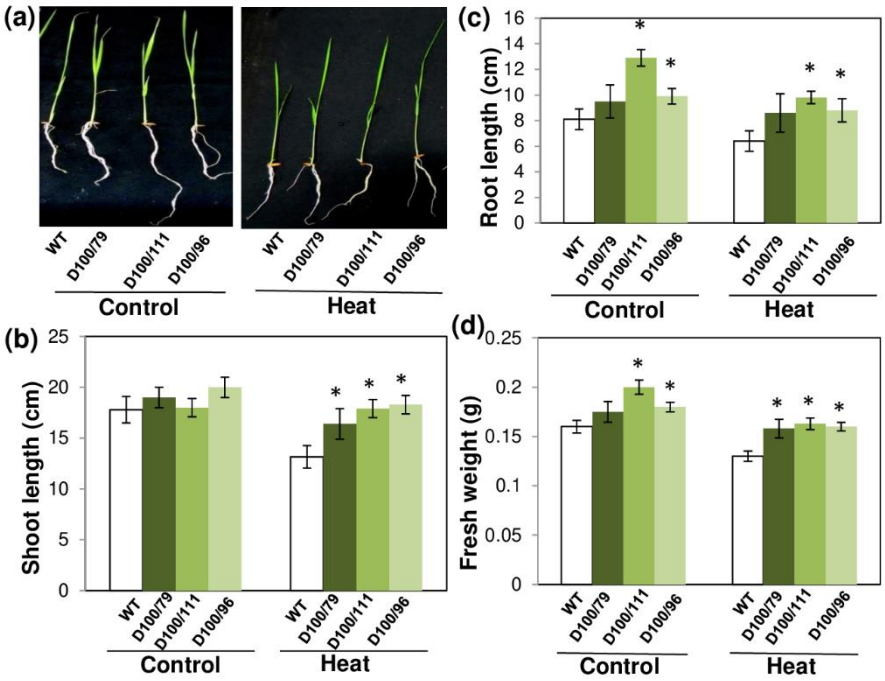

Fig 1. Phenotype analysis of three selected mutant (M_3) families under control conditions and after heat stress treatment (45ºC for 12h). (a) Picture of three selected mutant lines along with the WT under control conditions and after heat stress treatment; Determination of (b) shoot length (c) root length and (d) fresh weight of selected mutant seedlings in comparison to WT under control conditions and after heat stress treatment. Vertical column represents mean of three biological replicates ± SE. '*' denotes significant difference ($P<0.05$) as compared to WT plants.

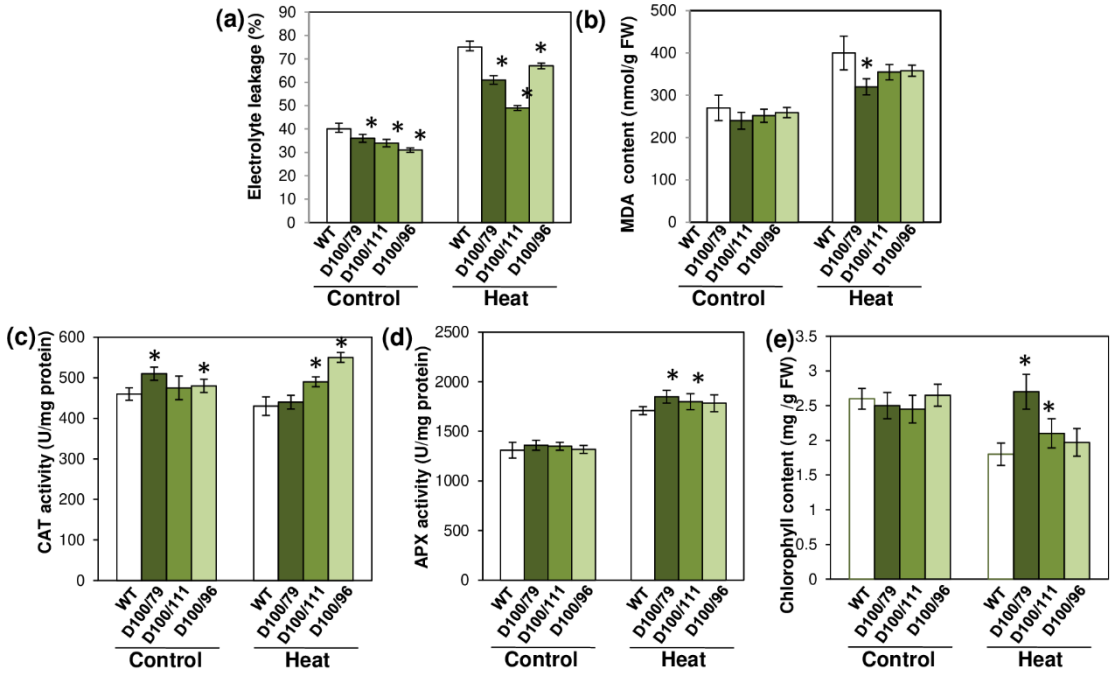

Fig 2. Analysis of physiological and biochemical parameters in WT and three selected heat-tolerant mutant (M_3) families under control conditions and after heat stress treatment (45 ºC for 12h). Study of (a) electrolyte leakage, (b) MDA content, (c) catalase activity, (d) APX activity and (e) chlorophyll content in selected mutant lines in comparison to WT under control conditions as well as after heat stress treatment. Vertical column represents mean of three biological replicates ± SE. '*' denotes significant difference ($P<0.05$) as compared to respective WT plants.

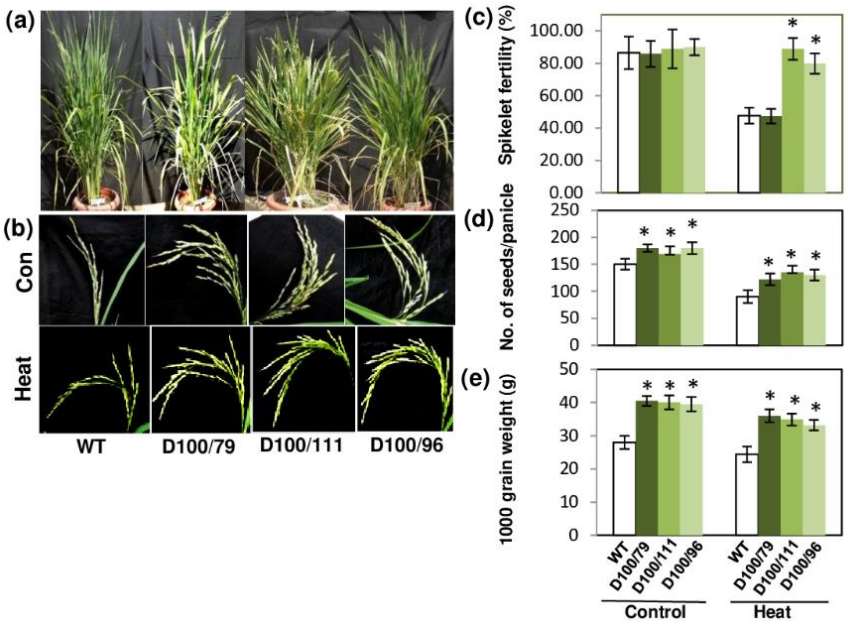

Fig 3. Analysis of morphological parameters in selected heat-tolerant mutant lines at mature (grain filling) stage after heat stress treatment (40 ºC for 4h). At the onset of flowering, plants were transferred to a pre-set growth chamber at 40 ºC for 4 h (0800 h to 1200 h) daily for three consecutive days to allow complete flowering on the panicle from the main tiller (a) Pictorial view of WT and heat-tolerant mutants before heat stress treatment. (b) Panicle phenotype of WT and heat-tolerant mutants under control conditions (upper panel) and after heat stress treatment (lower panel). (c) Percentage of spikelet fertility, (d) number of seeds per panicle and (e) 1,000 grain weight of heat tolerant mutant lines in comparison to WT under control conditions and after heat stress treatment. Vertical column represents mean of three biological replicates ± SE. '*' denotes significant difference ($P<0.05$) as compared to respective WT plants.

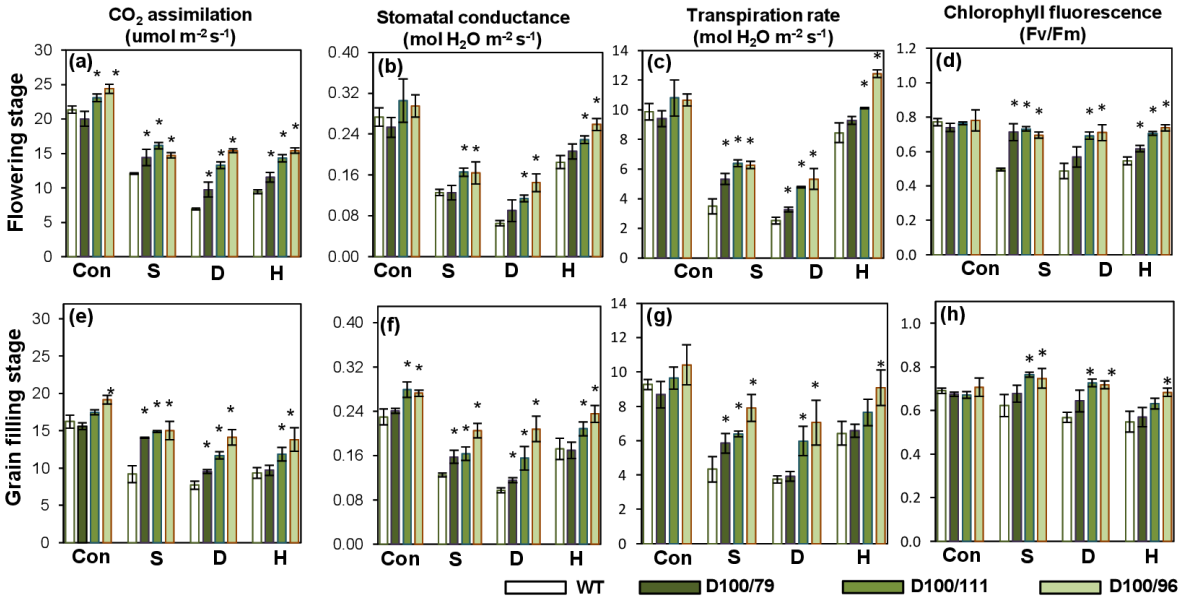

Fig 4. Analysis of gas exchange traits and chlorophyll fluorescence of WT and three selected heat tolerant mutant (M_3) families under control, drought, salinity and heat stress at anthesis (a-d) and post anthesis stage (e-h). Determination of (a) CO_2 assimilation, (b) stomatal conductance, (c) transcription rate and (d) chlorophyll fluorescence in flag leaves of control, drought, salinity and heat stress treated plants at anthesis. (e), (f), (g) and (h) represent CO_2 assimilation, stomatal conductance, transcription rate and chlorophyll fluorescence respectively in the flag leaves of control, drought, salinity and heat stress treated plants at the grain filling stage. Vertical column represents mean of three biological replicates ± SE. '*' denotes significant difference ($P<0.05$) as compared to WT plants.

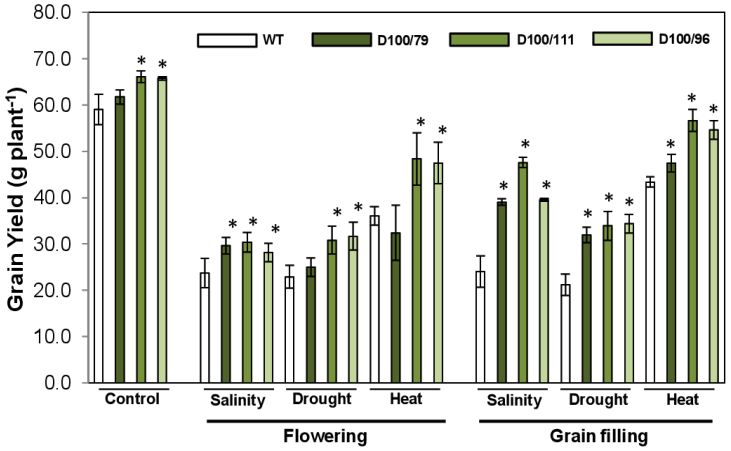

Fig 5. Grain yield per plant in WT and three selected heat-tolerant mutant (M_3) families under control, salinity, drought and heat stress at flowering and grain filling (GF) stages. Vertical column represents mean of four biological replicates ± SE. '*' denotes significant difference ($P<0.05$) as compared to respective control plants.

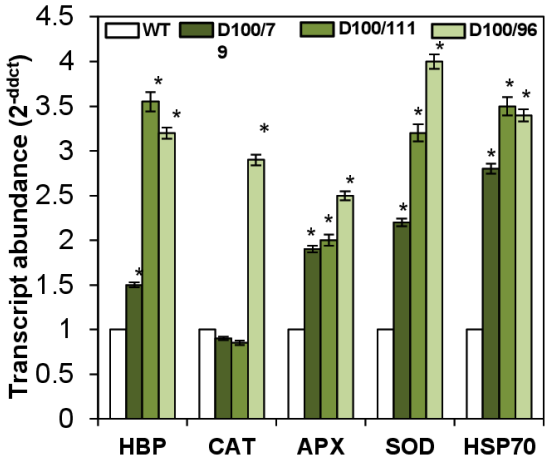

Fig 6. Molecular characterization of three selected heat-tolerant mutant (M_3) families through expression analysis of stress responsive transcripts. (a) Transcript abundance (fold change) has been shown for histone-gene binding protein (HBP), catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD) and heat shock protein-70 (HSP70) in WT and heat-tolerant mutant lines in unstressed conditions. Vertical column represents mean of three biological replicates ± SE. '*' denotes significant difference ($P<0.05$) as compared to respective WT plants.

In the present study, the selected mutant family (D100/79, D100/111 and D100/96) seedlings were found to possess better membrane stability as evident from lower electrolyte leakage, lower MDA levels, higher CAT and APX activity, and higher chlorophyll content that WT seedlings after heat stress treatment, which confirmed the lines to be superior than WT for tolerance towards high temperature (Fig 2). The three putative tolerant mutant lines were thus taken for a second round of testing for osmotic stress tolerance and characterization, where important morphological, physiological, biochemical and molecular parameters were taken into account.

Although rice is affected by high temperature at almost all growth stages (Pareek et al., 1995; Shah et al., 2011), the vegetative stage is relatively more tolerant to high temperatures. Temperatures beyond optimum thresholds reduce plant growth, alter flowering time and reduce yield (Yoshida et al., 1981). Stress coinciding with flowering and grain filing is more detrimental for grain yield (Wassmann et al., 2009; Masuduzzaman et al., 2016). Interestingly, high tolerance levels of selected mutant families to heat (45°C) at the seedling stage were consistent over the later phenological stages and we observed better plant phenotype, higher spikelet fertility (~80%), increased number of spikelets per panicle and higher grain weight in the selected mutant lines (D100/79, D100/111 and D100/96) compared to wild type plants after heat stress treatment were given at the flowering or early grain filling stage (Fig 3).

Abiotic stresses are inter-connected in the agricultural environment (Pareek et al., 2010; Rao et al., 2016; Lakra et al., 2015; Faralli et al., 2015). High temperature, salinity and drought are stresses which, besides having their own unique dimensions, share osmotic stress as a common denominator (Kissoudis et al., 2014). It is important to note that plants face more than one stress at a time or during the life cycle (Tester and Bacic, 2005; Mittler, 2006; Barnabas et al., 2007). Thus, tolerance to multiple abiotic stress is needed and is the most suitable approach to tailor climate resilient rice cultivars. Owing to the impressive progress of omics-based technologies, commonality in these stresses at biochemical and

molecular level has been established (Soni et al., 2015; Das et al., 2015b). Cross talk in signalling machineries has further established a strong inter-relationship between these abiotic stresses (Kissoudis et al., 2014; Das et al., 2015a). Thus, we were prompted to test the tolerance of these heat tolerant mutants for salinity and drought stress. Selected mutant lines recorded significantly higher CO_2 assimilation rate, better transpiration rate, higher stomatal conductance and higher chlorophyll fluorescence across all three stress conditions applied at flowering or grain filling stages (Fig4). Higher photosynthetic ability and maintenance of higher levels of stomatal conductance and transpiration rates represent simple methods of stress avoidance or escape routes.

It is evident that better growth rates and higher rates of photosynthesis under stress conditions could contribute to higher yield (Long et al., 2006; Makino et al., 2011). Significantly higher grain yields were found in all three mutant families (D100/79, D100/111 and D100/96) across the stress treatments (Fig 5). In general, higher tolerance levels have a cost (yield penalty) under non-stress conditions possibly due to shifts in metabolic activity towards defence related processes. Breeders are consistently putting their efforts into improving genotypes in a way that they should provide reasonable yields under stress but should be able to produce 'at par' under non-stress conditions. Interestingly, two of the selected mutant families (D100/111, D100/96) were able to produce high yields, both under optimum as well as stress conditions. This is an interesting finding and suggests that the underlying mechanisms are constitutive. This has also been found by Bado et al. (2016b) where mechanisms for salt tolerance in rice were found to be active in both control and salt-stressed treatments. Consequently, salt-tolerance may be screened for under normal conditions.

To investigate the mechanisms of improved stress tolerance in these mutant lines, we analyzed transcript abundance of key stress-responsive genes such as, HSP 70 and HBP. HSP 70 acts as a cellular thermometer and its over-expression is correlated with improved tolerance to heat stress (Park et al., 2015). Transcript abundance analysis for HSP 70 in WT and mutants clearly demonstrated >3-fold higher transcript in the mutants as compared to WT seedlings under non-stress conditions. Similarly, HBP is another protein, which results in higher stress tolerance upon over-expression in tobacco (Lakra et al., 2015). The qRT-PCR based expression analysis of HBP in WT and mutants revealed higher transcript abundance in the mutants, than in WT seedlings. Transcripts corresponding to antioxidant enzymes such as, CAT, APX and SOD were also found to behave in a similar fashion where higher constitutive expression was seen in the mutants as compared to WT plants (Fig6a). However, *CAT* expression was found to be altered in only one mutant family i.e. D100/96 thereby suggesting multiple pathways/mechanisms operative in these mutants which may contribute towards the observed tolerance. This is in corroboration with many previous findings where abiotic stress response has shown to be highly complex and a multigenic trait (Kumari et al., 2009; Das et al., 2015b; Soda et al. 2016). Since higher constitutive expression of a representative sample of key stress response genes was found to be the possible reason for the observed differences in stress tolerance between the WT and mutant plants. Similar mechanism of stress tolerance has been shown recently to operate in natural land races of rice (Lakra et al., 2017).

From these results, we propose that these 'mutants resulted from higher constitutive expression of the representative sample (and possibly others) of key stress responsive genes and proteins, which may, in turn, be a result of deletion or modification of some unknown inhibitors of these genes due to exposure to gamma radiations. However, the findings are in good corroboration with a host of reports where differential accumulation of transcripts and proteins between contrasting genotypes has been reported for these genes (Kumari et al., 2009) and constitutive higher abundance of these genes/proteins has been reported in the tolerant genotype (Nutan et al., 2016; Soda et al., 2013; Karan et al., 2009; Bado et al., 2016b; Lakra et al 2017). Hence, we propose that the well preparedness of the mutants, to handle the stress in anticipation, seems to be a key mechanism of tolerance. Nonetheless, these mutant families will serve as useful germplasm for areas affected by a combination of stresses and the detailed molecular basis of tolerance for those mutants is yet to be revealed.

Materials and Methods

Plant materials, growth conditions and stress treatments

Rice (cv. IR64) seeds were subjected to 100Gy of ^{60}Co gamma dose in a gamma chamber (BARC, India) to obtain M_1 mutant seed. These were germinated and survivingM_1 mutant plants (2,000) were advanced by selfing to produceM_2 and then M_3 generations. Selection of the M_3 heat-tolerant mutant families (D100/7, D100/96 and D100/111) was done by phenotypic, biochemical and molecular analyses of the plants at seedling as well as reproductive stages.

To optimise the heat stress treatment and recovery at the seedling stage, seven-day-old wild type (WT) seedlings grown under optimum growth conditions (12 h day/night, 28 ^{o}C, 10,000 Lux light and 70% humidity) were subjected to high temperature (45 ^{o}C for 8, 10 or 12 h) in a growth chamber (Daihan Labtech Co. Ltd, India), followed by different durations of recovery (40, 60 or 72 h) under optimum growth conditions. Based on these results, seven-day old seedlings were subjected to 12 h of heat shock at 45 ^{o}C followed by a 72-h recovery period at normal temperature. Under these conditions, 50% of WT seedlings failed to recover (LD_{50} for WT)**.** Following the stress treatment and recovery, leaf tissues from each line were taken for detailed experimental analysis.

Similarly, for heat stress at the flowering stage, 20 days old mutant seedlings were first transferred from hydroponic to soil-filled pots and maintained ibbenign growth conditions in a greenhouse. At the onset of flowering, plants were transferred to a pre-set growth chamber at 40 ºC for 4 h (0800 h to 1200 h) daily for three consecutive days to allow complete flowering on the panicle from the main tiller. As the rice inflorescence is very sensitive to high temperature (Jagadish et al., 2007), the temperature and duration of stress was reduced at the flowering stage, as compared to the seedling stage to avoid any severe effect on reproductive structures and seed set. Immediately after heat stress, the plants were transferred to recovery conditions (28°C for 72h). Following recovery, physiological and biochemical analyses were carried out. Similar to high temperature stress, salinity and drought stresses were imposed at flowering and early grain filling stages using independent sets of plants from the M_3 families. For the salinity treatment, the soil of the potted mutant plants was saturated with half-strength Yoshida solution (Yoshida et al*.,* 1981) supplemented with 200mM NaCl for seven days and subsequently allowed to recover by watering with half-strength Yoshida solution. For drought, responses of mutant plants were observed by water-withdrawal from the respective pots at the onset of flowering or at the early grain filling stage, the soil was allowed to dry until the soil-tensiometer reading reached 70-80 kPa. Plants were then re-watered with half-strength Yoshida solution for recovery. In parallel, WT plants were grown without any stress and served as control.

Phenotypic and yield component analysis
Plant phenotyping (such as root length, shoot length and fresh weight) was carried out at the seedling stage while yield component analysis (such as number of tillers/plants, spikelet fertility, grain weight and grain yield/plant) was done using data from mature plants.

Electrolyte leakage
Analysis of electrolyte leakage was carried out following the protocol of Bajji et al. (2002). Leaf samples from control and heat-treated mutant seedlings were harvested and washed with distilled water to remove any surface adhering ions. 100mg tissue was immediately dipped into 20ml of de-ionized water. After incubating the leaf tissues at 37ºC for 2h, the electrical conductivity (E_1) of the immersion solution was measured using a conductivity meter (ELEINS, Inc., India). To determine the total conductivity (E_2), samples with immersion solution (effusate) were autoclaved for 15 min at 121ºC and the conductivity of the effusate was measured after cooling to room temperature. Relative electrical conductivity was measured by the following formula: percentage of electrolyte leakage = $E_1/E_2 \times 100$.

Lipid peroxidation assay
Lipid peroxidation was estimated by measuring the formation of malondialdehyde (MDA). MDA content was quantified by thiobarbituric acid reactive substances assay (Heath and Packer, 1968). For this purpose, about 100mg of leaf tissue from the control and heat-treated seedlings was homogenized in 5ml of 5% (w/v) trichloroaceticacid and the homogenate was centrifuged at 12,000×g for 10 minat room temperature. The supernatant was mixed with an equal volume of thiobarbituric acid [0.5% in 20% (w/v) trichloroaceticacid] and the mixture boiled for 25min at 100ºC, followed by centrifugation for 5min at 7500g. Absorbance of the supernatant was measured at 532nm. MDA equivalents were calculated using the extinction coefficient of $155 mM^{-1} cm^{-1}$.

Measurement of total proteins
Total soluble proteins were extracted from the leaves (100mg) of control and heat-treated seedlings using Zivy's buffer (Zivy et al.,1983). Amount of soluble proteins was estimated by Bradford'sassay (1976) by reading from a standard curve prepared by using different concentrations of Bovine Serum Albumin.

Antioxidant enzyme activity assay
About 100mg leaf material from control and heat-treated seedlings were homogenized in ice-cold 50mM K_2PO_4 buffer (pH 7.5) containing 2mM ethylenediaminetetraacetic acid (EDTA) and 0.1mM phenylmethylsulphonylfluoride (PMSF) The homogenizing buffer for ascorbate peroxidase (APX) additionally contained 2 mM of Na-ascorbate. The homogenate was centrifuged at 12,000×g for 10 min at 4 °C and the supernatant was used for enzyme assay. Total protein content in supernatant was determined following Bradford (1976). APX activity was measured following Nakano and Asada (1981). The reaction mixture in a total volume of 1ml consisted of 50 mM (pH 7.5) K_2PO_4 buffer, 0.1mM EDTA, 0.25 mM ascorbate, 10 mM H_2O_2 and enzyme extract. H_2O_2-dependent oxidation of ascorbate was followed spectrophotometrically by recording the decrease in absorbance at 290 nm. Slope value of absorbance in 290 nm was considered for rate calculation. Catalase (CAT) activity was measured following the method described by Aebi (1984), by measuring the decrease in absorbance at 240 nm due to decomposition of H_2O_2. The slope value of the rapid decline in 240nm absorption was considered for rate calculation. The reaction mixture in 1ml contained 50 mM K_2PO_4 buffer (pH 7.0) with leaf extract equivalent to 5 µg total protein. The reaction was initiated by adding H_2O_2 to a final concentration of 20mM.

Analysis of photosynthesis parameters
Chlorophyll fluorescence, photosynthesis (µmol CO_2 m^{-2} s^{-1}), leaf stomatal conductance (mol H_2O m^{-2} s^{-1}) and transpiration rate (mmol H_2O m^{-2} s^{-1}) of the flag leaf were monitored using an InfraRed Gas Analyser (IRGA, LICOR-6400XT). Chlorophyll *a* fluorescence and photosynthesis of the leaves of stress-treated or untreated plants (both WT and mutant) were monitored at 25 ºC under supplied CO_2 concentration (400 ppm). Leaves were dark adapted for 30 min prior to measurement of chlorophyll fluorescence. The minimal level of dark fluorescence (F_0) was measured under weak modulated light and the maximal intensity of fluorescence (F_m) was evoked by application of a short saturating light pulse (10,000 µmol m^{-2} s^{-1}). The maximal steady-state photochemical efficiency (i.e., intrinsic quantum yield under dark adapted condition) was indicated by (F_v / F_m), where $F_v = F_m - F_0$.

Quantitative real-time-PCR (qRT-PCR) analysis
Total RNA was isolated from the flag leaf of mature (at flowering stage) plants using Tri-Reagent (Sigma-Aldrich). Elimination of genomic DNA contamination was carried out by addition of DNase I (Epicentre), as per the manufacturer's instructions. RNA samples were reverse transcribed using first strand cDNA synthesis kit (Fermentas, United States). Real-time PCR was performed using Power SYBR Green PCR Master Mix (ABI) on ABI prism 7900 Real-Time PCR system. Primer pairs (Table 1) for transcript expression analysis of stress responsive genes was designed using ABI primer designer. Actin was used as the reference gene in the expression analysis. The 2^{-ddCT} method was used to analyse the fold change in transcript expression (Livak and Schmittgen, 2001).

Statistical analysis
Physiological and biochemical experimental analyses were repeated three times using three independent mutant plants from each mutant family. All the experiments were analysed as a completely randomized design (CRD) with two-way ANOVA. Means were compared using least significant difference (LSD) at $P<0.05$.

Conclusions

Three selected mutant M_3 families (D100/79, D100/111 and D100/96) were found to have higher transcript and protein abundance**,** better plant phenotype, better antioxidant response, higher spikelet fertility, higher photosynthetic activity and higher grain yield under heat, salinity and drought stress as compared to the wild type parental line. It has been confirmed from the study that gamma irradiation could create useful mutations in the rice genome which could ultimately enhance tolerance to multiple abiotic stress conditions. The region(s) of mutation in these novel lines is yet to be discovered which will further open inroads of molecular basis of stress tolerance. The selected mutant lines provide useful germplasm for use in breeding programmes for abiotic stress tolerance in rice.

Acknowledgements

Authors are thankful to International Atomic Energy Agency (IAEA) for financial support to carry out the work. Research in the laboratory of AP is also supported from funds received from Ministry of Science and Technology, India and Resource Network Program from University Grants Commission (UGC-RNW) through the University. PD is thankful to University Grants Commission (UGC) for Dr D. S. Kothari Fellowship awarded to her.

References

Aebi H (1983) Catalase. In: Bergmeyer HU (ed) Methods of Enzymatic Analysis, Academic Press, Inc, New York, NY, USA. 273-288.

Ahloowalia BS, Maluszynski M, Nichterlein K (2003) Global impact of mutation-derived varieties. Euphytica. 135: 187-204.

Ayeneh A, Van Ginkel M, Reynolds MP, Ammar K (2002) Comparison of leaf, spike, peduncle and canopy temperature depression in wheat under heat stress. Field Crops Res. 79: 173-184.

Bado S, Forster BP, Ghanim AMA, Jankowicz-Cieslak J, Berthold G, Gregorio GB, Luxiang L (2016a) Protocols for pre-Field screening of mutants for salt tolerance in rice, wheat and barley. Springer Open Access, ISBN 978-3-319-26588-9.

Bado S, Forster BP, Nielen S, Ali AM, Lagoda PJL, Laimer M (2015) Plant Mutation Breeding: Current Progress and Future Assessment. In: Janick J (ed) Plant Breeding Reviews,39(Chapter 2): 23-88.

Bado S, Forster BP, Padilla-Alvarez R, Resch C, Migliori A, Diawara Y, Jaksic M and Laimer M (2016) Prediction of salt tolerance in rice (*Oryza sativa*) based on shoot element content in non-stressed conditions. J Mater Sci Eng. A6: 1-16.

Bahuguna RN, Jha J, Pal M, Shah D, Lawas LM, Khetarpal S, Jagadish KS (2015) Physiological and biochemical characterization of NERICA-L-44: a novel source of heat tolerance at the vegetative and reproductive stages in rice. Physiol plant. 154(4): 543-559

Bajji M, Kinet JM, Lutts S (2002) The use of the electrolyte leakage method for assessing cell membrane stability as a water stress tolerance test in durum wheat. Plant Growth Regul. 36: 61-70.

Baloch AW, Soomro AM, Javed MA, Bughio HR, Alam SM, Bughio MS, Mohammed T, Mastoi NN (2003) Induction of salt tolerance in rice through mutation breeding. Asian J Plant Sci. 2: 273-276.

Barnabas B, Jager K, Feher A (2008) The effect of drought and heat stress on reproductive processes in cereals. Plant cell environ. 31(1): 11-38.

Bradford MM (1976) A dye binding assay for protein. Annals Biochem. 72: 248-54.

Cassells AC, Doyel BM (2003) Genetic engineering and mutation breeding for tolerance to abiotic and biotic stresses: science, technology and safety. Bulgarian J Plant Physiol (Special Issue). 52-82.

Chhun T, Taketa S, Tsurumi S, Ichii Z (2003) Interaction between two auxin-resistant mutants and their effects on lateral root formation in rice (*Oryza sativa* L.). J Exp Bot. 54: 2701-2708.

Das P, Mishra M, Lakra N, Singla-Pareek SL, Pareek A (2014) Mutation breeding: a powerful approach for obtaining abiotic stress tolerant crops and upgrading food security for human nutrition. In: Tomlekova N, Kojgar I, Wani R (eds) Mutagenesis: Exploring Novel Genes and Pathways, Wageningen Academic Publisher, Wageningen. 15-36.

Das P, Nutan KK, Singla-Pareek SL, Pareek A (2015a) Oxidative environment and redox homeostasis in plants: dissecting out significant contribution of major cellular organelles. Front Environ Sci.2: 70.

Das P, Nutan KK, Singla-Pareek SL, Pareek A (2015b) Understanding salinity responses and adopting 'omics-based' approaches to generate salinity tolerant cultivars of rice. Front Plant Sci. 6: 712.

Faralli M, Lektemur C, Rosellini D, Gürel F (2015) Effects of heat shock on salinity tolerance in barley (Hordeum vulgare L.): plant growth and stress-related gene transcription. Biol Plant. 59(3):537-546.

Grover A, Minhas D (2000) Towards production of abiotic stress tolerant transgenic rice plants: issues, progress and future research needs. Proc IndianNatl Sci Acad, Part B. 66(1): 13-32.

Hayashi Y, Takehisa H, Kazama Y, Kanba C, Ohbu S, Ryuto H, Fukunishi N, Tokairin H, Ohkoshi K, Yoshini Y, Sato T, Abe T (2008) Characterization of salt tolerant mutants of rice induced by heavy ion irradiation. RIKEN Accelerator Progress Report. 41: 234.

Hayashi Y, Takehisa H, Kazama Y, Ichida H, Ryuto H, Fukunishi N, Abe T, Kamba C Sato T (2007). Effects of ion beam irradiation on mutation induction in rice. Paper presented in Eighteenth International Conference, Cyclotrons and Their Applications. Catania, Italy, 1-5 October 2007, 1-5.

Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplasts: I. Stoichiometry of fatty acid peroxidation. Arch Biochem Biophys. 125: 189-98.

Hoagland DR, Arnon DI (1938) The water-culture method for growing plants without soil. Circular. California Agricultural Experiment Station. 347: 32.

Jagadish SVK, Craufurd PQ, Wheeler TR (2007) High temperature stress and spikelet fertility in rice (*Oryzasativa* L.). J Exp Bot. 58: 1627-1635.

Jain MS, Suprasanna P, (2011) Induced mutations for enhancing nutrition and food production. Gene Conserve. 40: 201-215.

Joshi R, Prashat R, Sharma PC, Singla-Pareek SL, Pareek A (2016) Physiological characterization of gamma-ray induced mutant population of rice to facilitate biomass and yield improvement under salinity stress. Indian J Plant Physiol. 21:545-555.

Karan R, Singla-Pareek SL, Pareek A (2009) Histidine kinase and response regulator genes as they relate to salinity tolerance in rice. Funct Integr Genomics. 3: 411-417.

Kissoudis C, van de Wiel C, Visser RGF, Van Der Linden G (2014) Enhancing crop resilience to combined abiotic and biotic stress through the dissection of physiological and molecular crosstalk. Front Plant Sci. 5: 207.

Kumari S, Panjabi nee Sabharwal V, Kushwaha HR, Sopory SK, Singla-Pareek SL, Pareek A (2009) Transcriptome map for seedling stage specific salinity stress response indicates a specific set of genes as candidate for saline tolerance in *Oryza sativa* L. Funct Integr Genomics. 9(1): 109-123.

Laborte AG, Paguirigan NC, Moya PF, Nelson A, Sparks AH, Gregorio GB (2015) Farmers' preference for rice traits: Insights from farm surveys in central luzon, Philippines. Plos One. 10: 1966-2012.

Lakra N, Kaur C, Anwar K, Singla-Pareek SL, Pareek A (2017) Proteomics of Contrasting Rice Genotypes: Identification of Potential Targets for Raising Crops for Saline Environment. Plant Cell Environ. DOI: 10.1111/pce.12946.

Lakra N, Nutan KK, Das P, Anwar K, Singla-Pareek SL, PareekA (2015) A nuclear-localized histone-gene binding protein from rice (OsHBP1b) functions in salinity and drought stress tolerance by maintaining chlorophyll content and improving the antioxidant machinery. J plant physiol: 176:36-46.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{[-\text{Delta Delta C(t)}]}$ method. Methods. 25: 402-8.

Lobell DB, Gourdji S(2012) The influence of climate change on global crop productivity. Plant Physiol. 160: 1686-1697.

Long SP, ZhuXG, Naidu SL, Ort DR (2006) Can improvement in photosynthesis increase crop yields? Plant Cell Environ. 29: 315-330.

Makino A, (2011) Photosynthesis, grain yield, and nitrogen utilization in rice and wheat. Plant physiol. 155(1): 125-129.

Masuduzzaman ASM, Ahmad HU, Haque M, Ahmed MME (2016) Evaluation of rice lines tolerance to heat during their flowering stage. Rice Research-Open Access. 4: 170.

Mittler R (2006) Abiotic stress, the field environment and stress combination. Trends Plant Sci. 11: 15-19.

Nakano Y, Asada K (1981) Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol. 22: 867-80.

Nguyen NV, Ferrero A (2006) meeting the challenges of global rice production. Paddy Water environ. 4: 1-9.

NutanKK, Kushwaha HR, Singla-Pareek SL, Pareek A (2017) Transcription dynamics of Saltol QTL localized genes encoding transcription factors, reveals their differential regulation in contrasting genotypes of rice. Funct Integr Genomics.17(1): 69-83.

Pareek A, Singla-Pareek SL, Grover A (1995) Immunological evidence for accumulation of two high molecular weight (104 and 90 kDa) HSPs in response to different stresses in rice and in response to high temperature stress in diverse plant genera. Plant Mol Biol**.** 29: 293-301.

Pareek A, Singla-Pareek SL, GroverA (1997) Short term salinity and high temperature stress-associated ultrastructural alterations in young leave cells of *Oryza sativa* L. Annals Bot. 80: 629-639.

Pareek A, Sopory SK, BohnertHJ, Govindjee (2010) Abiotic Stress Adaptation in Plants: Physiolgical, Molecular and Genomic Foundation. Publisher: Springer, The Netherlands.

Park HJ, Jung WY, Lee SS, Song JH, Kwon S-Y, Kim HR, Kim CW, Ahn JC, Cho HS (2013) Use of heat stress responsive gene expression levels for early selection of heat tolerant cabbage (*Brassica oleracea*L.) International JMol Sci. 14: 11871-11894.

Parry MAJ, Madgwick PJ, Bayon C, Tearall k, Lopez AH, Baudo M, Rakszegi M, Hamada W, Al-Yassin A, Ouabbou H, Labhilili M, Phillips AL (2009) Mutation discovery for crop improvement. JExp Bot. 60:2817-2825.

Sakata T, HigashitaniA (2008) Male sterility accompanied with abnormal anther development in plants-genes and environmental stresses with special reference to high temperature injury. Int J Plant Dev Biol. 2: 42-51.

Saleem MY, Mukhtar Z, Cheema AA, Atta BM (2005) Induced mutation and in vitro techniques as a method to induce salt tolerance in Basmati rice (*Oryza sativa* L.). Int J environ Sci Technol. 2: 141-145.

Shah F, Huang J, Cui K, Nie L, Shah T, Chen C, Wang K (2011) Impact of high-temperature stress on rice plant and its traits related to tolerance. J Agr Sci. 149: 545-556.

SinghBD (2000) Plant Breeding-Principles and Methods. Kalyani Publisher. Narosa Publishing House. India.

Soda N, Kushwaha HR, Soni P, Singla-Pareek SL, Pareek A (2013) A suite of new genes defining salinity stress tolerance in seedlings of contrasting rice genotypes. Funct Integr Genomics. 13: 351-365.

Soda N, SharanA, Gupta BK, Singla-Pareek SL, Pareek A **(**2016) Evidence for nuclear interaction of a cytoskeleton protein (OsIFL) with metallothionein and its role in salinity stress tolerance. Sci Rep.6: 34762.

Soni P, Nutan KK, Soda N, Nongpiur RC, Roy S, Singla-Pareek SL, Pareek A (2015) Towards understanding abiotic stress signaling in plants: convergence of genomics, transcriptomics, proteomics and metabolomics approaches. In: Pandey GA (ed) Elucidation of Abiotic Stress Signaling in Plants, Springer, New York, USA. 3-40

Sullivan CY (1972) Mechanisms of heat and drought resistance in grain sorghum and methods of measurement. In: Rao NGP and House LR (eds. Sorghum in The Seventies, pp. 247-264. Oxford and IPH, New Delhi, India.

Tester M, Bacic A (2005) Abiotic stress tolerance in grasses. From model plants to crop plants. Plant Physiol. 137: 791–793.

Wassmann R, Jagadish SVK, Heuer S, Ismail A, Redona E, Serraj R, Singh RK, Howell G, Pathak H, Sumfleth K (2009) Climate Change Affecting Rice Production: The Physiological and Agronomic Basis for Possible Adaptation Strategies. In: Donald LS (ed) Advances in Agronomy, Burlington. 59-122.

Yokoi S, Quintero FJ, Cubero B, Ruiz MT, Bressan RA, Hasegawa PM, Pardo JM (2002) Differential expression and function of *Arabidopsis thaliana* NHX Na^+/H^+ antiporters in the salt stress response. Plant J. 30: 529-539.

Yoshida S, Satake T, Mackill D (1981) High temperature Stress. IRRI Research Paper. 67: 1–15.

Zivy M, Thiellement H, Vienne D, Hofmann JP (1983) Study on nuclear andcytoplasmic genomic expression in wheat by two-dimensional gel electrophoresis. TheorAppl Genet. 66: 1-7.

DOI: 10.21475/ajcs.21.15.09.sp-2

Australian Journal of Crop Science AJCS

Analysis of proline accumulation, antioxidant capacity and HSP expression in mutant rice lines with different heat tolerance

Jian-Zhong Huang[1,2], Shou-Ling Xu[1], Ting-Chen Ma[3], You-Fa Li[4], Hao-Wei Fu[4], Ze-Fu Li[3], Qing-Yao Shu[2]*

[1]Institute of Nuclear Agricultural Sciences, Zhejiang University, Hangzhou 310058, China
[2]National Key Laboratory of Rice Biology, Institute of Crop Sciences, Zhejiang University, Hangzhou 310058, China
[3]Rice Research Institute, Anhui Academy of Agricultural Sciences, Hefei 230031, China
[4]Jiaxing Academy of Agricultural Sciences, Jiaxing314016, Zhejiang, China

***Corresponding author: qyshu@zju.edu.cn**

Abstract

Three mutant rice (*Oryza sativa* L.) lines (AG1, AG2 and AG3) were selected as heat tolerant mutants from a gamma-ray-irradiated population of a heat-susceptible line (AG), based on their floret fertility grown under high temperatures. They were subjected to heat stress treatment (45°C, 22 hrs) at the 5-leaf stage, together with a heat-tolerant cultivar N22 and AG. Analysis of seedling root growth by WinRHIZO scanning revealed that N22 and AG3 were more heat-tolerant than the other lines (AG being the most heat susceptible). Following heat stress, a significantly higher level of oxidative damage, as indicated by TBARS, was observed in AG than in N22 and AG3. The proline accumulation was significantly higher in N22 and AG3（12- to 13.5- fold）than AG (2.5- fold). Similarly, significantly greater increases of total antioxidant capacity (T-AOC) and superoxide dismutase (SOD) activity were observed in N22 and AG3 than in AG. The expression of four heat shock proteins was also investigated using qPCR: *OsHSP16.9*, *OsHSP80.3* and *OsHSP100.9*were induced by heat stress to various levels while *OsHSP72.6*was down-regulated in all tested lines. The heat-induced expression of *OsHSP16.9* and *OsHSP100.9* in N22 and AG3 was about twice that of AG. Higher proline accumulation and expression of the three *HSP* genes, as well as elevation of T-AOC and SOD activity were observed in the heat-tolerant N22 and the mutant line AG3 under heat stress.

Keywords: heat-tolerance; rice; *Oryza sativa*; proline accumulation; antioxidant system; heat-shock protein.
Abbreviations: HSPs_heat shock proteins; POD_peroxidase; SOD, superoxide dismutase; ROS, reactive oxygen species; T-AOC, total antioxidant capacity; TBARS, thiobarbituric acid reactive substances.

Introduction

Rice (*Oryza sativa* L.) is a staple food crop and a principal calorie source for Asian people (Khush, 2001). The optimal temperature for rice grain formation and yield is around 25°C, and pollen viability and production begin to decline as daily maximum temperatures exceeds 33°Cand completely fail at 40°C (Luo, 2011); temperatures exceeding 35°C can cause a high percentage of spikelet sterility (Yoshida, 1981). The irreversible damage caused by high temperatures to seedling growth, leaf photosynthesis, and spikelet fertility contribute to yield reduction in rice. Under global warming scenarios, creating new rice cultivars (or germplasm for breeding) provides a sustainable solution to reduce the risk of high temperatures on world food security.

Overwhelming evidence indicates that heat stress is accompanied by increased levels of reactive oxygen species (ROS), which can cause oxidative damage to the plant cells. Antioxidant capacity of a plant, therefore, can be important in the tolerance of heat stress (Suzuki and Mittler, 2006; Kotak et al., 2007; Nouri and Komatsu, 2013). As an adaptive mechanism, plants grown under high temperature often accumulate osmo-protectants such as proline and soluble sugars (Wahid et al., 2007). Proline is believed to be beneficial to protect cellular structures from damage and maintain function under extreme temperatures (Bita and Gerats, 2013). In addition, cells from virtually all organisms respond to heat stress by the rapid synthesis of heat shock proteins (HSPs). In higher plants, HSPs are induced under heat shock at any stage of development and major HSPs are highly conserved among distinct organisms (Vierling, 1991). HSPs belong to multigene families encoding molecular chaperones involved in various processes including maintenance of protein homeostasis as a requisite for optimal development and survival under stress conditions. These proteins play important roles in the development of thermotolerance and protection from cellular damage associated with stress in higher plants (Wahid et al., 2007). Over-expression of heat shock proteins in rice plants has been shown to increase thermotolerance (Murakami et al., 2004; Fragkostefanakis et al., 2015; Wang et al., 2015a).

In this study, we mutated a heat-susceptible line (Fu 296) with gamma ray irradiation and identified three mutant lines (AG1, AG2 and AG3) with enhanced floret fertility when grown under high temperatures. To investigate the physiological and molecular basis of increased thermotolerance of the rice mutants, i.e., AG1, AG2 and AG3, we analyzed the levels and activities of antioxidants, oxidative stress (TBARS), proline accumulation and the expression of four heat-shock proteins under heat stress. The results were compared to plant performance in terms of shoot and root growth, especially seedling root growth which provided a good indicator of heat stress tolerance.

The results suggested that higher proline accumulation and HSP expression as well as the elevated T-AOC and SOD activity under heat stress may have contributed to the increased thermotolerance of the mutant lines.

Results

Plant growth under heat stress

After heat treatment at 45°C and 60% humidity for 22 hours, slight but insignificant decrease in plant height was observed in N22 and all tested rice lines at the leaf 5 stage (Fig. 1a). However, root growth was more affected, with significantly reduced total root length (-9%, Fig. 1b), reduced average root diameter (-20%, Fig. 1c) and reduced total root surface area (data not shown) in the relatively heat susceptible progenitor line AG, whereas the decreases in the heat-tolerant cultivar N22 and mutant line AG3 were insignificant (Fig. 1b, 1c). Significant declines were also observed in AG1 (-5% and -14% for total root length and root average diameter, respectively) (Fig. 1b, 1c) and in AG2 (-9% of root average diameter) (Fig. 1c).

Changes in seedling root parameters under heat stress indicated that N22 and AG3 were more heat-tolerant than AG, AG1 and AG2, consistent with observations of their fertility performance in paddy field. However, heat-inhibited growth in the relatively heat susceptible line AG was indistinguishable from that of AG1, which showed improved heat-tolerance in terms of fertility. This was probably due to the insufficient time following heat stress for plants to manifest the difference, both in the case of shoot (Fig. 1a) and root growth (Fig. 1b, 1c).

Oxidative damage in rice lines with different thermotolerance

High levels of TBARS indicate high levels of oxidative damage and often the heat–susceptibility of plants (Larkindale et al., 2005). Consistent with this, the heat-tolerant cultivar N22 and the relatively heat-tolerant line AG3 had relatively lower levels (150-180% of control) of TBARS following heat stress. By contrast, the heat-susceptible progenitor line AG had the highest level (270% of control) of TBARS. TBARS levels of the moderate heat-tolerant lines AG2 and AG3 were also intermediate (210-220% of control; Fig. 1d).

Proline levels and antioxidant activities following heat stress

Afterheat stress, proline content in leaves was significantly increased in all tested lines (Fig. 2a). The heat-induced proline increases in N22 and AG3 was about 12- to 13.5- fold, significantly higher than those of the three other less heat-tolerant lines (2.5- to 7.5) (Fig. 3a).

T-AOC levels were also increased significantly after heat stress. The heat-induced increase of T-AOC was much higher in N22 (2.4-fold) and AG3 (1.9-fold) than in the other three lines (1.3- to 1.7- fold) (Fig. 2 b).

Following heat stress, POD activity declined in the test lines except in the AG (Fig. 2c). However, the activity of SOD was significantly increased in N22 and AG3 exposed to heat stress and was significantly lower in the heat-susceptible line AG (Fig. 2d).

Expression of four heat-shock protein genes

To examine the heat-induced response of molecular chaperones, the expression of four *HSP* genes encoding, respectively, small HSP(OsHSP16.9), HSP70 (OsHSP72.6), HSP90(OsHSP80.3) and HSP100(OsHSP100.9), were analyzed (Table 1). Expression of the three of *HSP* genes, i.e., *OsHSP16.9*, *OsHSP80.3*and *OsHSP100.9*, was increased by 1.5- to 2.9-, 1.3- to 1.9- and 11- to 22- folds respectively, while that of *OsHSP72.6* decreased by 1.1- to 1.7- folds following heat stress (Fig. 3).

The largest increase of *OsHSP16.9* expression occurred in the heat tolerant rice cultivarN22 (about 2.9- fold), while the least in the heat-susceptible line AG (about 1.5-fold). The expression levels of *OsHSP16.9* in the relatively heat-tolerant line AG3 and moderate tolerant lines AG1 and AG2 were increased by 2.5-, 2.1- and 2.4-fold, respectively (Fig. 3). The most dramatic increase was seen in the expression of *OsHSP100.9*with 22-fold increase in N22 and AG3, 15- to 17-fold in AG1 and AG2, and 11-fold in AG (Fig. 3).

Discussion

Global warming has been evident throughout the world in the past few decades with an increased frequency of occurrence of extreme temperatures in increasing land areas (Hansen et al., 2012). Development of rice cultivars which could withstand high temperatures will be achieved in part through understanding the molecular basis of heat stress responses in this crop.

At the cellular level, high temperatures can cause perturbations in membrane-linked processes due to increased fluidity of membrane lipids and alteration of permeability (Bischof et al., 1995). In addition, heat-induced protein denaturation and aggregation can cause enzyme inactivation. Membrane and protein damage lead to metabolic imbalances and the production of

toxic compounds and ROS (Wahid et al., 2007).

Compatible low molecular osmolytes such as proline and soluble sugars accumulate under heat stress (Wahid et al., 2007). Although Lv et al. (2011) found that growth of the proline-overproduced Arabidopsis seedlings were inhibited during heat stress, other reports

showed that proline accumulation is necessary to regulate osmotic activities and protect cellular structures from increased temperatures by maintaining the cell water balance, membrane stability, and by buffering the cellular redox potential (Dobra et al., 2010; Bita and Gerats, 2013). Field trials with proline-overexpressed soybean lines showed improved drought performance and higher heat tolerance compared to wild type cultivars (de Ronde et al., 2004; Verbruggen and Hermans, 2008). It is likely that the effects of proline accumulation in plants can be species- and organ- specific. This is in accordance with our finding in that the heat-tolerant rice cultivar N22 and the relatively heat-tolerant mutant line AG3 had the highest level of proline among the tested rice lines exposed to heat stress. Multiple signaling pathways are implicated in response to heat-stress. Protection of plant from the oxidative damage by elevation of antioxidant activity in stressed tissues is an important component of a plant under heat stress (Kotak et al., 2007). In general, the T-AOC reflects the enzyme and non-enzyme antioxidant system as a whole. SOD among others is a major antioxidant enzyme in plants. They are responsible for the removal of free radicals such as $O^{2\bullet-}$, thus reducing the injury caused by lipid oxidation of cell membrane (Moller et al., 2007). The higher levels of T-AOC and SOD activity in N22 and AG3 can be important to their heat-tolerance. Although most HSPs are induced to be synthesized by heat stress, *OsHSP* genes exhibited very diverse expression patterns in different rice tissues (Vierling, 1991; Ye et al., 2012). Large variations exist in the literature with respect to the expression levels of specific *OsHSP* genes. For instance, while Ye et al. (2012) found that*Loc_Os11g47760* (HSP70 family) in seedling of rice cv. IRAT109 was upregulated 6- fold under 42°C for 12h.

Table 1. Primer sequences used for qPCR.

Gene	Family; predicted MW*	Primer sequences	Size (bp)
LOC_Os01g04370	sHSP; 16.9 kDa	qHSP1-F: AAGATAGAGCAACCATGTCG qHSP1-R: CTTCACCTCCTCCTTCTTGA	221
LOC_Os03g02260	HSP70; 72.6 kD	qHSP2-F: GCTGACAACCAGACGCAAGT qHSP2-R: CGATCTCGGAGGCAACCT	409
LOC_Os04g01740	HSP90; 80.3 kD	qHSP3-F: AGCAGTGAACTGAAATGGCG qHSP3-R: GAGAGAGTCTTGGAGGGCTT	268
LOC_Os05g44340	HSP100; 100.9 kD	qHSP4-F: GTCATGCAGGAGGTGAGGAG qHSP4-R: TCGATGTACACCGTGCAGTT	320

*Abbreviations (using MW) for the four HSP genes are *HSP16.9*, *HSP72.6*, *HSP80.3* and *HSP100.9*, respectively.

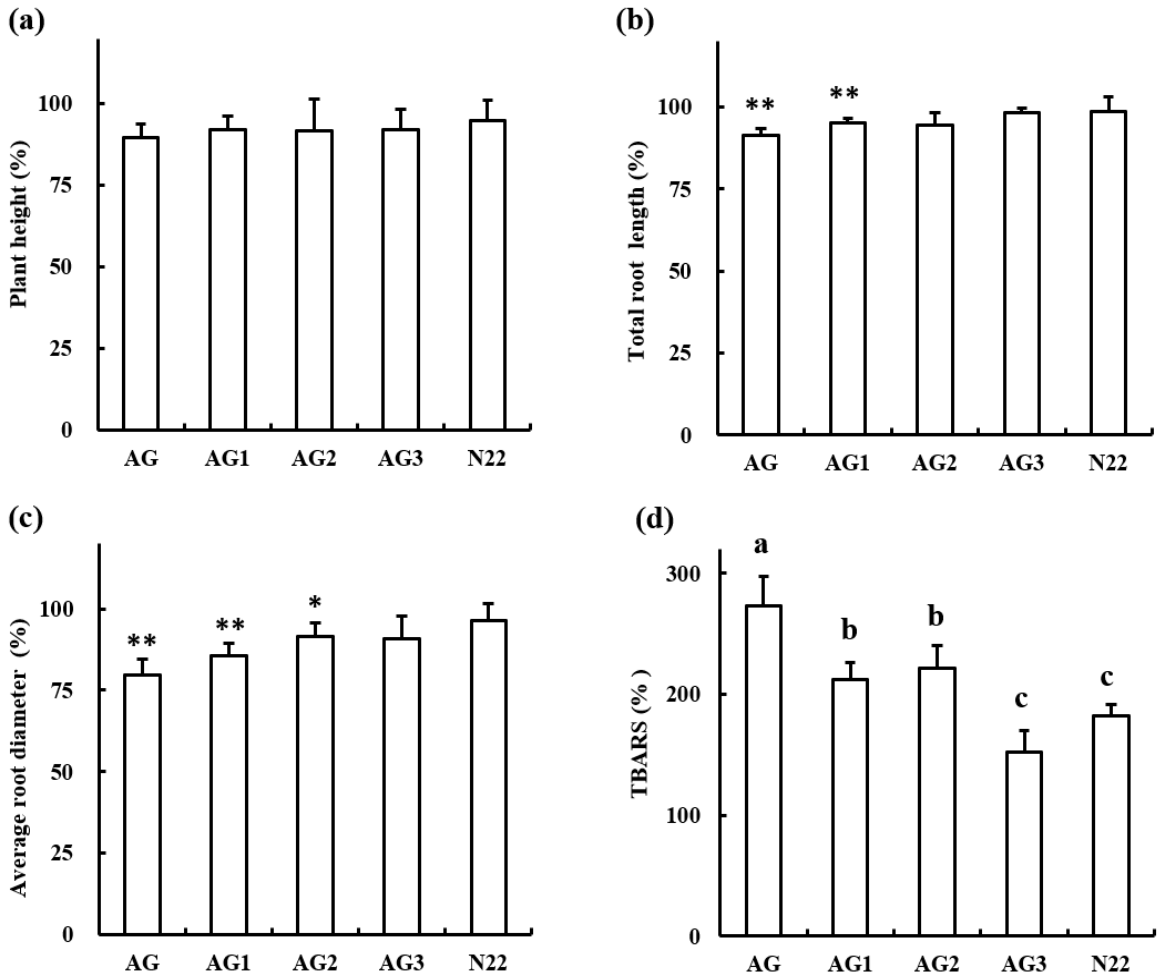

Fig 1. Effect of high temperature (45°C, 22 hrs) on the growth and oxidative damage in rice seedlings. Plant height (a), total root length (b) and average root diameter (c) were relative to those of the same linegrown without heat stress. (a) plant height; (b) total root length; (c) average root diameter; (d) TBARS levels. Root parameters were obtained using WinRhizo root image analysis system. (a, b, c) Bars with * indicate significant difference compared to respective control at P≤0.05 and ** at P≤0.001; (d) Bars with the same lower-case letter are not significantly different at P ≤ 0.05. Statistical significance was determined by using the Student's t-test. Experiments were performed in triplicate, each with 12 seedlings per line harvested from the same container.

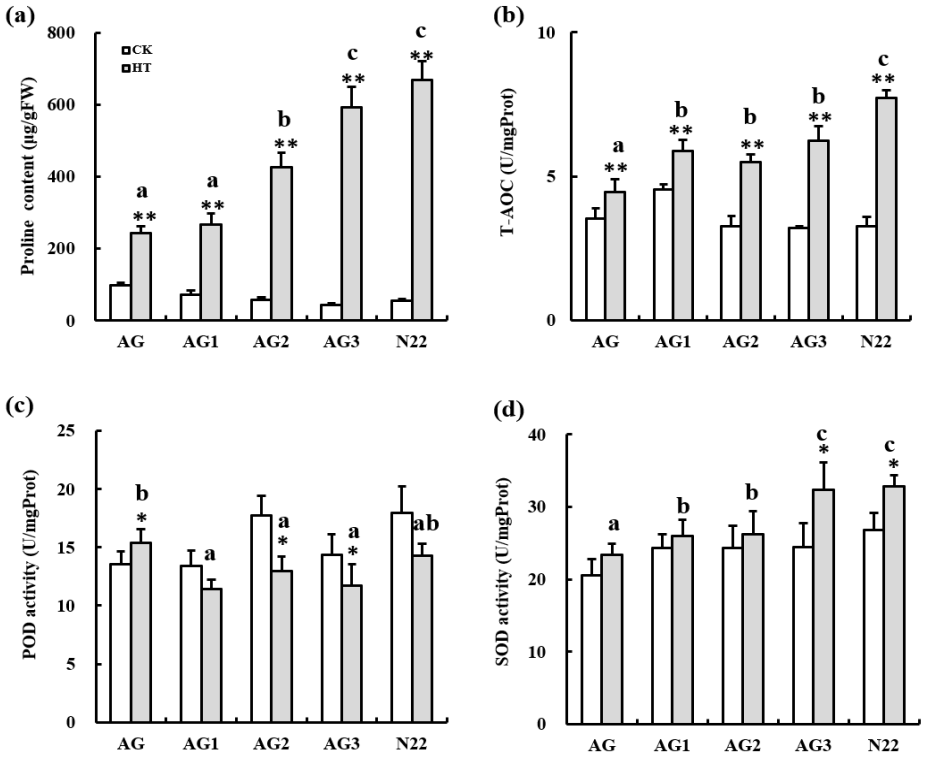

Fig 2. Leaf proline and T-AOC levels and activities of antioxidant enzymes (POD and SOD) in rice seedlings with different thermotolerance before (white bars) and after heat stress (grey bars). Bars with * indicate significant difference with respective control at P≤0.05 and ** at P≤0.001. Bars with the same lower-case letter are not significantly different at P ≤ 0.05. Experiments were performed in triplicate, each with 12 seedlings per line harvested from the same container.

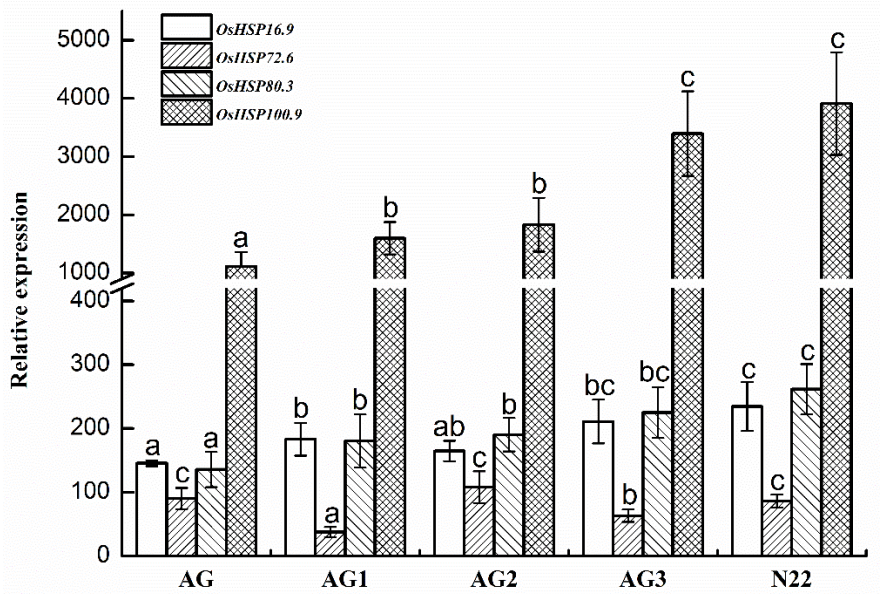

Fig 3. Expression of four HSP genes in rice seedlings with different thermotolerance following heat stress. Total RNA was extracted from rice leaf tissue at the 5-leaf stage from 12 seedlings per line harvested from the same container. The experiment was done in triplicates. Expression level of the progenitor parental line AG without heat stress was set as 100%. A. *OsHSP16.9*(LOC_Os01g04370); B. *OsHSP72.6* (LOC_Os03g02260); C. *OsHSP80.3* (LOC_Os04g01740); D. *OsHSP100.9* (LOC_Os05g44340). Bars with * indicate significant difference with control at P≤0.05 and ** at P≤0.001. Bars with the same lower-case letter are not significantly different at P ≤ 0.05.

Hu et al. (2009) found it downregulated in rice cv. ZH11. Similar contradictory results were obtained regarding the expression of *LOC_Os01g08860* (sHSP family) under heat stress by different research groups.

The expression of this gene was found to be up-regulated by heat treatment in rice seedlings from > 4-fold (Hu et al., 2009), >8-fold (Jung and An, 2012) and even to >227-fold (Ye et al., 2012). However, it was not detected under 25°C, 37°C and 42 °C by Ouyang et al. (2009).

Using proteomics, Jagadish et al. (2011) found that heat shock proteins were significantly upregulated under heat stress in the highly heat-tolerant cultivar N22 during rice anthesis. The high expression levels of *OsHSP16.9*, *OsHSP80.3* and *OsHSP100.9*in N22 following heat stress were well correlated with the high heat tolerance of this rice variety. The expression levels of these three *HSPs* in the relatively heat tolerant mutant line AG3 were also upregulated to similar level as those of N22. On the other hand, the relatively heat-susceptible line AG exhibited the least increase in all these genes among the tested rice lines.

The ability to withstand and to acclimate to supra-optimal temperatures can involve numerous traits at morphological, physiological and molecular levels. Development and maintenance of thermotolerance in plants will have to do with the antioxidant activity, membrane lipid unsaturation, gene expression and translation, protein stability, and accumulation of compatible solutes (Larkindale et al., 2005; Bita and Gerats, 2013). Constitutive elevation of osmolytes and antioxidants seems to be happening under non-stress conditions, which is potentially useful to plant growth; Although an episode of high temperature at an earlier developmental stage may not directly affect the heat response of a subsequent stage (Wollenweber et al., 2003), it is likely that the response of the osmolyte and antioxidant mechanisms at seedling stage happens at later growth as well, although not examined in the current experiments. Therefore, overall elevation of antioxidant capacity and membrane stabilization and protein protection by compatible osmolytes and molecular chaperones are certainly beneficial to both vegetative and reproductive stages in amelioration of heat-induced injury in plants.

Materials and methods

Plant materials

For generation of heat tolerant mutants, rice seeds of cv. Fu 296 were irradiated with γ rays (350 Gy) and their progenies were subjected to screening for improved heat tolerance from M_2 to M_4 in paddy fields in the summers of 2012 to 2015 in Hangzhou, China (30.42°N, 120.12°E). Fu 296 is an early inbred indica rice cultivar, developed through the pedigree breeding from γ ray-irradiation of F_1 dry seeds Z96-03 × Guangchangzhan. Improved traits include: erect leaves, sturdy stems, large panicle and a higher filled seed-set, higher and stable grain yield. It is about 95 cm of height and requires ~111 d from sowing to harvest. The cultivar was registered in Hunan Province in China in 2008 (Liu et al., 2010). Only mutant lines showing the same phenotype with the cultivar's characteristic traits were selected from the γ -irradiated progenies of Fu296. A number of mutant lines showing improved heat tolerance were selected at anthesis with increased fertilities at natural high temperatures. We selected three mutant lines (AG1, AG2 and AG3) with different thermotolerance for the following experiment: AG3 was more tolerant than AG1 and AG2, which were in turn more tolerant than the progenitor parental line Fu296 (hereafter AG) (Supplemental Table 1).

Heat treatment

Rice seedlings (M_5) consisting of three mutant lines and their progenitor, and a reference heat-tolerant cultivar (N22; Yoshida et al., 1981; Jagadish et al., 2011), were raised in soil culture in plastic containers (Wang et al., 2009). Twelve plants per line and four lines each container was grown in a container (length/wide/depth: 60/40/25cm) with water logged paddy soil. Containers were maintained at field saturation capacity and irrigation was done when required. At 5-leaf stage, part of one-month old seedlings was subjected to heat stress by incubation in controlled growth chambers at 45°C and 60% humidity for 22 hours and a 16 h photoperiod under 160 □ mol m^{-2} s^{-1} light. The remaining seedlings of each line or cultivar were kept at ~30°C as controls. Upon the completion of stress treatment, the fifth leaf of each plant was cut for immediate assays of antioxidants, or frozen in liquid nitrogen and stored under -70°C until required for further investigation. Experiments were performed in triplicate, each with 12 seedlings per line harvested from the same container.

Plant growth measurement

Before and immediately following heat treatment, six plants from each rice line were uprooted carefully from the soil. Plant height was measured, and seedling roots taken by cutting off the culms. Root samples were then washed carefully on a 0.5-mm mesh screen. After removal of debris, roots were spread out on a glass plate and scanned using the root scanner WinRHIZO system (WinRHIZO, Regent Instrument, Montreal, QC, Canada). Total root length, total root surface area and average root diameter were then obtained.

Proline, TBARS and antioxidants assay

Leaf samples were homogenized using a chilled mortar and pestle with ice-cold extraction buffer supplied with the kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China). Homogenate was centrifuged at 10,000 ×*g* for 10 min at 4°C. The supernatant was used for measurement of thiobarbituric acid reactive substances (TBARS), proline, total antioxidant capacity (T-AOC), soluble protein, peroxidase and superoxide dismutase (SOD) using corresponding assay kits. All reagents used were provided with the supplier's kits.

Heat-shock protein expression

For quantitative PCR (qPCR) analysis, total RNA was extracted from rice leaf tissue at the 5-leaf stage using the Qiagen Spin Plant RNA Mini Kit (Qiagen, Germany). cDNAs were reverse transcribed from 100ng total RNA using the oligo-dT_{18} primer and GoScript™ Reverse Transcription System Kit (Promega, USA) according to the manufacturer's instructions. qPCRs were performed using a SYBR Green GoTaq® qPCR Master Mix kit (Promega, USA). One primer pair each was designed for qPCR analysis of the four*HSP*genes (qHSP1–4, Table 1). All qPCRs were performed in 20 μL volumes with 1μL cDNA in 1× GoTaq® qPCR Master Mix (containing PCR buffer, $MgCl_2$, dNTPs, Taq DNA polymerase), and 0.4 μM of each primer. The following programme was used for all qPCRs: 10 min at 95 °C, 40 cycles of 30 s at 95 °C, 30 s at 55 °C, and 60 s at 72 °C. The rice ubiquitin gene was used as an internal reference (primersUbq-F, 5'-GCTCCGTGGCGGTATCAT-3'; Ubq-R, 5'-CGGCAGTTGACAGCCCTAG-3'; Wang et al., 2015b). Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$method (Livak and Schmittgen, 2001). qPCRs were repeated with three biological replicates, each with RNA extracted from two individual seedlings.

Conclusions

Heat stress treatment at the 5-leaf stage demonstrated that the mutant line AG3 had increased heat tolerance compared with its heat-susceptible parental line Fu 296. Higher proline accumulation and expression of *OsHSP16.9* and *OsHSP100.9*, as well as elevated T-AOC and SOD activity might constitute part of the mechanisms that underpin its increased thermotolerance of AG3. The mutant line could be used for breeding heat tolerant rice cultivars in future breeding programmes.

Acknowledgements

This work was supported by the FAO/IAEA Coordinated Research Project CRP 23029, Research Contract No16818.

References

Ahloowalia BS, Maluszynski M (2001) Induced mutations – a new paradigm in plant breeding. Euphytica. 118:167–173.

Bischof JC, Padanilam J, Holmes WH, et al (1995) Dynamics of cell membrane permeability changes at supraphysiological temperatures. Biophys J. 68: 2608–2614.

Bita CE, Gerats T (2013) Plant tolerance to high temperature in a changing environment: scientific fundamentals and production of heat stress-tolerant crops. Front Plant Sci. 4: 273.

de RondeJA, Laurie RN, Caetano T, et al (2004) Comparative study between transgenic and non-transgenic soybean lines proved transgenic lines to be more drought tolerant. Euphytica. 138: 123–132.

Dobra J, Motyka V, Dobrev P, et al (2010) Comparison of hormonal responses to heat, drought and combined stress in tobacco plants with elevated proline content. Plant Physiol. 167: 1360–1370.

Fragkostefanakis S, Röth S, Schleiff E, Scharf KD (2015) Prospects of engineering thermotolerance in crops through modulation of heat stress transcription factor and heat shock protein networks. Plant Cell Environ.38: 1881–1895.

Hansen J, Sato M, Ruedy R (2012) Perception of climate change. P Natl Acad Sci USA.109: E2415–E2423.

Hu W, Hu G, Han B (2009) Genome-wide survey and expression profiling of heat shock proteins and heat shock factors revealed overlapped and stress specific response under abiotic stresses in rice. Plant Sci.176: 583–590.

Jagadish SVK, Muthurajan R, Rang ZW, et al (2011) Spikelet proteomic response to combined water deficit and heat stress in rice (*Oryza sativa* cv. N22). Rice.4: 1–11.

Jung KH, An G (2012) Application of MapMan and RiceNet drives systematic analyses of the early heat stress transcriptome in rice seedlings. Plant Biol.55: 436–449.

Khush GS (2001) Green revolution: the way forward. Nat Rev Genet.2: 815–822.

Kotak S, Larkindale J, Lee U, et al (2007) Complexity of the heat stress response in plants. Curr Opin Plant Biol.10: 310–316.

Larkindale J, Hall JD, Knight MR, Vierling E (2005) Heat stress phenotypes of Arabidopsis mutants implicate multiple signaling pathways in the acquisition of thermotolerance. Plant Physiol.138: 882–897.

Liu Q, Yan W, Yu F, Qi Y, Wu G, Jin W, Jin Q (2010) Breeding of 'Fu 296'rice with high yield and good quality by ^{60}Coγ-rays irradiaton dry seeds of hybrid F1. J Nucl Agr Sci. 24**:** 744–747.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods. 25: 402–408.

Luo Q (2011) Temperature thresholds and crop production: a review. Climatic Change.109: 583–598.

Lv WT, Lin B, Zhang M, Hua XJ (2011) Proline accumulation is inhibitory to Arabidopsis seedlings during heat stress. Plant Physiol.156: 1921–1933.

Moller IM, Jensen PE, Hansson A (2007) Oxidative modifications to cellular components in plants. Annu Rev Plant Biol.58: 459–481.

Murakami T, Matsuba S, Funatsuki H, et al (2004) Over-expression of a small heat shock protein, sHSP17.7, confers both heat tolerance and UV-B resistance to rice plants. Mol Breeding.13: 165–175.

Nouri MZ, Komatsu S (2013) Subcellular protein overexpression to develop abiotic stress tolerant plants. Front Plant Sci.4: 2.

Ouyang Y, Chen J, Xie W, Wang L, Zhang Q (2009) Comprehensive sequence and expression profile analysis of Hsp20 gene family in rice. Plant Mol Biol.70: 341–357.

Suzuki N, Mittler R (2006) Reactive oxygen species and temperature stresses: a delicate balance between signaling and destruction. Physiol Plant. 126: 45–51.

Verbruggen N, Hermans C (2008) Proline accumulation in plants: a review. Amino Acids.35: 753–759.

Vierling E (1991) The roles of heat-shock proteins in plants. Annu Rev Plant Physiol Plant Mol Biol.42: 579–620.

Wahid A, Gelani S, Ashraf M, Foolad MR (2007) Heat tolerance in plants: an overview. Environ Exp Bot.61: 199–223.

Wang A, Yu X, Mao Y, et al(2015a) Overexpression of a small heat-shock-protein gene enhances tolerance to abiotic stresses in rice. Plant Breeding.134: 384–393.

Wang C, Zhang X, Fan Y, et al(2015b) XA23 is an executor R protein and confers broad-spectrum disease resistance in rice. Mol Plant.8: 290–302.

Wang H, Huang J, Ye Q, Wu D, Chen Z (2009) Modified accumulation of selected heavy metals in Bt transgenic rice. J Environ Sci.21: 1607–1612.

Wollenweber B, Porter JR, Schellberg J (2003) Lack of interaction between extreme high-temperature events at vegetative and reproductive growth stages in wheat. J Agron Crop Sci. 189: 142–150.

Ye S, Yu S, Shu L, Wu J, Wu A, Luo L (2012) Expression profile analysis of 9 heat shock protein genes throughout the life cycle and under abiotic stress in rice. Chin Sci Bull.57: 336–343.

Yoshida S (1981) Fundamentals of rice crop science. IRRI, Los Baños, 269.

Yoshida S, Satake T, Mackill DS (1981) High temperature stress in rice. IRRI Res Pap Ser 67.

DOI: 10.21475/ajcs.21.15.09.sp-3

Australian Journal of Crop Science AJCS

Identification of small open reading frames (sORFs) associated with heat tolerance in nitrogen-fixing root nodules of *Phaseolus vulgaris* wild-type and cv BAT93

Alejandra Zayas-del Moral[1], Damián Martínez-Reyes[2], Carmen Quinto[1], Federico Sanchez† and Claudia Díaz-Camino[1]*.

[1]Departamento de Biología Molecular de Plantas Instituto de Biotecnología, Universidad Nacional Autónoma de México Av. Universidad 2001, CP 62210, Cuernavaca, Morelos, México
[2]Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México AP 565A Cuernavaca, Morelos, México
† In loving memory.

***Corresponding author: claudia@ibt.unam.mx**

Abstract

Common bean is an important legume crop and a major source of protein for low-income groups around the world. Legumes have the ability to engage symbiotic interactions with nitrogen-fixing soil bacteria. In this study, next-generation sequencing technology was used to perform transcriptome analyses of a yet unexplored group of peptides encoded by small open reading frames (sORFs; < 150 codons) in nitrogen-fixing symbiotic nodules of two heat-tolerant genotypes of common bean (*Phaseolus vulgaris* L): the cultivar BAT93 and a wild genotype (named *P. vulgaris* 7) from the south of Mexico. After heat stress, total RNA was isolated and used for transcriptome analysis. Sixty differentially expressed sORFs were identified between control and heat stress treatments. The expression profiles of these sORFs suggest that, regardless the evolutionary closeness between *P. vulgaris* BAT93 and *P. vulgaris* 7, each genotype has independently adapted their molecular signaling pathways to survive heat stress. The dataset developed may provide a useful resource for future genetic and genomic studies in these species.

Keywords: Heat stress, small open reading-frames, common bean, legume-rhizobia symbiosis, biological nitrogen-fixation, next-generation sequencing, transcriptome analysis.
Abbreviations: sORFs - short open reading frames, SPs - small proteins.

Introduction

The world´s human population is expected to reach 9.1 billion in 2050 (Food and Agriculture Organization of the United Nations, http://www.fao.org/). Over-population is associated with increasing global consumption of resources, food security and climate change. Recent climate models estimate that the global surface temperature is likely to rise by 4.8 °C in the worst-case scenario (IPCC, 2014). In semi-arid and tropical regions, which are among the most populated and under-developed, the increase in surface temperatures will severely affect crop production (IPCC, 2014). Legumes include important grain, pasture, and agroforestry species, and are second to cereal crops in agricultural importance based on area harvested and total production (http://www.fao.org/). Pulses (dry seeded legumes) are protein rich and affordable foods, and an important component in human sustenance, especially in the dietary pattern of low-income people in developing countries. In addition to their nutritional value, most legumes have symbiotic associations with nitrogen-fixing bacteria mainly belonging to the Rhizobiaceae family (rhizobia) (Dénarié et al., 1996). This remarkable biological interaction culminates with the formation of specialized root organs, the symbiotic nodules, where biological fixation of atmospheric nitrogen takes place. Nodulated legumes produce substantial amounts of organic nitrogen fertilizer and play a key role in sustainable agriculture in tropical and temperate climates (Peoples et al., 1995; Tate, 1995). Numerous studies have established that high temperatures (30°C to 40°C depending on species) have negative impacts on Rhizobium soil survival, root bacterial attraction and infection, and also nodule development (Lebrazi & Fikri Benbrahim, 2014; Abd-Alla et al., 2014). Knowledge of nodule functioning after heat stress, such as those experienced by legume plants in the field during a day is limited.

Small proteins (SPs) have emerged as an important class of signaling molecules involved in nodulation (Batut et al., 2011), and also in growth, development, and in response to stress (Hanada et al., 2012; Marmiroli & Maestri, 2014). SPs are encoded by short open reading frames (sORFs) and distinguished from other ORFs by their sizes (30-150 codons in length). Although many sORFs play important roles as regulators of diverse biological processes, this gene group usually escapes gene annotation because they are particularly difficult to predict by computational biology due to their small size. Thus, sORFs have been studied in only a few plant species and their biological importance is little understood. Here a comparative analysis was made of the expression of sORFs of the root-nodule transcriptome of two *P. vulgaris* genotypes under control and stress treatments (sudden and prolonged heat exposure). Computational strategies were deployed to identify sORFs that were up-regulated in active nitrogen-fixing nodules under heat stress. This information may be relevant in selecting new bean genotypes able to harbour active nitrogen-fixing nodules resilient to heat stress.

Results

Phenotypic and molecular responses to heat stress in P. vulgaris heat-stress resistant genotypes

To evaluate the ability of *P. vulgaris* cv. BAT93 and *P. vulgaris* 7 to adapt to sudden heat stress (without any priming, known as basal thermo-tolerance), the plants of each genotype were subjected to heat stress for 6 continuous hours. After stress, the aerial plant parts of *P. vulgaris* BAT93 and *P. vulgaris* 7 were photographed (Figure 1a and 1b). *P. vulgaris* BAT93 wilted (Fig 1a), and only a third of the plants subjected to heat were able to recover after one week in benign conditions. Recovered plants of BAT93 just produced one pod, in general with 1 seed (Table 1). In contrast *P. vulgaris* 7 showed no differences in foliar turgor nor seed production between control and stress treatments (Table 1). There were no differences in nodule size and number of nodules per root plant (Figure 1e), but the results show a significant and similar increase in chaperone transcript accumulation (Figure 1c), an indication that nodules of both genotypes responded to heat stress. Interestingly, the rate of nitrogen-fixation in nodules elicited by *R. tropici* CIAT899 in *P. vulgaris* BAT93 was severely reduced in heat-shock treated plants compared to control, but this effect was not observed in nodules of the wild *P. vulgaris* 7 (Figure 1 d). The data indicate that the basal thermo-tolerance of *P. vulgaris* 7 is higher than *P. vulgaris* BAT93, and that the biological nitrogen fixation process is not altered in *P. vulgaris* 7 by heat stress.

Distribution of sORFs in P. vulgaris BAT93 and G19833 genotypes and in other model legumes

Some 64,692 and 31,638 ORFs from *P. vulgaris* genotypes Mesoamerican BAT93 and Andean G19833, respectively (Vlasova et al., 2016, Schmutz et al., 2014), 88,647 ORFs from *Glycine max* (Schmutz et al., 2010), 10,979 ORFs from *Lotus japonicus* (Sato et al., 2008) and 62,319 ORFs from *Medicago truncatula* (Young et al., 2011) were collected from Phytozome version 11 (www.phytozome.net; Goodstein et al., 2012) and from miyakogusa.jp version 3.0 for *L. japonicus* (http://www.kazusa.or.jp/lotus). The ratio of sORFs (30 to 150 amino acids length) *versus* the total number of ORFs reported for each genome version was calculated (Figure 2 and Table 2). Although the annotations of total ORFs have changed in recent years in all the genomes of listed legumes (Guillén et al., 2013), the highest frequency of sORFs was found in the best-studied genomes of leguminous plants, i.e. *M. truncatula* and *L. japonicus* (0.2 and 0.3, respectively), while the proportion of sORFs/ORFs annotated in the *P. vulgaris* genomes fluctuates between 0.11 to 0.14, a slight difference that may be due to annotation systems used in these genomes.

Differential expression analysis of sORFs under heat-shock conditions in P. vulgaris

Out of 235 differentially expressed ORFs in *P. vulgaris* BAT93 under heat-stress (data not shown), 16 (6.8%) were sORFs. Most differentially expressed sORFs could not be assigned to any gene ontology (GO) category (Table 3 and 4), so these were analyzed by the MEME Suite (Figure S1), and also by BLASTP, which was found to be the most informative algorithm. In stressed root nodules of *P. vulgaris* BAT93, a histone and a thymidine kinase domain are present in three down-regulated sORFs (Figure 3 and Table 3). Five sORFs in *P. vulgaris* BAT93 nodules under heat-stress were up-regulated, andwe identified known protein domains in two of them (Figure 3a and Table 3): a domain found in SL33 plant splicing factors (PHASIBEAM10F006374), and a cytocrome-c oxidase domain (PHASIBEAM10F012744). In *P. vulgaris* 7 significant expression changes were detected in 1,064 ORFs, and 44 (4.1%) of them were identified as sORFs. A GO associated function could be annotated in 26 sORFs (Table 4), but in this case, neither the use of MEME (Figure S1), nor the BLASTP algorithm gave additional information over the putative biological function of some other sORFs of this group (Table 4). In heat-stressed nodules of *P. vulgaris* 7, 13 sORFs were down-regulated (Figure 3 b and Table 4). Most protein domains of these sORFs are yet unknown, or belong to proteins with no described biological function. However, some protein domains found in stress-related proteins were identified (Phvul.008G112900.1, Phvul.008G189400.1, Phvul.009G027600.1.) in a growth factor (Phvul.003G233400.1) (Yang et al., 2001), cytochrome b5 (Phvul.006G115900.1) and in proteins responsive to gibberellic acid (Phvul.008G235300.1), respectively. Up-regulated sORFs in these root nodules included calmodulin-like domains present in proteins involved in the signaling of calcium (Phvul.001G155400.1, Phvul.001G260700.1, Phvul.003G115800.1, Phvul.007G111200.1, Phvul.007G278900.1), and in phyto-hormone responsive proteins, such as ethylene, auxin or gibberellin (Phvul.007G193400.1, Phvul.007G219700.1, Phvul.009G015900.1, Phvul.010G019700.1).

Discussion

Small proteins encoded by small open reading frames (sORFs, 30 to 150 codons) have been shown to be relevant in legume-rhizobia interactions as well as in plant growth and development, and in response to stress (Batut et al., 2011; Hanada et al., 2012; Marmiroli & Maestri, 2014). sORFs identification can be predicted by bioinformatics approaches, such as web-based tools [sORFfinder (Hanada et al., 2010), HAltORF (Vanderperre et al., 2012), or uPEPperoni (Skarshewski et al., 2014)] by homology with other related-species, or by sequence analysis and clustering. Several molecular techniques are used to confirm sORFs gene expression, among these next-generation-sequencing technologies are reliable, e.g. RNA-seq. In this work, RNA-seq technology was used to gather relevant data on changes in gene expression of small proteins encoded by sORFs in root nodules of two *P. vulgaris* genotypes elicited by *R. tropici* CIAT899, a bacterium resistant to heat (Martínez-Romero et al. 1991), under prolonged heat stress conditions.

P. vulgaris BAT93, a representative cultivar of the Mesoamerican common bean gene pool, was bred for high productivity in tropical conditions at the Centro Internacional de Agricultura Tropical (CIAT), Colombia (Voysest, 1983, 2000). This breeding line has been well-studied, and its genome has been recently sequenced (Vlasova et al., 2016). Taken in consideration all these advantages, *P. vulgaris* BAT93 was chosen as the reference genotype to compare with *P. vulgaris* 7, which is a wild-type genotype collected from the south of México. Plant responses to heat stress in both common bean genotypes were confirmed by the strong induction of heat-shock proteins (HSps) (Figure 1 c) (Wang et al., 2004, Aparicio et al., 2005, Larkindale et al., 2005; Kim et al., 2011). Interestingly, although the induction of HSPs was similar in both common bean genotypes (Figure 1c), deleterious phenotypic effects at the whole plant level were observed only in *P. vulgaris* BAT93 (Figure 1a compared to 1b and Table 1).

Table 1. Phenotypic responses to heat stress in *Phaseolus vulgaris* cv. BAT93 and in *P. vulgaris* 7 genotypes.

	GENOTYPE			
FEATURES	*P. vulgaris* BAT93		*P. vulgaris* 7	
	CTRL	HS	CTRL	HS
Survival after heat stress (6h 37°C, 3 plants per replicate, 3 technical replicates)	NA	1/3	NA	3/3
Average Pods per plant	3	0.33	>3	>3
Average Seeds per pod	5	1	4	4
Average seed weight (g)	0.186 g	0.168 g	0.046 g	0.058 g
Average nodule number per plant	84.48		44.71	
Average nodule dry weight per plant (g)	0.02		0.0055	

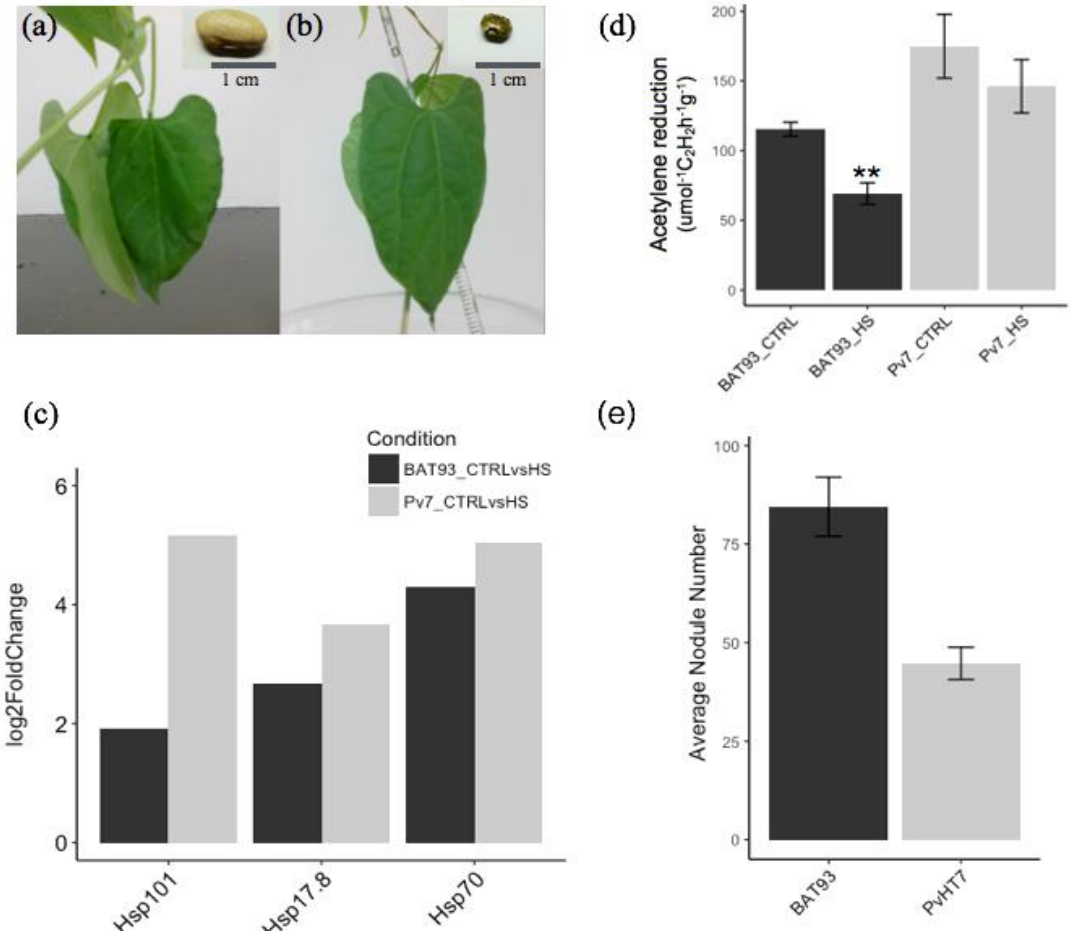

Fig 1. Heat-stress response in P. vulgaris cv. BAT93 and P. vulgaris 7 genotypes. (a) Foliar turgor changes observed in P. *vulgaris* BAT93 (a) and in P. *vulgaris* 7 plants after the heat-shock treatment (37°C/6 h). Insets in (a) and (b) show seeds of the corresponding bean genotypes. Bar size, 1 cm. (c) Expression ratio of HSP101, HSP17.8 and HSP70 chaperones in root nodules of P. *vulgaris* BAT93 (in black) and P. *vulgaris* 7 (in grey), either in control conditions (CTRL) or subjected to heat stress (HS). The fold change in expression was obtained by DESeq of each heat-stress molecular marker from root nodules of control plants versus its expression in root nodules of heat-stressed plants. Values in both graphs represent the Log2 fold change of three biological replicates. (d) Effects of the thermal shock on the nitrogenase activity of P. *vulgaris* BAT93 or P. *vulgaris* 7 root nodules, either in control conditions or after the heat-shock treatment. ** $P < 0.01$, n=15. (e) Average nodule number of each genotype at 20 dpi. n=15

Table 2. Comparison of total number of open reading frames (ORFs) and small open reading frames (sORFs) in *Phaseolus vulgaris* cv. BAT93 and G19833, *Glycine max*, *Lotus japonicus* and *Medicago truncatula*.

	Phaseolus vulgaris BAT93	*Phaseolus vulgaris* G19833	*Glycine max*	*Lotus japonicus*	*Medicago truncatula*
ORFs	64692	31638	88647	10979	62319
sORFs	7414	4560	14979	2195	18688
sORFs/ORFs ratio	0.11	0.14	0.16	0.19	0.29

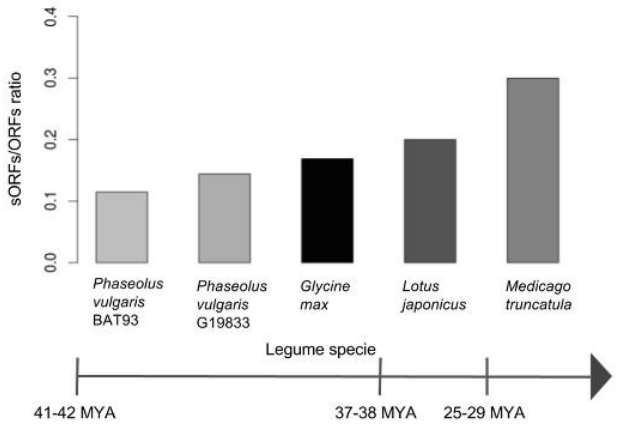

Figure 2

Fig 2. Proportion of sORFs detected in legume plant genomes. *P. vulgaris* G19833, *G. max*, *L. japonicus* and *M. truncatula* protein sizes in Phytozome version 1.11, and *P. vulgaris* BAT93 in *The Novo Genome Assembly and Annotation Team*. Arrow represents timeline evolution of these plant legumes based on archaeological and molecular data (Choi et al., 2004). Intersections indicate the time of divergence between clades. MYA, *million years ago*.

Table 3. List of differentially expressed sORFs in *P. vugaris* cv. BAT93 after heat-stress.

ID	Size	Associated protein	MOTIF MEME	BLASTP (Superfamilies)	Sequences producing significant alignments	Associated processes
PHASIBEAM10B038118 (T1)	103	N/A	N/A	No Putative Conserved Domains	Spidroin-1-like [Glycine max]	
PHASIBEAM10B045106 (T1)	103	N/A	N/A	H4 superfamily	Histone H3.2 [Cajanus cajan]	
PHASIBEAM10F001830 (T1)	103	N/A	N/A	No Putative Conserved Domain	Glutamine dumper 5-like [Cicer arietinum] transmembrane protein [Medicago truncatula]	
PHASIBEAM10F002901 (T1)	116	N/A	N/A	No Putative Conserved Domain	uncharacterized genes	
PHASIBEAM10F003368 (T1)	123	N/A	N/A	AAI_LTSS superfamily	Lipid transfer protein DIR1 [Vigna angularis, Medicago truncatula]	
PHASIBEAM10F004274 (T1)	140	N/A	N/A	No Putative Conserved Domain	Lysine-rich arabinogalactan protein 19-like [Vigna angularis] transmembrane protein, putative [Medicago truncatula]	
PHASIBEAM10F006225 (T1)	145	N/A	N/A	HMG-box superfamily	High mobility group B protein 7-like [Glycine soja] PREDICTED [Vigna radiata]/ HMGB-UBF_HMG-box, class II and III members of the HMG-box superfamily of DNA-binding proteins	
PHASIBEAM10F006374 (T1)	81	N/A	N/A	RRM_SF superfamily	Serine/arginine-rich SC35-like splicing factor SCL33 isoform X1 [Vigna radiata]	Hormonal control (Cruz et al., 2014, Suzuki et al., 2016)
PHASIBEAM10F007017 (T1)	137	Histone H2B.6	N/A	H2B superfamily	probable histone H2B.3 [Vigna radiata]	DNA package (Iliakis et al., 2008; Kim et al. 2015; Kantidze et al., 2016)

PHASIBEAM10F008866 (T3)	130	Thymidine kinase a	N/A	TK superfamily	Thymidine kinase-like [Vigna radiata]	Nucleotide synthesis (Wang & Liu, 2006; Garton et al., 2007); nucleotide salvage pathway (Moffat et al. 2002)
PHASIBEAM10F011464 (T1)	130	Histone H3.2	N/A	H4 superfamily	Histone H3.2 [Cajanus cajan] histone H3 [Triticum aestivus] core histone H2A/H2B/H3/H4	DNA package (Iliakis et al., 2008; Kim et al. 2015, Kantidze et al., 2016)
PHASIBEAM10F012744 (T1)(T2)	75(T1) 67(T2)	N/A	Motif B	COX7a_Cyt_c_Oxidase_VIIa superfamily	Cytochrome-c oxidases, electron carriers [Theobroma cacao]	Stress response (Gong et al. 1998, Huang et al. 2016))
PHASIBEAM10F019557 (T1)(T2)	88(T1) 93(T2)	N/A	Motif C/A	SANT_Superfamily/ Myb_DNA-Binding	PREDICTED: protein RADIALIS-like 3 [Vigna radiata] MYB transcription factor MYB142 [Glycine max]	
PHASIBEAM10F022486 (T1)	74	N/A	N/A	No Putative Conserved Domain	Transmembrane protein, putative [Medicago truncatula]	
PHASIBEAM10F025436 (T1)	72	N/A	N/A	No Putative Conserved Domain	Hypotetical protein LR48_Vigan08g167400 [Vigna angularis]	
PHASIBEAM10F026060 (T1)	144	N/A	N/A	Alpha-crystallin-HSPs_p23-like superfamily/IbpA	PREDICTED: 15.7 kDa heat shock protein, peroxisomal [Vigna angularis][Vigna radiata]	(Vierling et al. 1997)

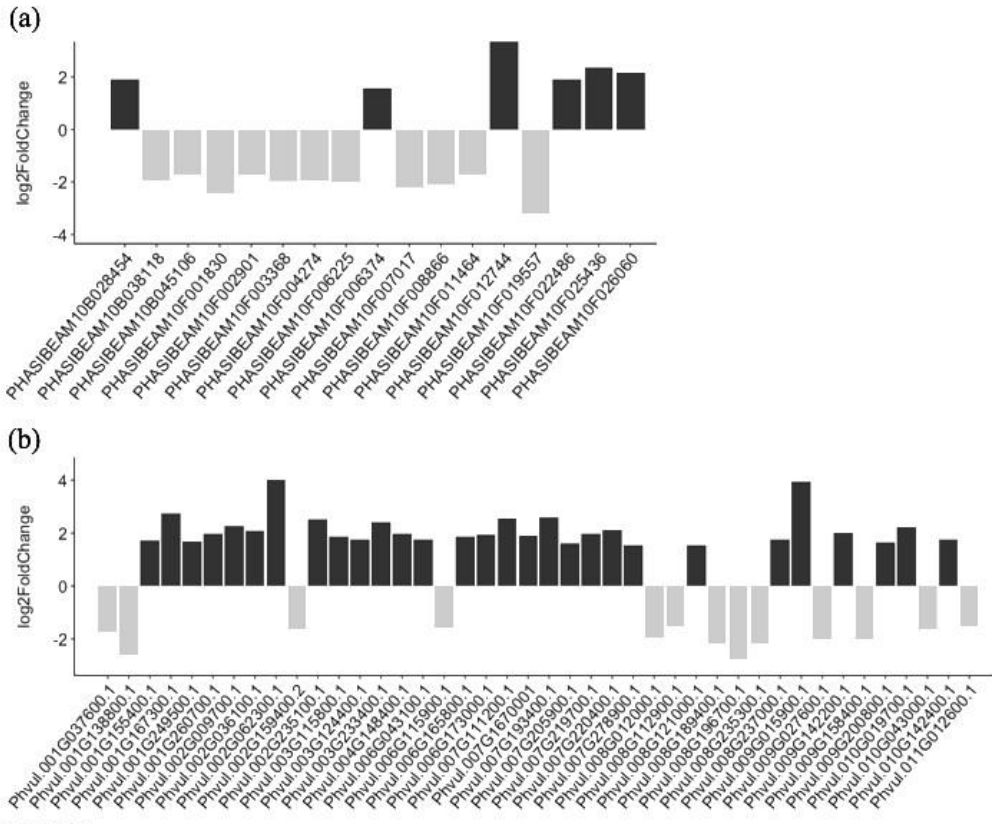

Figure 3

Fig 3. sORFs differentially expressed in (a) *P. vulgaris* BAT93 and (b) *P. vulgaris* 7. Gene expression values between control or heat-stressed 20 dpi nodules are expressed as the Log2 of the fold change. Accession numbers are indicated on the X axis.

Table 4. List of differentially expressed sORFs in *P. vugaris* 7 after heat-stress.

ID	Size	GO	MOTIF MEME	BLASTP (Superfamilies)	Sequences producing significant alignments	Associated process
Phvul.010G019700.1	112	Uncharacterised protein family SERF	N/A	4F5	Gibberellin regulated protein [Cynara cardunculus var. Scolymus]	Hormonal control
Phvul.010G142400.1	114	N/A	N/A	No Putative Conserved Domain	uncharacterized genes	
Phvul.010G043000.1	97	Domain of unknown function (DUF581)	N/A	zf-FLZ superfamily	uncharacterized genes	
Phvul.003G124400.1	74	N/A	N/A	No Putative Conserved Domain	uncharacterized genes	
Phvul.003G233400.1	75	phytosulfokine 4 precursor	N/A	PSK superfamily	phytosulfokines-like [Glycine max]	Growth
Phvul.003G115800.1	121	Ca2+-binding protein 1	Motif F/A	Efh superfamily (EF-hand7)	hypersensitivye reaction associated Ca2+-binding protein [Phaseolus vulgaris] calmodulin-like [Vigna angularis]	Calcium signaling (Liu et al., 2003; Al-Quaraan et al., 2010)
Phvul.009G200800.1	141	N/A	N/A	G_glu_transpept superfamily	transmembrane protein, putative [Medicago truncatula]	
Phvul.009G158400.1	58	N/A	N/A	No Putative Conserved Domain	aldo/keto reductase [Desulfitobacterium metallireducens]	
Phvul.009G015900.1	101	SAUR-like auxin-responsive protein family	N/A	Auxin_inducible superfamily	auxin-induced protein ARG7 [Cajanus cajan] Predicted: auxin-induced protein 15A [Vigna angularis]	Hormonal control
Phvul.009G142200.1	115	N/A	N/A	No Putative Conserved Domain	uncharacterized genes	
Phvul.009G027600.1	150	Heavy metal transport/detoxification superfamily protein	N/A	HMA_superfamily	Predicted: heavy metal-associated isoprenylated plant protein 22 [Vigna angularis]	Stress response
Phvul.011G012600.1	86	Domain of unknown function, DUF642	N/A	PLN03089/hypotetical	uncharacterized genes	
Phvul.008G196700.1	44	N/A	N/A	No Putative Conserved Domain	uncharacterized genes	
Phvul.008G121000.1	101	N/A	N/A	No Putative Conserved Domain	uncharacterized genes	
Phvul.008G235300.1	97	Gibberellin-regulated family protein	N/A	GASA superfamily	gibberellic acid - stimulated protein 1 [Glycine soja]	Hormonal control
Phvul.008G112900.1	101	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein	N/A	AAI_LTSS superfamily	predicted: putative ipid-transfer protein DIR1 [Vigna angularis]	Stress response
Phvul.008G189400.1	134	Heavy metal transport/detoxification superfamily protein	N/A	HMA_superfamily	Predicted: copper transport protein ATX1-like [Glycine max]	Stress response
Phvul.008G012000.1	137	Calcium-binding EF-hand family protein	N/A	EFh superfamily/EF-hand7	calcium-binding EF-hand protein [Medicago truncatula]	
Phvul.008G237000.1	144	HSP20-like chaperones superfamily protein	N/A	alpha-crystallin-HSPs_p23-like superfamily	Predicted: 15.7 kDa heat shock protein, peroxisomal [Vigna angularis]	
Phvul.004G148400.1	71	N/A	N/A	No Putative Conserved Domain	uncharacterized genes	

Phvul.007G220400.1	54	N/A	N/A	No Putative Conserved Domain	uncharacterized genes	
Phvul.007G205900.1	62	Low temperature and salt responsive protein family	N/A	No Putative Conserved Domain	Predicted: hydrophobic protein RCI2B [Vigna adiata]/Stress-induced hydrophobic peptide [Theobroma cacao]	
Phvul.007G111200.1	118	calmodulin-like 11	Motif D/F	EFh superfamily/EF-hand7	Predicted: calmodulin-like protein 11 [Vigna radiata]	Calcium signaling (Liu et al., 2003; Al-Quaraan et al., 2010)
Phvul.007G193400.1	147	Integrase-type DNA-binding superfamily protein	N/A	AP2 superfamily	Ethylene-responsive transcription factor ERF098 [Glycine soja]	Hormonal control
Phvul.007G167000.1	131	N/A	N/A	No Putative Conserved Domain	uncharacterized genes	
Phvul.007G278900.1	150	calmodulin-like 11	Motif D/F/G	EFh superfamily	Predicted: calmodulin-like protein 8 [Vigna radiata var. radiata]	Calcium signaling (Liu et al., 2003; Al-Quaraan et al., 2010)
Phvul.007G219700.1	96	SAUR-like auxin-responsive protein family	N/A	Auxin_inducible superfamily	Predicted: auxin-induced protein X15-like [Glycine max]	
Phvul.001G037600.1	119	Domain of unknown function (DUF3511)	N/A	DUF3511 superfamily	uncharacterized genes	
Phvul.001G138800.1	67	N/A	N/A	No Putative Conserved Domain	uncharacterized genes	
Phvul.001G167300.1	127	RmlC-like cupins superfamily protein	N/A	Cupin_3/Cupin_like superfamily	RmlC-like cupins superfamily protein	
Phvul.001G155400.1	148	calmodulin-like 11	Motif D/F/G	EFh superfamily	Predicted: calmodulin-3-like [Vigna radiata var. radiata]	Calcium signaling (Liu et al., 2003; Al-Quaraan et al., 2010)
Phvul.001G249500.1	67	N/A	N/A	No Putative Conserved Domain	uncharacterized genes	
Phvul.001G260700.1	84	N/A	N/A	No Putative Conserved Domain	uncharacterized genes/ F-box domain, cyclin-like protein [Cynara cardunculus var. scolymus]	Cell proliferation
Phvul.006G173000.1	93	N/A	N/A	No Putative Conserved Domain	uncharacterized genes	
Phvul.006G165800.1	127	jasmonate-zim-domain protein 8	N/A	tify_superfamily / CCT_2 superfamily	Predicted: protein TIFY 5A-like [Vigna radiata var. radiata]	
Phvul.006G043100.1	94	N/A	N/A	No Putative Conserved Domain	uncharacterized genes	
Phvul.006G115900.1	143	cytochrome B5 isoform E	N/A	Cyt-b5 superfamily	cytochrome b5-like [Vigna angularis]	
Phvul.002G062300.1	56	N/A	N/A	DUF4534 superfamily	uncharacterized genes	
Phvul.002G235100.1	73	N/A	N/A	DUF761 superfamily	uncharacterized genes	
Phvul.002G036100.1	113	cytochrome c-2	N/A	Cytochrom_C superfamily	Cytochrome c [Cajanus cajan][Medicago truncatula]	

Phvul.002G159400.1	99	SPIRAL1-like2	Motif E	No Putative Conserved Domain	Predicted: protein SPIRAL-like 5 [Vigna angularis]
Phvul.002G159400.2	99	SPIRAL1-like2	Motif E	No Putative Conserved Domain	Predicted: protein SPIRAL-like 5 [Vigna angularis]

The observed differences were accompanied by lower nitrogen-fixation levels (Figure 1d), supporting the hypothesis that reduced metabolic activity caused by heat stress reduces nitrogen fixation rates. Interestingly, compared to *P. vulgaris* BAT93, the average nodule number per root in plants of *P. vulgaris 7* was considerably lower (Figure 1e), although the level of nitrogen-fixation was higher (Figure 1d). This finding suggest that, compared to *P. vulgaris* BAT93, *P. vulgaris 7* root nodules are not only more resistant to heat stress but more efficient in fixing nitrogen.

To reveal the presence and quantity of any RNA in a biological sample by RNA-seq, statistical estimation of data is required. Three statistical methods were used to validate changes in gene expression, and only sORFs with a significant differential expression were considered. Compared to unstressed symbiotic nodules (control), 15 sORFs were differentially expressed in *P. vulgaris* BAT93 root nodules, whereas 44 sORFs were identified in *P. vulgaris* 7. Contrary to *P. vulgaris* 7, in *P. vulgaris* BAT93 most sORFs were down-regulated, with only a few being up-regulated (Figure 3 and Table 3). RNA-seq data on heat stressed nodules from both common bean genotypes suggest the involvement of phytohormones and antioxidant systems in the signaling for thermo-tolerance acquisition (Suzuki et al., 2016). However, the most remarkable difference at the molecular level observed among heat-stressed nodules of these genotypes was the notable abundance of sORFs transcripts related to calcium signaling in *P. vulgaris* 7 (Table 4). This finding suggests that, regardless of the evolutionary closeness of the domesticated *P. vulgaris* BAT93 and the wild *P. vulgaris* 7, each genotype has independently adapted their molecular responses to preserve the biological nitrogen fixation process under heat stress (Figure 3, and Tables 3 and 4). This ability becomes highly relevant in nitrogen deprived soils, such as those of tropical and temperate regions. In this sense, *P. vulgaris* 7 as well as other *P. vulgaris* wild relatives of Mexico are important reservoirs of genetic variation that could be sourced for crop improvement.

To our knowledge, this is the first report of a set of sORFs being associated with heat stress. Taking into consideration the highest resistance to heat stress shown by *P. vulgaris* 7

(Figure 1), induced sORFs under heat should be subject of further functional genomics studies. Although these studies are necessary to prove the biological function of each of these sORFs, the described procedure opens new possibilities to detect potentially relevant genes involved in heat stress response.

Materials and Methods

Plant growth and heat-stress treatments

Dry, mature seeds of *Phaseolus vulgaris* cv. BAT93 and a *Phaseolus vulgaris* wild heat-tolerant genotype (named *P. vulgaris* 7) were surface sterilized as previously described (Estrada-Navarrete et al., 2007). Sterilized seeds were transferred to sterile trays containing wet paper towels. Trays were covered with foil and incubated at 28°C for 2 days (Estrada-Navarrete et al., 2007). Two-day-old common bean sprouts were inoculated with *Rhizobium tropici* CIAT899 (Martínez-Romero et al., 1991) and grown at 28ºC/18ºC day/night temperature, 65% relative humidity, 180-300 µ-1mol photon m-2s-1 and 14 h photoperiod for 20 days in a growth chamber. Common bean plants were watered every third day with N-free sterile B&D nutrient solution (Broughton & Dilworth 1971). After this period, plants were subjected to a sudden heat-stress (37ºC), sustained for 6 h. Twenty days post-inoculation (dpi), root-nodules from 5 plants of each genotype were harvested, frozen in liquid nitrogen, and stored at -80 ºC.

Nitrogenase activity was evaluated from 20 dpi inoculated roots (following methods of Ramírez et al., 1999; Verdoy et al., 2004) under control and heat stress conditions in both bean genotypes. Nodulated roots were incubated in acetylene gas for 1 h and ethylene production was determined by gas chromatography (Varian model 3300). Specific activity was expressed as $\mu mol^{-1}C_2H_2h^{-1}g^{-1}$ nodule dry weight.

Bacterial strain and culture

The *Rhizobium tropici* CIAT899 strain was selected as it has known resistance to heat (37ºC; Martínez-Romero et al., 1991). Two-day-old bean sprouts were inoculated with *R. tropici* CIAT899 according to Ramírez et al. (2005) with some minor modifications. Briefly, *R. tropici* CIAT899 was grown in peptone yeast liquid medium [0.5% bactopeptone (w/v), 0.3% yeast extract (w/v), 7 mM $CaCl_2 \cdot 2H_2O$] supplemented with 20 g/mL nalidixic acid at 30 ºC to a cell density of 5 to 8×10^8 mL^{-1}. 1 mL was applied to the root.

RNA extraction, cDNA libraries preparation and sequencing using Illumina Hiseq2000

Twenty dpi symbiotic nodules were isolated, frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. The sample was immediately processed for total RNA isolation using the extraction kit ZR Plant RNA MiniPrep (Zymo Research, USA) according to manufacturer's instructions. Total RNA in each sample was more than 5 µg. RNA integrity was confirmed using a 2100 Bioanalyzer (Agilent Technologies, Inc.) with a minimum RNA integrity number (RIN) value of 7.0. cDNA library templates from 3 biological replicates of each genotype, and from both control and heat-stress conditions (24 cDNA libraries in total), were prepared using a Truseq™ RNA Sample Prep Kit (Illumina) according to the manufacturer's recommendations at the University Unit for Massive Sequencing (UUSM) from the Universidad Nacional Autónoma de México (UNAM). These libraries were sent to Macrogen Inc. (Korea; www.macrogen.com) for sequencing by Illumina Hiseq2000 (http://www.illumina.com).

Strategy for large-scale discovery of putative sORFs in P. vulgaris BAT93 and G19833 genotypes

sORFs (30 to 150 aa in length) of *P. vulgaris* BAT93 were gathered from CoGe and *The Novo Genome Assembly and Annotation Team* (CoGe database [https://genomevolution.org/CoGe/] and [http://denovo.cnag.cat/genomes/bean/], genome ID 20365) while the sORFs from *P. vulgaris* G19833 were collected from Phytozome (Phytozome version 11 database [www.phytozome.net]; *P. vulgaris* v1.0), respectively. In both cases, all sORFs with no initial methionine were discarded to avoid truncated transcripts.

Gene expression and motif-based analysis of P. vulgaris sORFs

In order to estimate transcript abundance for each experimental condition tested, raw sequence data from Illumina Hiseq2000 were analyzed using FASTQC software (www.bioinformatics.babraham.ac.uk/projects/). The short sequence reads obtained (of around 100 bp in length) were aligned to the reference genome; *P. vulgaris* BAT93 (CoGe database [https://genomevolution.org/CoGe/], genome ID 20365) or *P. vulgaris* G19833 (*P. vulgaris v1.0;* Phytozome version 11 database [www.phytozome.net]) to uncover their identity. The SMALT software (http://www.sanger.ac.uk/science/tools/smalt-0) was used to this purpose. Finally, differential expression was estimated with DESeq, an R/Bioconductor package performing a pairwise differential expression analysis (Anders & Huber, 2010, Bioconductor V3.3, R V 3.3, http://bioconductor.org/packages/2.11/bioc/, Robinson 2010). Only *P. vulgaris* sORFs validated by this method with a 2-fold change and a *P*-value < 0.05 between control and the heat stress condition of each common bean genotype were considered for study. Selected *P. vulgaris* sORFs were classified according the GO annotation (http://www.agbase.msstate.edu/cgi-bin/tools/GOanna.cgi, McCarthy et al., 2006), and further analyzed by the MEME Suite (http://meme-suite.org/tools/meme, Bailey et al., 2009), and by the BLASTP algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi, Altschul et al., 1990).

Acknowledgments

We acknowledge Alfonso Leija (CCG, UNAM) for technical support with the acetylene reduction analysis. We are very grateful to Jorge Acosta, INIFAP - CE Bajío and Alfonso Delgado, Instituto de Biología, UNAM for supplying *Phaseolus vulgaris* seeds. We are thankful to the University Unit for Massive Sequencing (UUSM) from the National University of Mexico (UNAM) for Illumina sample sequencing and data analyisis.

This work was funded by FAO/IAEA CRP 23029 Research Contract 16601, PAPIIT IN206415 and IN206815 from Universidad Nacional Autónoma de México and CONACYT No. 177744 and 177207. Eunice Alejandra Zayas-Del Moral is a doctoral student from Programa de Doctorado en Ciencias Biomédicas (UNAM) and received fellowship No. 317533 from CONACYT.

References

Abd-Alla MH, Issa AA, Ohyama T (2014) Impact of harsh environmental conditions on nodule formation and dinitrogen fixation of legumes. Advances in biology and ecology of nitrogen fixation. ISBN *978-953-51-1216-7*, P.978-953.

Al-Quraan NA, Locy RD, Singh NK (2010) Expression of calmodulin genes in wild type and calmodulin mutants of *Arabidopsis thaliana* under heat stress. Plant Physiol Biochem. 48 (8): 697-702.

Altschul, SF, Gish W, Miller W, Myers EW, and Lipman DJ (1990) Basic local alignment search tool. J Mol Biol. 215: 403-410.

Anders S, and Huber W (2010) Differential expression analysis for sequence count data. Genome Biol. 11: R106.

Aparicio F, Thomas CL, Lederer C, Niu Y, Wang D, and Maule AJ (2005) Virus induction of Heat Shock Protein 70 reflects a general response to protein accumulation in the plant cytosol. Plant Physiol. 138: 529-536.

Bailey TL, Boden M, Buske FA, Frith M, Grant CE, Clementi L, Ren J, Li WW, and Noble WS (2009) MEME SUITE: tools for motif discovery and searching. Nucleic Acids Res. 37 (Web Server Issue): W202-W208.

Batut J, Mergaert P, and Masson-Boivin C (2011) Peptide signalling in the rhizobium-legume symbiosis. Curr Opin Microbiol. 14(2): 181-187.

Broughton WJ, and Dilworth MJ (1971) Control of leghaemoglobin synthesis in snake beans. Biochem J. 125: 1075-1080.

Denarié J, Debellé F, and Promé JC. (1996) Rhizobium lipochitooligosaccharide nodulation factors: signaling molecules mediating recognition and morphogenesis. Annu Rev Biochem. 65: 503-35.

Estrada-Navarrete G, Alvarado-Affantranger X, Olivares JE, Guillén G, Díaz-Camino C, Campos F, Quinto C, Gresshoff PM, and Sanchez F (2007) Fast, efficient and reproducible genetic transformation of *Phaseolus spp*. by *Agrobacterium rhizogenes*. Nature Prot. 2: 1819-1824.

Garton S, Knight H, Warren GJ, Knight MR, and Thorlby GJ (2007) Crinkled leaves 8-a mutation in the large subunit of ribonucleotide reductase–leads to defects in leaf development and chloroplast division in Arabidopsis thaliana. Plant Journal 50: 118–127.

Goodstein, D.M., Shu, S., Howson, R., Neupane, R., Hayes, R.D., Fazo, J., Mitros, T., Dirks, W., Hellsten, U., Putnam, N., et al. (2012) Phytozome: a comparative platform for green plant genomics. Nucleic Acids Res. 40: D1178-D1186.

Guillén, G., Díaz-Camino, C., Loyola-Torres, C.A., Aparicio-Fabre, R., Hernández-López, A., Díaz-Sánchez, M., and Sanchez, F. (2013) Detailed analysis of putative genes encoding small proteins in legume genomes. Front. Plant Sci. 4: 208.

Hanada, K., Akiyama, K., Sakurai, T., Toyoda, T., Shinozaki, K., and Shiu, S.H. (2010) sORF finder: a program package to identify small open reading frames with high coding potential. Bioinformatics. 26(3): 399-400.

Hanada, K., Higuchi-Takeuchi, M., Okamoto, M., Yoshizumi, T., Shimizu, M., Nakaminami, K., Ohashi, C., Ilda, K., Tanaka, M., Horii, Y., et al. (2012) Small open reading frames associated with morphogenesis are hidden in plant genomes. Proc Acad Nat Sci. 110(6): 2395-2400.

Huang YC, Niu CY, Yang CR, Jinn TL (2016) The heat-stress factor HSFA6b connects ABA signaling and ABA-mediated heat responses. Plant Physiology Preview. 172(2):1182-1199

Iliakis G, Wu W, and Wang M (2008) DNA double strand break repair inhibition as a cause of heat radiosensitization: re-evaluation considering backup pathways of NHEJ. Int J Hyperthermia. 24 (1): 17-29.

IPCC (2014) Climate Change 2014: Impacts, Adaptation, and Vulnerability. Part A: Global and Sectoral Aspects. Contribution of Working Group II to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change [Field CB, Barros VR, Dokken DJ, Mach KJ, Mastrandrea MD, Bilir TE, Chatterjee M, Ebi KL, Estrada YO, Genova RC, Girma B, Kissel ES, Levy AN, MacCracken S, Mastrandrea PR, and White LL (Eds.)]. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA, p. 1132.

Kantidze OL, Velichko AK, Luzhin AV, and Razin SV (2016) Heat stress-induced DNA damage. Acta Naturae. 8(2):75-78.

Kim DH, Xu ZY, Na YJ, Yoo YJ, Lee J, Sohn EJ, and Hwang I (2011) Small heat shock protein HSP17.8 functions as an AKR2A cofactor in the targeting of chloroplast outer membrane proteins in Arabidopsis. Plant Physiol. 157(1): 132-146.

Kim JM, Sasaki T, Ueda M, Sako K, and Seki M (2015) Chromatin changes in response to drought, salinity, heat, and cold stresses in plants. Front Plant Sci. 6: 114.

Larkindale J, Hall JD, Knight MR, and Vierling E. (2005) Heat stress phenotypes of Arabidopsis mutants implicate multiple signaling pathways in the acquisition of thermotolerance. Plant Physiol. 138: 882-897.

Lebrazi S & Fikri Benbrahim K (2014) Environmental stress conditions affecting the N2 fixing *Rhizobium*-legume symbiosis and adaptation mechanisms. African Journal of Microbiology Research. 8(53):4053-4061

Liu HT, Li B, Shang ZL, Li XZ, Mu RL, Sun DY, and Zhou RG (2003) Calmodulin Is Involved in Heat Shock Signal Transduction in Wheat. Plant Physiology, 132:1186–1195.

Marmiroli N, and Maestri E (2014) Plant peptides in defense and signaling. Peptides 56: 30-44.

Martínez-Romero E, Segovia L, Martins MF, Franco AA, Graham P, and Pardo MA (1991) *Rhizobium tropici*, a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena sp.* trees. Int J Syst Bacteriol. 41(3): 417-426.

McCarthy FM, Wang N, Magee GB, Nanduri B, Lawrence ML, Camon EB, Barrell DG, Hill DP, Dolan ME, Williams WP et al. (2006) AgBase: a functional genomics resource for agriculture. BMC Genomics. 7: 229.

Moffatt BA, Stevens YY, Allen MS, Snider JD, Pereira LA, Todorova MI, Summers PS, Weretilnyk EA, Martin-McCaffrey L, Wagner C (2002) Adenosine kinase deficiency is associated with developmental abnormalities and reduced transmethylation. Plant Physiology 128: 812–821

Peoples MB, Herridge DF, and Ladha JK. (1995) Biological nitrogen fixation: an efficient source of nitrogen for sustainable agricultural production. Plant Soil. 174: 3-28.

Ramírez M, Graham MA, Blanco-López L, Silvente S, Medrano-Soto A, Blair MW, Hernández G, Vance CP, and Lara M (2005) Sequencing and analysis of common bean ESTs: Building a foundation for functional genomics. Plant Physiol. 137: 1211-1227.

Ramírez M, Valderrama B, Arredondo-Peter R, Soberón M, Mora J, and Hernández G (1999) Rhizobium etli genetically engineered for the heterologous expression of Vitreoscilla sp. Hemoglobin: effects on free-living and symbiosis. MPMI. 12(11):1008-1015

Robinson MD, McCarthy DJ, and Smyth GK (2010) edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. 26(1): 139-140.

Sato S, Nakamura Y, Kaneko T, Asamizu E, Kato T, Nakao M, Sasamoto S, Watanabe A, Ono A, Kawashima K et al. (2008) Genome structure of the legume, *Lotus japonicus*. DNA Res. 15: 227-239.

Schmutz J, Cannon SB, Schlueter J, Ma J, Mitros T, Nelson W, David L, Hyten L, Song Q, Jay J et al. (2010) Genome sequence of the palaeopolyploid soybean. Nature. 463: 178-183.

Schmutz J, McClean PE, Mamidi S, Wu GA, Cannon SB, Grimwood J, Jenkins J, Shu S, Song Q, Chavarro C, Torres-Torres M, Geffroy V, et al. (2014) A reference genome for common bean and genome-wide analysis of dual domestications. Nature Genetics. 46:707-713

Skarshewski A, Stanton-Cook M, Huber T, Al Mansoori S, Smith R, and Beatson SA (2014) uPEPperoni: an online tool for upstream open reading frame location and analysis of transcript conservation. BMC Bioinformatics. p. 15-36

Suzuki N, Bassil E, Hamilton JS, Inupakutika MA, Zandalinas SI, Tripathy D, Luo Y, Dion E, Fukui G, Kumazaki A, Nakano R, Rivero RM, Verebeck GF, Azad RK, Blumwald E, Mittler R (2016) ABA is required for plant acclimation to a combination of salt and heat stress. PLoS One. e0147625.

Tate RL (1995) Soil microbiology (symbiotic nitrogen fixation). New York, N.Y. John Wiley & Sons, Inc. p. 307-333.

Vanderperre B, Lucier JF, Roucou X (2012) HAltORF: A database of predicted out-of-frame alternative open reading frames in human. Database (Oxford). Bas025.

Verdoy D, Lucas MM, Manrique E, Covarrubias AA, De Felipe MR, Pueyo JJ (2004) Differential organ-specific response to salt stress and water deficit in nodulated bean (*Phaseolus vulgaris*). Plant, Cell & Environment. 27(6):757-767

Vierling E. (1997) The small heat shock proteins in plants are members of an ancient family of heat induced proteins. Acta Physiol Plant. 19:539-547.

Vlasova, A., Capella-Gutiérrez, S., Rendón-Anaya, M., Hernández-Oñate, M., Minoche AE, Erb J, Câmara F, Prieto-Barja P, Corvelo A, Sanseverino W, et al. (2016) Genome and transcriptome analysis of the Mesoamerican common bean and the role of gene duplications in establishing tissue and temporal specialization of genes. Genome Biol. 17: 32.

Voysest O (1983) Variedades de frijol en América Latina y su origen. Centro Internacional de Agricultura Tropical (CIAT), Cali, CO. p. 87 (20823).

Voysest O (2000) Mejoramiento genético del frijol (*Phaseolus vulgaris* L.): Legado de variedades de América Latina 1930-1999. Centro Internacional de Agricultura Tropical (CIAT), Cali, CO. p. 195 (Publicación CIAT no. 321) (SB 327.V69).

Wang W, Vinocur B, Shoseyov O, and Altman A (2004) Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. Trends Plant Sci. 9: 244-252.

Wang C, Liu Z (2006) Arabidopsis ribonucleotide reductases are critical for cell cycle progression, DNA damage repair, and plant development. Plant Cell. 18: 350–365.

Wang F, Dong Q, Jiang H, Zhu S, Chen B, Xiang Y (2012) Genome-wide analysis of the heat shock transcription factors in Populus trichocarpa and Medicago truncatula. Mol Biol Rep. 39: 1877-1886.

Yang H, Matsubayashi Y, Nakamura K, Sakagami Y (2001) Diversity of Arabidopsis Genes Encoding Precursors for Phytosulfokine, a Peptide Growth Factor1. Plant Physiology. 127(3):842-851.

Young ND, Debellé F, Oldroyd GE, Geurts R, Cannon SB, Udvardi MK, Benedito VA, Mayer KF, Gouzy J, Schoof H, et al. (2011) The Medicago genome provides insight into the evolution of rhizobial symbioses. Nature. 480: 520-524.

Supplementary information

Fig S1. Motifs identified by MEME in the differentially expressed sORFs in cv. BAT93 (a) and *Phaseolus vulgaris* 7 (b) heat stress transcriptomes.

DOI: 10.21475/ajcs.21.15.09.sp-4

Australian Journal of Crop Science AJCS

Expression of heat shock protein (*HSP*) genes and antioxidant enzyme genes in hybrid rice II YOU 838 during heat stress

Yan. Wang[1,2], Min. Huang[2], Peng. Gao[2], Hao. Chen[2], Yu. Zheng[2], Chenming. Yang[2], Zhirong. Yang[1] & Qun. Sun[1]

[1]Key Laboratory of Bio-resource and Bio-environment of the Ministry of Education, College of Life Science, Sichuan University, Chengdu, Sichuan 610064, P.R. China
[2]Key Laboratory of Irradiation Preservation of Sichuan Province, Sichuan Institute of Atomic Energy, Chengdu, Sichuan 610066, P.R. China

***Corresponding author: qunsun@scu.edu.cn**

Abstract

II YOU 838 (*Oryza sativa* subsp. *indica*), crossed by the maternal II-32A and paternal Fu Hui 838, was one of the most widely cultivated hybrid rice in China. Fu Hui 838, which has resistance to high temperature, was generated by mutation technology in 1990. Previous field-testing showed that II YOU 838 had tolerance to high temperature stress and this was confirmed in the present study. The mechanism of heat tolerance of II YOU 838 is not understood. The present study reports gene expression of a representative sample of heat-responsive proteins in II YOU 838 flag leaves subjected to heat stress during flowering. Differential expression of the heat shock protein 70 (HSP70), heat shock protein 90 (HSP90), small heat shock protein (smHSP), superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) were studied under heat stress and optimum temperatures in flag leaves of II YOU 838. All six genes studied were responsive to high temperatures. Quantitative real-time PCR showed increased expression of the heat shock protein genes and antioxidant enzyme genes in flag leaves under heat stress. With increasing number of days gene expression decreased under high temperature. Peak expression of *SOD*, *POD, hsp70* and *hsp90* was on Day 2 under 39 ℃. On Day 3, the expression of *CAT* under 39 ℃ was the highest. The expression of *smhsp* was highest on Day 3 under 27 ℃, followed by that on Day 2 under 27 ℃. The maximum expression values were observed on Day 2 or Day 3 after beginning of heat stress. This suggests that *hsp90, hsp70, SOD* and *POD* are principally involved in early responses to heat in rice flag leaves, and that *smhsp* may play a role in the recovery mechanism in rice after heat stress. This may provide insights into the mechanism of heat-tolerance in rice.

Key Words: II YOU838; antioxidant enzyme; hybrid rice; high-temperature stress; heat shock protein; quantitative real time-PCR
Abbreviation: SSH_ Suppression subtractive hybridization; RT-PCR_ Reverse transcription polymerase chain reaction; HSPs_ Heat shock proteins; ROS_ Reactive oxygen species; SOD_ Superoxide dismutase; CAT_ Catalase; POD_ Peroxidase.

Introduction

High temperature is considered as one of the major stresses in crop production (Grover et al. 2000). Rice is very sensitive to high-temperature stress at almost all the stages of growth and development, especially anthesis is the most susceptible stage. Suppression subtractive hybridization (SSH) allows genes involved in heat stress response to be identified and their activity monitored (Huang et al. 2009). The *smhsp*, *hsp70,* and *hsp90, SOD*, *POD* and *CAT* genes were differentially expressed by SSH. Several stresses induce the production of a group of proteins called heat shock proteins (HSPs) (Chandel et al. 2013). It has been suggested that HSPs, as molecular chaperones, regulate the folding and accumulation of proteins as well as their localization and degradation in plants ((Hu et al. 2009; Sottile and Nadin 2018). HSPs protect other proteins from damage and participate in re-folding proteins, thus ensuring the maintenance of correct protein structure during heat stress conditions (Panaretou and Zhai 2008). HSPs protect cells from injury and facilitate recovery and survival after a return to normal growth conditions. The HSPs in plants are grouped into HSP100, HSP90, HSP70, HSP60, HSP40 and smHSPs families according to their molecular weight, amino acid sequence homologies and functions (Gupta et al. 2010).

It is believed that the diversification of these proteins reflects an adaptation to heat stress. HSP70, smHSPs and HSP90 proteins are the predominant forms of chaperones expressed under high temperature stress conditions (Wang et al. 2004). HSP70 is a cytosolic chaperone which facilitates protein folding, degradation, complex assembly, and translocation, as well as assembly and disassembly of multimeric structure in plants (Chen et al. 2017). HSP70 may also suppress programmed cell death in rice protoplasts (Qi et al. 2011). The smHSPs may prevent irreversible aggregation of partially unfolded or denatured proteins by binding to hydrophobic sites on their surface to maintain them in a re-foldable conformation (Baniwal et al. 2004; Chen et al. 2014; Smykal et al. 2000). HSP90 is a highly conserved molecular chaperone contributing to the folding, maintenance of structural integrity and proper regulation of a subset of cytosolic proteins (Csermely et al. 1998; Young et al. 2001). The most well-known function of HSP90 is its involvement in the maintenance of key proteins such as steroid receptors and protein kinases by forming specific complexes. The heat shock response is characterized by a rapid and robust increase in HSPs upon exposure to protein-damaging stresses.

This evolutionarily conserved cellular protection mechanism is primarily regulated at the level of transcription (Hietakangas and Sistonen 2006). The transcriptional responses of the different *HSPs* to different intensities of heat stress are unclear. The aim here is to compare their expression under a range of heat stress treatments, in order to gain a better understanding of their response to heat stress in rice.

Exposure to heat stress can give rise to excessive accumulation of reactive oxygen species (ROS) in plant cells (Cheng et al. 2007; Zhao et al. 2018). ROS are potentially harmful to the cell, as they can raise the level of oxidative damage through loss of cellular structure and function. Cells possess antioxidants and antioxidative enzymes to interrupt the cascades of uncontrolled oxidation in cellular organelles. Among the antioxidative enzymes, superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) play key roles in detoxification. SOD, the first enzyme in the detoxifying process, converts superoxide anion radicals (O_2^-) to hydrogen peroxide (H_2O_2), then POD and CAT reduces H_2O_2 to water (Asada 1999). H_2O_2 is an important signal molecule in the signal transmission network of many stresses. Previous work has demonstrated that CAT functions in plant disease, heat, water, salt, and light stress reactions. The homeostasis of H_2O_2 is mostly due to CAT scavenging and regulating functions. How SOD, POD and CAT genes regulate the various aspects of heat damage is still unclear, but the information accumulated so far is a promising starting point for further studies to identify their functions and to investigate potential applications /targets, e.g. in molecular plant breeding. II YOU 838 exhibited superiority for multiple agronomic traits, including yield, wide adaptability, especially the resistance to high temperatures, thus it has been widely cultivated in China (Su et al. 2013; Wang et al. 2016). Its pedigree goes back to the rice restorer line, Taiying I hao, which was irradiated with ^{60}Co gamma-rays in 1976 (Deng et al. 2009). The mutant rice cultivar Fuhui 06 was obtained by selection in the fourth mutant generation (M_4). Fuhui 06 was crossed with glutinous rice 80182 in 1982 to obtain the glutinous rice restorer line 226, selected in the 5th generation (M_5). Seed of the rice restorer line Minghui 63 were irradiated with 380 Gy ^{60}Co gamma-rays in 1984 to develop the mutant rice cultivar γ552 by selection at varying temperatures in artificial climate chambers. In 1990, the Fu Hui 838, which was selected from the sixth generation (M_6), was crossed with a maternal mutation rice (γ552) and paternal mutation rice (226). Fu Hui 838 was bred and released by combined techniques of radiation induced mutation, conventional hybridization, and temperature tolerance screening. Fu Hui 838, which is a major restorer line in China, it has good resistance and produces good vigour in hybrids when crossed with certain rice lines. II YOU 838, which was crossed by II-32A as female parent and Fu Hui 838 as male parent, was examined and approved by the Sichuan provincial variety committee in the Sichuan Institute of Atomic Energy in 1995.

The objective of the present study was to evaluate the expression patterns of *hsp90*, *hsp70, smhsp, SOD, POD* and *CAT* in II YOU 838 at different heat-stress intensities, and to evaluate the potential of these genes as heat resistance molecular biomarkers. If there is a correspondence between the heat resistance /injury and the expression of these genes, we can analyze the expression of these genes to clarify the degree of heat stress in rice. There are many studies involving these genes, but insufficient data to prove that these genes can be used as molecular bio-markers for rice heat stress in field. This may provide a better understanding of the molecular mechanisms involved in heat tolerance and may facilitate the development of heat-tolerant rice cultivars.

Results

Standard curve development of hsp90, hsp70, smhsp, SOD, POD and CAT

Gel-electrophoresis and a melting curve analysis of PCR products showed that very little or no primer-dimers or other non-specific amplification products were generated during the runs. Ten-fold serial PCR product dilutions were tested and used to construct the standard curve by plotting the PCR products. The generated standard curve covered a linear range of 1×10^2 to 1×10^8 copies per reaction for *smhsp*, *hsp70*, *hsp90*, *POD*, *SOD*, *CAT*, *18s rRNA* and *actin-1*. Samples were repeated three times. The average mean of the three PCR reaction efficiencies was used. The efficiency of the reaction of 18s*rRNA* primers, *actin-1* primers, *smhsp* primers, *hsp90* primers, *hsp70* primers, *SOD* primers, *POD* primers and *CAT* primers was 87.2%, 97.3%, 76.4%, 82.9%, 84.2%, 92%, 88.9%, 95.4%, respectively. The standard curves are credible when the $R^2 > 0.980$, so the standard curves of the five genes are considered credible.

Expression of hsp70, hsp90 and smhsp genes after heat treatment

During the flowering stage, both the heat treatment group and the control group samples were collected over 24-h intervals for four days. Samples for the heat-stress treatment of 27 °C were collected at 08:00 h and those for 39 °C were collected at 16:00 h. We compared the expression of *HSPs* between the heat treatment group and the control group by quantitative real-time RT-PCR. The results were normalized against the mean for the reference genes. The normalized expression of *hsp70, hsp90* and *smhsp* were significantly increased after heat treatment (Fig. 1), and higher than the control group samples and that of samples before heat treatment. There were significant differences between the heat treatment group and the control group ($p < 0.05$), except in case of the 39 °C heat treatment group of *hsp70* and *hsp90* at Day 4. There was no significant difference in the expressions of *smhsp*, *hsp70* and *hsp90* among the control group from Day 1 to Day 4. After heat treatment, the expressions of *hsp70* and *hsp90* under 39 °C on Day 2 were the highest of any other heat treatment group sampled under 39 °C. The expression of *smhsp* under 27 °C on Day 3 was the highest of any other heat treatment and could be more than one hundred times higher than the control group. When the temperature cycle increased from 27 °C to 39 °C, the expression level of *smhsp* did not increase, but decreased on Day 2 or Day 3. With respect to comparisons of gene expression of *hsp70*, *hsp90* and *smhsp*, we found that the transcription level of *smhsp* was much higher than that of *hsp70* and *hsp90* under the same heat stress. Thus, *smhsp* may play an important part in response to heat stress in rice flag leaves. During the heat treatment cycle, the expression level of *smhsp*, *hsp70* and *hsp90* genes always changed with the trend of temperature, but the changes became smaller over time. The maximum expression values appeared on Day 2 or Day 3. The time points of Day 2 and Day 3 might be the key points for II YOU 838 responses to high temperature.

Expression of SOD, POD and CAT genes after heat treatment

We compared the expression of *SOD*, *POD* and *CAT* genes between samples of the heat treatment group and the control group over four days at 27 °C (sampled at 08:00 h) and 39 °C (sampled at 16:00 h) by quantitative real-time RT-PCR. The results were normalized by the mean for the reference genes *actin-1* and *18s rRNA*. The normalized expression of *SOD*, *POD* and *CAT* genes were significantly increased after heat treatment (Fig. 2). They were notably higher than the control

group samples and the samples prior to the imposition of heat stress, except for *SOD* in the 39 °C heat treatment group on Day 4. The expression of of *SOD* under 39 °C was significantly lower than that under 27 °C on Day 4 ($p < 0.05$). When the temperature cycle increased from 27 °C to 39 °C, the expression level of *SOD* decreased on Day 4. There were no significant differences in the expressions of *SOD*, *POD* and *CAT* genes among the control group over four consecutive days ($p < 0.05$). After heat treatment, the expression of *SOD* and *POD* under 39 °C on Day 2 were the highest of any other heat treatment group samples at this temperature. The expression of *CAT* under 39 °C on Day 3 was the highest of any other heat treatment group samples. The expression of *POD* and *CAT* genes still increased significantly until the Day 4. The expressions of *POD* and *CAT* were very consistent with the change of temperature. It could be deducing that the *SOD* was involved in early responses to heat, and the *CAT* and *POD* were involved in later responses to heat in the rice flag leaf. The time points of Day 2 and Day 3 might be the important transition for II YOU 838 responses to high temperature. During the heat treatment cycle, the expression levels of *SOD*, *POD* and *CAT* changed with temperature, but the change range became smaller with time. Thus, the three antioxidant protective enzyme genes appeared to be sensitive to the high temperature.

Discussion

Heat stress is a major abiotic stress limiting rice growth and yield in many areas of the world. Rice hybrids have shown resistance to high temperatures, which may be due to heterosis to some extent (Hochholdinger and Baldauf 2018). To overcome the challenges presented by global warming, it is important to understand how rice hybrids perceive and respond to high temperatures. II YOU 838 is a hybrid rice which was developed by irradiation mutation breeding of Fu Hui 838 (male parent) and II-32A (female parent). As a hybrid, II YOU 838 has notable resistance to high temperatures (Su et al. 2013; Wang et al. 2016; Wang et al. 2015). The field identification results showed that seed set and yield are highest in II YOU 838 compared to its parents under natural high temperature stress. The heat tolerance of II YOU 838 may be also associated with heterosis.

We performed expression analysis of *smhsp*, *hsp70*, *hsp90*, *SOD*, *POD* and *CAT* genes in II YOU 838 by quantitative real-time RT-PCR in response to different heat stress to analyze the connection between heat resistance /injury and expression of these 6 genes. The results showed that the expression of *smhsp*, *hsp70*, *hsp90*, *SOD*, *POD* and *CAT* genes responded to the different heat-stress intensities. The *smhsp*, *hsp70*, *hsp90*, *SOD*, *POD* and *CAT* genes were very sensitive to temperature changes during the long-term heat stress. Based on these findings, we propose these genes as potential heat resistance molecular biomarkers in the control of heat stress response.

The expression analysis of *hsp70*, *hsp90* and *smhsp* in II YOU 838 rice flag leaves under heat treatment revealed that the HSP family genes are activated and available to play protective roles in rice cells. The expression of the *smhsp* gene had the largest change under a range of heat stress treatments within four days. It is consistent with previous studies in that the heat-responses of smHSPs were remarkably and continuously elevated during heat shock, and may work efficiently in heat stress response in rice flag leaf (Jagadish et al. 2010; Lee et al. 2010; Zhang et al. 2013). The data demonstrated that the expression of *smhsp* increased after a night of recovery and increased more with the accumulation of heat stress days. It suggested that it may play a significant role in the cellular recovery mechanism by regulating the level of transcription in rice after heat stress.

Most HSP90 and HSP70 family genes were significantly up-regulated under early heat treatment (Hahn and Scharf 2011; Zhang et al. 2013), which is similar to the expression pattern in the rice panicle (Zhang et al. 2012). The protective HSP70 and HSP90 had elevated levels of protein and gene expression after heat treatment in wild rice (Scafaro et al. 2010). Earlier studies in a range of species, showed that HSP70 protein interacts with un-folded proteins (Bhattacharya et al. 2009) and this is crucial for the survival of bacteria, yeast and plants under stress conditions (Mogk et al. 2008). The investigation suggested that mitochondrial HSP70 may suppress programmed cell death in rice protoplasts by inhibiting the amplification of ROS (Qi et al. 2011). OsHsp90 from rice when expressed in *E. coli* maintained its growth at hydroperoxides to harmless molecules (Ighodaro and Akinloye, 2017).

The expression of *hsps* and antioxidant enzyme genes had initially alleviated some of the heat stress in rice. Then the expression of *hsps* and antioxidant enzyme genes tend to decline after the maximum during the four consecutive days of heat treatment. There was a threshold between Day 2 and Day 3 under long-term heat stress. These time points might be the transition and key points for II YOU 838 responses to high temperature. The crop's response to heat stress is associated with a complex molecular genetic regulatory network. The difference in *smhsp*, *hsp70*, *hsp90*, *SOD*, *POD* and *CAT* expressions, might lead to different functions in rice under heat stress. This fueld further research on the transcriptional changes of *smhsp*, *hsp70*, *hsp90*, *SOD*, *POD* and *CAT* genes in response to heat stress in rice. Although the expression of *smhsp*, *hsp70*, *hsp90*, *SOD*, *POD* and *CAT* responded to different heat-stress intensities, the transcription of these six genes was limited. However, it should be a focus of the further investigation of their possible application in the improvement of heat tolerance in rice. high temperatures (Liu et al. 2009). In contrast, over-expression of AtHsp90.3 impaired plant tolerance to heat stress, which suggested that proper homeostasis of HSP90 is critical for cellular stress response and /or tolerance in plants (Xu et al. 2010). Compared with the wild type, the OsHsp90 family gene-overexpressing plants had significantly higher SOD activity, which probably acts through the modulation of ROS homeostasis in response to abiotic stresses (Xiang et al. 2018). It could be deduced that the *HSP90* and *HSP70*, which modulated the ROS homeostasis, are involved in a versatile regulatory regime for the early responses to heat stress in the rice flag leaf. As the first enzyme in the detoxifying process, the *SOD* gene was greatly up regulated at the early stage of heat stress (Asada 1999). *SOD* may play a central role in the regulation of total SOD activity and ROS detoxification in rice anther as affected by high temperature exposure at meiosis stage (Zhao et al. 2018). The expression of *POD* genes is regulated in response to biotic and abiotic stresses as well as during plant development (Andréia et al. 2012). It implicates peroxidases as key players during the whole life cycle of a plant, and particularly in cell wall modifications, in roles that can be antagonistic depending on the developmental stage (Passardi et al. 2004). This diversity of functions derives in part from two possible catalytic cycles of peroxidases involving the consumption or release of H_2O_2 and reactive oxygen species. CAT, a major peroxisome protein, plays a critical role in removing peroxisome-generated reactive oxygen species (ROS) produced by peroxisome enzymes (Lee et al. 2018). SOD, POD and CAT are efficient in neutralizing any molecule with the potential of developing into a free radical or any free radical with the ability to induce the production of other radicals. These enzymes dismutate superoxide radical, and breakdown hydrogen peroxides, respectively.

Materials and Methods

Samples, heat stress and flag leaf collection

Ⅱ YOU 838 rice plants were transplanted from the experimental field in Sichuan Institute of Atomic Energy in China to controlled environment cabinets (PGX-300B light

Table 1. The Primers were used in quantitative real-time RT-PCR analysis.

Target	Forward primer (5'– 3')	Reverse primer (5'– 3')	Product length (bp)	Annealing temperature (°C)
actin-1	GAACTGGTATGGTCAAGGCTG	ACACGGAGCTCGTTGTAGAAG	250	58
18s rRNA	CGTCCCTGCCCTTTGTACAC	CGAACACTTCACCGGATCATT	65	58
HSP70	CATCTTCTCAATCTCCTCCTTGCT	GCTCCCAGGGGTGTTCCA	95	64
HSP90	ACTGCTCGTCGTCGTTGTGC	GCTCGCTGCTGGTGCTGA	100	60
smHSP	ACAAACCAGGCCAAAGCGAAAA	TCCCCGACAACGCCAAGC	90	70
SOD	CCCTTTCCACTCGCTCCG	GGTGACGACTCCCTCAACCTG	133	58
POD	GTGCTGCTCAGCTCCACCG	CAGCTTCGCCTTCACCC	103	70
CAT	CATCCAGACCATCGACCCC	TGAGCACCATCCTCCCCAC	116	67

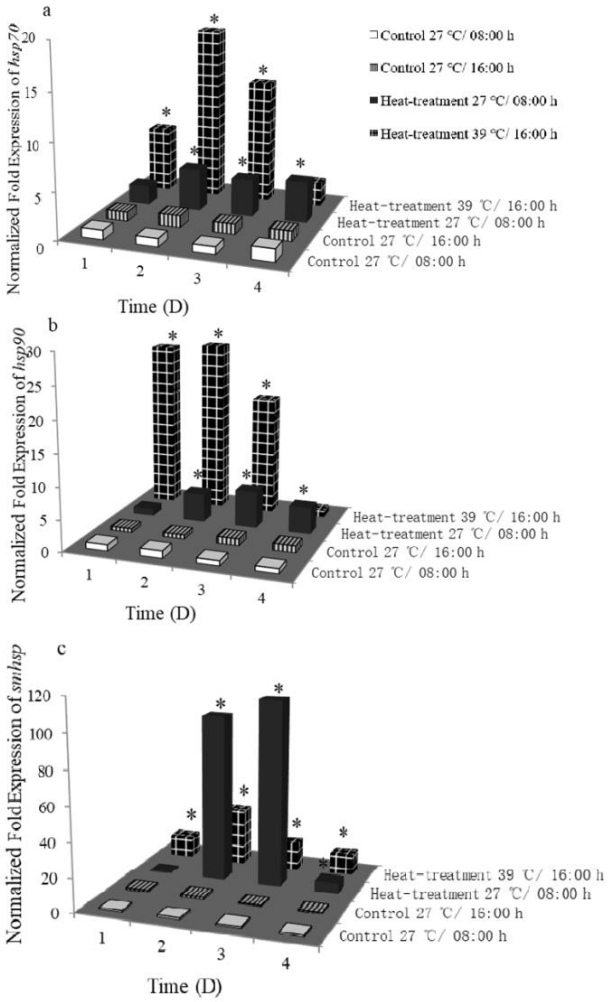

Fig 1. Mean normalized fold expression levels for heat-shock protein genes. Mean normalized fold expression levels for *hsps* under 27 °C at 08:00 and 39 °C at 16:00 between the heat treatment group and the control group over four consecutive days, normalized by the geometric mean for the reference genes. a. *hsp70*, b. *hsp90*, c. *smhsp*. The data are the means ± standard deviations, n=3. Date with the "*" makers were significantly different ($p < 0.05$) as the heat treatments compared with the controls on the same day.

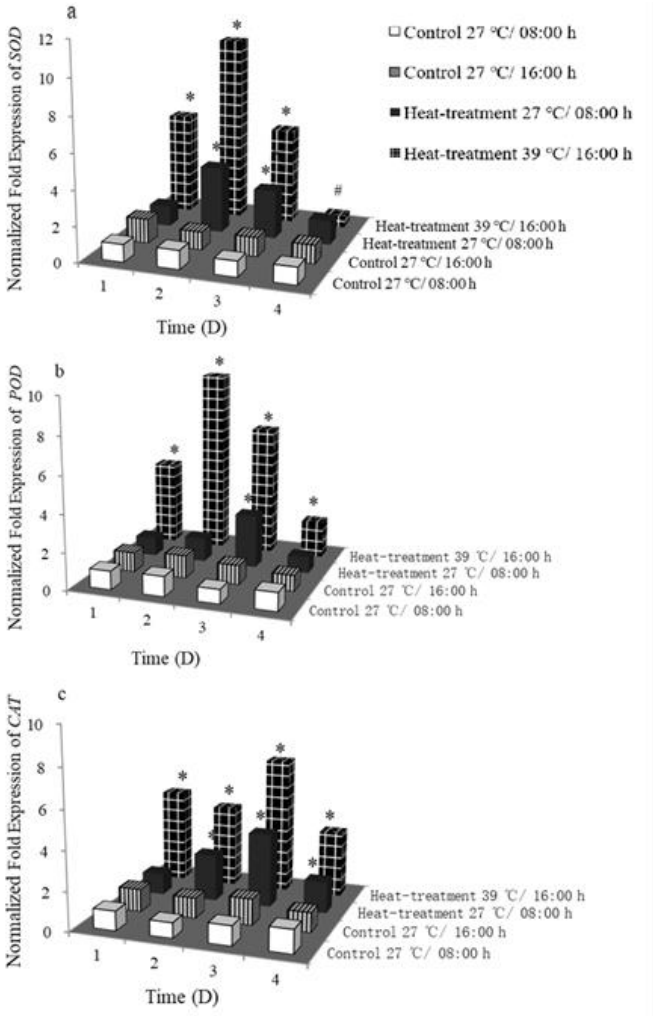

Fig 2. Mean normalized fold expression levels for *SOD, POD* and *CAT* genes. Mean normalized fold expression levels for antioxidant enzyme genes under 27 °C at 08:00 and 39 °C at 16:00 between the heat treatment group and the control group in four days, normalized by the geometric mean for the reference genes. a. *SOD* gene, b. *POD* gene, c.*CAT* gene. The data are the means ± standard deviations, n=3. Date with the "*" makers were significantly different ($p < 0.05$) as the heat treatments compared with the controls on the same day.

incubator). The rice plants were grown in a growth chamber under the conditions of 600 μmol m^{-2} s^{-1} artificial illumination (13h photoperiod) and 75%-80% humidity. Temperature stresses were applied during rice anthesis, the daily average temperature was 34 °C (the temperature cycle was 08:00-10:00 at 35 °C; 10:00 -12:00 at 37 °C; 12:00 -19:00 at 39 °C; 19:00-21:00 at 37 °C; 21:00 -24:00 at 34 °C; 24:00 -03:00 at 30 °C; 03:00-08:00 at 27 °C). The control group was maintained at a constant 27 °C. The heat treatment group and control group had the same light intensity, humidity, water and fertilizer conditions.

The flag leaves of Ⅱ YOU 838 rice were harvested during anthesis at 08:00 and 16:00 over four consecutive days. They were at 08:00 minimum temperature of 27 °C and at16:00 maximum temperature of 39 °C. The heat treatment group and the control group flag leaves were collected at the same time,

and were frozen immediately in liquid nitrogen. The process was repeated three times for three batches of rice materials. Flag leaf samples from three independent biological replicates were harvested for RNA isolation.

RNA isolation and quantitative real-time RT-PCR analysis

Total RNA was isolated from 100mg of flag leaf of II YOU838 using an RNA prep pure Plant Kit (TIANGEN BIOTECH, Beijing, China) as described by the manufacturer. Genomic DNA was eliminated by RNase-free DNase I (TIANGEN BIOTECH, Beijing, China) treatment during the isolation procedure. 2μg RNA was extracted from every sample and reverse transcribed at 37 °C for 60 min using a blend of oligo $(dT)_{15}$ primer and 100 U of Quant reverse transcriptase and a Quant script RT Kit (TIANGEN BIOTECH, Beijing, China). Gene quantification was performed with a iQ™5 Real Time PCR Detection System (Bio-Rad, U.S.). Primers were designed from GenBank sequences with the aid of Primer-Blast. The sequences of the primers were shown in Table 1. For each gene, three technical replicates and three independent biological replicates were used at each sampling time point.

Each PCR was performed in a RealMasterMix, containing SYBR Green I and 0.5 U of Hotmaster Taq DNA polymerase (TIANGEN BIOTECH, Beijing, China). The final concentrations of primers and Mg^{2+} were 0.5μM and 4mM, respectively. The two internal reference genes, *actin-1* and *18s rRNA*, were used to correct six genes of interest. They are used to eliminate sample-to-sample variation of RNA isolation and reverse transcription (Radonic et al., 2004; Vandesompele et al., 2002). The thermal cycling conditions of *18s rRNA*, *actin-1* and *SOD* were as follows: 95 °C for 2 min; followed by 40 cycles of 10 s at 95 °C, 10 s at 58 °C, and 15 s at 68 °C. Data collection was performed during each extension phase. The thermal cycling conditions of other genes were the same with *SOD,* except the annealing temperature during 40 cycles. The annealing temperature of other genes was showed in Table 1.

After the PCR programme, the fluorescent real-time PCR data of all samples were tested by software tool iQ™5 Optical System Software Version 2.1 (Pfaffl et al., 2002).

Statistical analysis

The data obtained from real-time PCR analysis were subjected to One-way Analysis of Variance (ANOVA) followed by LSD (L) test to determine differences in the mean values among the treatments. Significance was concluded at $p < 0.05$ (n=3). Statistical analysis was performed using SPSS 17.0 for Windows.

Conclusion

Quantitative real-time RT-PCR showed significant expression of *smhsp, hsp90, hsp70,* CAT*, SOD* and *POD* in flag leaves under heat stress. The six genes were responsive and differentially to high temperatures. The maximum expression values were observed on Day 2 or Day 3 after beginning of heat stress. These were the critical periods for response of heat stress in II YOU 838 under long-term heat stress. The process is associated with a complex net of regulation of gene expression. It is imperative to gain a better understanding of the molecular basis of heat tolerance as well as the identification of HSPs and antioxidant enzymes for future studies.

Acknowledgment

This work was in part supported by IAEA's Coordinated Research Project: Climate proofing of food crops (No. 16592). And this work was also in part supported by Chinese projects: Applied Basic Science of Sichuan province (No. 2018JY0531, 18YYJC0284 and No. 2018RZ0045, 2018GZ0003) and Science and Technology Support of Sichuan province (No. 2016SZ0075, No. 2018JY0009 and No. 2018SZ0308).

References

Andréia C, Gisele P, Silvia Barcellos R, Carolina Werner R, Fernanda L, Márcia MP (2012) Plant responses to stresses: Role of ascorbate peroxidase in the antioxidant protection. Genetics & Molecular Biology. 35:1011-1019

Asada K (1999) The water-water cycle in chloroplast: Scavenging of Active Oxygens and Dissipation of Excess Photons. Annual Review of Plant Physiology and Plant Molecular Biology. 50:601-639. doi:10.1146/annurev.arplant.50.1.601

Baniwal SK et al. (2004) Heat stress response in plants: a complex game with chaperones and more than twenty heat stress transcription factors. J Biosci. 29:471-487

Bhattacharya A, Kurochkin AV, Yip GN, Zhang Y, Bertelsen EB, Zuiderweg ER (2009) Allostery in Hsp70 chaperones is transduced by subdomain rotations. Journal of Molecular Biology. 388:475-490 doi:10.1016/j.jmb.2009.01.062

Chandel G, Dubey M, Meena R (2013) Differential expression of heat shock proteins and heat stress transcription factor genes in rice exposed to different levels of heat stress. Journal of Plant Biochemistry and Biotechnology. 22:277-285. doi:10.1007/s13562-012-0156-8

Chen X et al. (2014) Expression and interaction of small heat shock proteins (sHsps) in rice in response to heat stress. BBA - Proteins and Proteomics. 1844:818-828

Chen X, Shi L, Zhu L, Shi J, Xu J (2017) Molecular Evolution Characteristics and Expression Pattern Analysis of the Heat Shock Protein 70(HSP70) Gene Superfamily in Plant. Genomics & Applied Biology.

Cheng C et al. (2007) An early response regulatory cluster induced by low temperature and hydrogen peroxide in seedlings of chilling-tolerant japonica rice. BMC Genomics. 8:175

Csermely P, Schnaider T, Soti C, Prohaszka Z, Nardai G (1998) The 90-kDa molecular chaperone family: structure, function, and clinical applications. A comprehensive review. Pharmacol Ther. 79:129-168 doi:S0163725898000138 [pii]

Deng D, Chen H, Deng W, Feng L, Yang X, Ma A, Liu M (2009) Development of a Indica rice restorer line FUHUI838 and its derivative lines with strong restoring ability and their utilization. Journal of Nuclear Agricultural Sciences. 23:175-179

Grover A, Agarwal M, Katiyar-Agarwal S, Sahi C, Agarwal S (2000) Production of high temperature tolerant transgenic plants through manipulation of membrane lipids. Curr Sci India. 79:557-559

Gupta SC, Sharma A, Mishra M, Mishra RK, Chowdhuri DK (2010) Heat shock proteins in toxicology: how close and how far? Life Sci. 86:377-384 doi:S0024-3205(10)00004-4 [pii]10.1016/j.lfs.2009.12.015

Hahn A, Scharf KD (2011) Crosstalk between Hsp90 and Hsp70 chaperones and heat stress transcription factors in tomato. Plant Cell. 23:741-755

Hietakangas V, Sistonen L (2006) Regulation of the heat shock response by heat shock transcription factors. Topics in Current Genetics. 16:1-34

Hochholdinger F, Baldauf JA (2018) Heterosis in plants. Curr Biol. 28:R1089-R1092 doi:10.1016/j.cub.2018.06.041

Hu W, Hu G, Han B (2009) Genome-wide survey and expression profiling of heat shock proteins and heat shock factors revealed overlapped and stress specific response under abiotic stresses in rice. Plant Science. 176:583-590 doi:10.1016/j.plantsci.2009.01.016

Huang M et al. (2009) Screening and Identification of Heat Tolerance Genes of Hybrid Rice Southwest China. Journal of Agricultural Sciences. 22:1199-1203

Ighodaro OM, Akinloye OA (2017) First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. Alexandria Journal of Medicine. S2090506817301550

Jagadish S, Cairns J, Lafitte R, Wheeler TR, Price A, Craufurd PQ (2010) Genetic analysis of heat tolerance at anthesis in rice. Crop Science. 50:1633-1641

Lee DG, Ahsan N, Lee SH, Kang KY, Bahk JD, Lee IJ, Lee BH (2010) A proteomic approach in analyzing heat-responsive proteins in rice leaves Proteomics 7:3369-3383

Lee JN et al. (2018) Catalase inhibition induces pexophagy through ROS accumulation. Biochem Biophys Res Commun. 501:696-702 doi:10.1016/j.bbrc.2018.05.050

Liu D, Lu Z, Mao Z, Liu S (2009) Enhanced thermotolerance of E. coli by expressed OsHsp90 from rice (Oryza sativa L.) Current microbiology 58:129-133

Mogk A, Haslberger T, Tessarz P, Bukau B (2008) Common and specific mechanisms of AAA+ proteins involved in protein quality control Biochemical Society transactions 36:120-125 doi:10.1042/BST0360120

Panaretou B, Zhai C (2008) The heat shock proteins: Their roles as multi-component machines for protein folding Fungal Biology Reviews 22:110-119 doi:10.1016/j.fbr.2009.04.002

Passardi F, Penel C, Dunand C (2004) Performing the paradoxical: how plant peroxidases modify the cell wall Trends in Plant Science 9:0-540

Qi Y, Wang H, Zou Y, Liu C, Liu Y, Wang Y, Zhang W (2011) Over-expression of mitochondrial heat shock protein 70 suppresses programmed cell death in rice FEBS letters 585:231-239

Scafaro AP, Haynes PA, Atwell BJ (2010) Physiological and molecular changes in Oryza meridionalis Ng., a heat-tolerant species of wild rice Journal of experimental botany 61:191-202

Smykal P, Hrdy I, Pechan PM (2000) High-molecular-mass complexes formed in vivo contain smHSPs and HSP70 and display chaperone-like activity Eur J Biochem 267:2195-2207 doi:ejb1223 [pii]

Sottile ML, Nadin SB (2018) Heat shock proteins and DNA repair mechanisms: an updated overview Cell Stress Chaperones:1-13

Su P, Feng H, Chen H, Yang C, Wu X, Huang Q (2013) Influences of Natural High-temperature Stress on Heat Resistance of Rice Southwest China Journal of Agricultural Sciences 26:1387-1391

Wang W, Vinocur B, Shoseyov O, Altman A (2004) Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response Trends Plant Sci 9:244-252 doi:10.1016/j.tplants.2004.03.006

S1360138504000603 [pii]

Wang Y, Gao P, Fu Y, He J, Sun Q, Huang M (2016) Identification of Heat Resistance in Radiation Breed Hybrid Rice II YOU 838 Hubei Agricultural Sciences:6061-6066

Wang Y, Gao P, Huang M, Chen H, Yang Z, Sun Q (2015) Effects of High Temperature on the Activity and Expression of Antioxidative Enzymes in Rice Flag Leaves during the Flowering Stage Plant Science Journal 33:355-361

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

Xu X, Song H, Zhou Z, Shi N, Ying Q, Wang H (2010) Functional characterization of AtHsp90.3 in Saccharomyces cerevisiae and Arabidopsis thaliana under heat stress Biotechnol Lett 32:979-987 doi:10.1007/s10529-010-0240-x

Young JC, Moarefi I, Hartl FU (2001) Hsp90: a specialized but essential protein-folding tool J Cell Biol 154:267-273

Zhang X et al. (2012) Expression profile in rice panicle: insights into heat response mechanism at reproductive stage PLoS One 7:e49652 doi:10.1371/journal.pone.0049652

Zhang X et al. (2013) Transcriptome profile reveals heat response mechanism at molecular and metabolic levels in rice flag leaf Gene 530:185-192 doi:10.1016/j.gene.2013.08.048

Zhao Q et al. (2018) Relationship of ROS accumulation and superoxide dismutase isozymes in developing anther with floret fertility of rice under heat stress Plant Physiol Biochem 122:90-101 doi:10.1016/j.plaphy.2017.11.009.

DOI: 10.21475/ajcs.21.15.09.sp-5

Australian Journal of Crop Science AJCS

Evaluation of mutant rice genotypes for tolerance to high temperature

María Caridad González Cepero[1], Elizabeth Cristo[1], Noraida Pérez[1], Yanelis Reyes[1], Dayné Horta[1], Rodolfo Guillama[1], Guillermo Blanco[1], Mario Varela[1], Madeleine Spencer[2] and Fatma Sarsu[2]

[1]National Institute of Agricultural Science, Cuba
[2]Joint FAO/IAEA Division of NAFA, Plant Breeding and Genetics Section, IAEA, Vienna, Austria

***Corresponding author: mcaridad09@yahoo.es**

Abstract

Rice *(Oryza sativa L.)* is planted in about a tenth of the arable area around the world and is the largest source of food energy for half of humanity. Climatic change with increasing frequency of severe and prolonged drought periods and significant increases in air temperature has affected global rice production. Therefore, generating mutant rice cultivars tolerant to high temperatures and low water supplies is of utmost importance. Advanced mutant rice lines which were derived from irradiated Amistad -82 and J-104 were evaluated in the field under high temperatures and low water supply conditions using Amistad-82 variety as control. The genotypes with the best and worst field performances were compared using physiological parameters such as cell membrane thermostability, pollen viability, lipid peroxidation, and peroxidase and catalase activity under high temperature conditions. Three mutant lines, 8852, 8552 and LP-12 showed high yielding under high temperatures and low water supplies conditions in the field andalso showed better pollen viability, cell membrane thermo stability, lipid peroxidation and peroxidase than LP-16 mutant lines and the control cv. Amistad-82.These results show that the physical irradiation of seeds with protons followed by subsequent *in vitro* embryo culture using 2,4D maygenerate genetic variability for tolerance to high temperatures.The variation observed for the physiological and biochemical indicators evaluated could be used for the early selection of high temperature tolerant rice genotypes.

Key Words: Heat stress, rice, mutation, biochemical indicators, proton beam, selection, pollen viability.
Abbreviation: A-82, Amistad-82, FAO – Food and Agricultural Organization, CAT– catalase, CMCT–cell membrane thermo-stability, 2-4D – diclorofenoxiacetic acid, J-104, Jucarito-104, MDA – manoldialdehide, POX– peroxidase, Kin – Kinetine.

Introduction

Rice (*Oryza sativa L.*) is one of the most important crops produced worldwide and is the staple food for more than a half of the world's population (Yang and Zhang, 2006). With respect to yield, rice is the second most produced crop after maize (*Zea mays* L.) and is the second highest crop after wheat with respect to the harvested area. Rice provides more calories than other cereals (Acevedo et al., 2006). Therefore, rice is widely grown in many over-populated countries including China, India, Pakistan, Indonesia, Philippines and Japan, where it accounts for about 92% of the world's total rice production (Kondamudi et al., 2012).

Rice is a main crop in Cuba where annual consumption is about 70kg per person, which is well above that of other countries of the American continent. However, rice yields in Cuba are low, around 3t/ha and do not meet the national demand (Morejón et al., 2012). Studies revealed that low rice yields in Cuba are associated with different types of abiotic and biotic stresses. Heat has been identified as the most important environmental stress limiting yield. Heat stress alters molecular and physiological processes affecting crop growth and yield (Mittler et al., 2012; Hasanuzzaman et al., 2013). Plants can modify their metabolism in various ways in response to heat stress, notably by generating compatible solutes that are able to help maintain proteins and cellular structures, maintain cell turgor pressure, and modify anti-oxidant mechanisms to re-establish cellular redox homeostasis (Munns and Tester, 2008; Janska et al., 2010). Key physiological and biochemical indicators of heat stress tolerance include electrolyte leakage and lipid peroxidation levels i.e. Malondialdehyde (MDA) content and anti-oxidant enzyme activity (Campos et al., 2003; Heath et al., 1986; Bajji et al., 2001; Nakano and Asada, 1981; Oberley and Spitz, 1985).

Mutation induction can contribute to plant improvement when combined with efficient screening methods. Plant mutation breeding has been very successful in rice and has produced around 850 mutant rice cultivars (FAO /IAEA Mutant variety database, 2017). Mutation breeding in rice has not only been successful in higher yielding cultivar production, notably the semi-dwarfs, but has also produced mutants for biotic and abiotic stress tolerance. Gamma irradiation in mutation breeding is an effective technique in creating new variation for the rapid breedingof new cultivars (Tembo and Munyinda, 2015). In Cuba, irradiation with protons has been used to generate genetic variability and a new rice cultivar with good productive potential and salinity tolerance has been developed at the National Institute of Agricultural Sciences (INCA) (González et al., 2009). The objective of the current study was to evaluate mutant lines generated byproton beam and in vitro culture to select for high temperature tolerance, which may better adaptto climate change stressors.

Results

Evaluation of advanced mutant lines under high temperature condition in the field

The mutant lines and cv. A-82 showed difference as compared to the LP-16 mutant line in all evaluated characters. All mutants except for LP-16 out-performed the control cv. A-82 in yield, number of panicles per plant and number of full grains per panicle. The mutants,8552, 8553 and LP-12 out-performed

the rest of the mutant lines as well as the A-82 Cuban cultivar (parent) in the number of panicles per m^2, the number of full grains per panicle, the weight of 1,000 grains and the yield under high temperature and low water supply conditions. A poor performing genotype under field conditions was from the mutant line LP-16 which recorded several negative traits for yield and yield components. This mutant has good agronomical performance in the winter season, but it is susceptible to high temperature which has strong negative effects on grain filling. Three of the best-performing mutants under the high temperature condition were8552, 8553 and LP-12, and these were selected for evaluation in farmer's fields with high scale production in multi-locational trials. The mutant LP-16 and A-82, which showed poor performance in those conditions, were selected as negative controls for the physiological and biochemical evaluations.

Effect of high temperature on physiological and biochemical parameters

Two physiological parameters, cell membrane thermostability and pollen viability, were used to characterise the mutants (LP-12, 8552, and 8553) for tolerance to high temperatures and compared to LP-16 and cv. A-82.

The susceptible LP-16 showed lower cell membrane thermostability than the other genotypes used for the analysis under heat stress conditions. Highest cell membrane thermostabilities under heat stress, however, were observed for 8553, 8552 and LP-12 mutants. These mutant lines also showed remarkably better performance than cv. A-82.

As for the pollen viability (Table 1), significant differences were observed among the genotypes. When the genotypes were exposed to the same heat stress, the effect was very strong on the susceptible LP-16 genotype, as its performance also coincided with the field data at high temperatures. Among the four lines tested, the lowest pollen viability was observed for LP-16 mutant. In contrast, the pollen viability of the LP-12, 8552 and 8553 mutants were two-fold higher than LP-16 line. Moreover, in the high temperature treatment, the pollen viability of LP-12, 8552 and 8553 mutants were only slightly lower thanthat of the control.

Biochemical evaluation of the selected mutant lines and the susceptible genotypes

For biochemical evaluation, we analysed catalase activity, peroxidase activity and malondialdehyde content among the selected mutant genotypes. The susceptible LP-16 and cv. A-82 were used as controls during the analyses.

Fig 1. shows the catalase activity difference between two treatments, 25°C as control and 40°C for 3 hoursas a heat stress. Compared to the control temperature, the catalase activity appearedin the mutant LP-12, 8552 and 8553 genotypesat the high temperature treatment of 40°C. In general, catalase activity among the mutants was significantly higher, and the highest value was observed in the mutant 8553 genotypes (Figure 2). The lowest level was observed in the mutant LP-16 genotype after heat treatment.

A similar pattern was observed among the mutant genotypes for peroxidase activity. These values are also given in figure 2. Compared to the control treatment, heat stress treatment resulted in higher peroxidase activity in the mutant LP-12, 8552 and 8553 genotypes. The peroxidase activity of these three mutants was higher than the control and the highest value was recorded for the mutant 8553. The lowest peroxidase activity among the mutants was- measured in the mutant LP-16 genotype, and this value was also significantly lower than that of the control treatment (Figure 3).

Various biochemical differences were observed in terms of the malondialdehyde content among the genotypes evaluated. Considering the high heat stress treatment, while the LP-16 genotype showed an increase in malondialdehyde content, the mutant 8553 genotypes did not show differences between the control and high temperature treatment. The 8552 and LP-12 genotypes, however, showed a slight decrease under stress conditions (Figure 4).

Discussion

Yield is the end product of plant cultivation and is the result of genotype interactions with the environmental abiotic and biotic factors. Hence, environmental stresses such as heat have become a yield limiting factor in many areas in the world (Wang et al., 2003). The increase in temperature has exposed most crops to heat stress during various stages of their life cycle. It is difficult topredict future climate change effects on agricultural productions (Watanabe and Kume, 2009).

Rice is relatively a tolerant plant to high temperatures during the vegetative stage; however, it is extremely susceptible during the reproductive stage, and particularly during the flowering stage (Jagadish et al., 2010). High or low temperatures during the flowering stage can affect fertilization and seed production of rice, and consequently yield (Das et al., 2014). Rice is mostly grown in those regions where current temperatures are close to optimum for rice production. Therefore, any variation, and particularly increases above optimum production temperatures even for short periodswill reduce grain yield.

Recent studies have estimated that rice yields may be reducedby 41% by the end of the 21st century due to high temperatures (Ceccarelli et al.,2010). For example, reduction in tillering and plant height during the vegetative stage and number of panicles and grain filling during maturation in the reproductive stagemay beexpected under high temperatures (Yoshida, 2006). Increasing temperatures also hinder translocation of photosynthats to grains due to elevated respiration rates (Morita, 2003; Zakaria et al., 2004). The optimum temperature range is 27 to 32°C (Yin et al.,1996) and the maximum temperatures that canbe endured is around 35°C; thus, the evaluation of genotypeperformances at 40°C in this studyis a good bench mark to differentiate the susceptible and tolerant mutant genotypes.

Genetic improvement represents a major tool in developing heat tolerant rice cultivarsand mutation induction is of importance in this respect. The IAEA Mutant Variety Database lists 85 drought-tolerant rice mutant cultivars, 31 salinity-tolerant rice genotypesand 11 high-temperature tolerant rice mutant cultivars (FAO/IAEA database, 2017). Most of these mutants have been induced by gamma irradiation, and no rice mutant cultivar has yet beenreported from proton treatments, which has been successfully used in Cuba to generate mutants tolerant to salinity and drought (González et al., 2008, González et al., 20l6). The present study also verified that the use of protons isadequate to induce tolerance to high temperatures.

Effect of high temperature on physiological and biochemical parameters

Pollen viability at high temperatures is a crucial factor for better grain set and yield. In riceplants, the flowering stage is highly sensitive to heat stress as drastic temperature fluctuations within the day and even at night affects fertility and seed production, and consequently, causes yield loss (Das et al.,2014; Ziska and Manalao, 1996). However, the selected mutant lines studied here produced pollen with high viability, thus guaranteeingfertilization and seed set under high temperatures. Furthermore, these selected mutants produced a high number of filled grain and better yield than their donor cultivar at high temperature conditions.

In terms of electrolytic conductance, this is correlated with the degree of heat stability of photosynthetic activity in isolated chloroplasts and with the degree of heat tolerance at the whole plant level (Sullivan, 1977). Hence, cell membrane thermostability can be considered as a significant indicator for heat tolerance. In this study, LP-16 showed a remarkablyhigh

level of damage in cell membrane thermostability relative to other genotypes and this genotype is known to be very susceptible to high temperature in field conditions.

We analysed three biochemical parameters: catalase activity, peroxidase activity and malondialdehyde content. Our study revealed that malondialdehyde content is not a prominent heat stress indicator, as selected mutants showed no significant differences underhigh temperatures. The malondialdehyde content increased in the susceptible LP-16 and donor A-82 genotype (this is an indicator of oxidative damage caused by the heat stress). However, the performances of the selected mutants LP-12, 8552 and 8553 were not so different relative to the donor A-82 genotype, i.e. there were no significant difference in malondialdehyde content after high stress treatment.

In contrast to the malondialdehyde content, the study revealed that peroxidase activity and catalase activity were significantly affected by heat stress and may be considered as indicators of heat stress (Dolatabadian and Saleh, 2009). Between the two indicators, peroxidase activity was the most distinctive as it showed the most dramatic differences. The reasons may be due to heat effects on cellular homeostasis, e.g. by the formation of reactive oxygen species (ROSs), which damage normal plant metabolism (Shao, 2008; Imlay, 2003). The actions of ROSs lead to non-selective oxidation of proteins and lipids (Joseph and Jini, 2011). To limit oxidative damage, plants have developed a series of detoxification systems, which are composed of enzymatic and non-enzymatic mechanisms. The enzymatic system involved in superoxide dismutase (SOD) eliminates oxygen-free (O_2^-) radicals and molecular oxygen, and finally produces hydrogen peroxide (H_2O_2).

The H_2O_2 is removed by the actions of catalases and peroxidases (Dolatabadian and Saleh, 2009). Several authors suggest that increased peroxidase and other enzyme activities in many plants are involved in the elimination of ROSs and provide protection from oxidative stress (Kartashov et al., 2008). Here the increase in peroxidase activity in the 8552, 8553 and LP-12 mutants can be associated with the response of these mutant genotypes to remove the H_2O_2 formed as a consequence of high temperature (Figure 1 and 2) (Blokina et al., 2003).

The evaluation of the physiological and biochemical indicators allowed associations to be made with field performance under high temperature stress. The mutants 8552, 8553 and LP-12 showed good agronomic performance under high temperature conditions in the field and this was associated with some specific physiological and biochemical responses (pollen viability, catalase and peroxidase activity) which may explain their tolerance. This study has highlighted associations between specific biochemical mechanisms and field performance in rice subject to heat stress. These indicators can differentiate between mutant lines and may be used more broadly as a pre-screen for heat tolerance before field evaluation.

Materials and methods

Seed of rice (Oryza sativa L.) cultivars J-104 and A-82 were treated with proton beam 20 Gy in DUBNA (González et al., 2009), in a reactor type IBR-2 of 1.5 Mev and a dose power of 0.36 Gy/min. The M1 seed matured embryos, and Amistad-82 (control) seeds matured embryos were in vitro cultured (2,4D (2mg.L-1) and BAP (2mg.L-1) to obtain callus and applied for regeneration in medium (Kin (4mg.L-1) and AIA (2mg.L-1). Selected in vitro derived mutant plantsmultiplied in green house condition as indicated in Figure 1. Seeds of best mutants were selected individually harvested for each generation, the best mutant lines were selected in under drought and heat stress conditions. This study evaluated 6 advanced lines (LP-7, LP-8, LP-9, LP-10, LP-12, LP-16) derived cv. Amistad-82 and together with five advanced M6 mutant lines: 8551, 8552, 8553, 8554 8555 obtained from the cv. J-104 and the local rice cultivar Amistad-82 (A-82) was used as a control (standard), LP-16 is also known as a susceptible line. Evaluation of agronomical performance of advanced mutant lines under high temperature condition in the field.

The advanced mutant lines and the varieties were grown in the field under high temperature (Fig. 1) using a randomized block design with three replications. Irrigation was provided with a drip water system. At harvest, plant height, number of panicles per m², full grains per panicle, unfilled grains per panicle, 1,000 grain weight and yield per m² were evaluated.

Evaluation of physiological and biochemical parameters of mutant lines under high temperature

Selected lines with the best performances in the field (LP-12, 8552, 8553) as well as the genotype with the worse performance (LP-16) and the control cv. Amistad-82 were tested under laboratory conditions for heat stress associated physiological and biochemical indicators to validate/support results and to identify heat stress tolerant genotypes.

Cell membrane conductivity

The 30-day-old leaf samples of rice genotypes were placed in vials with distilled water. The samples were placed in a temperature block at 40°C for 90 minutes, and controls at 25°C, with three replications per treatment. Electric conductivity solution of each vial was determined. Complete death of leaf samples was carried out by autoclaving and then tested for electric conductivity, as this factor is needed to determine cell membrane thermo-stability (CMT) according to following formula:

Table 1. Results of yield and yield related characters in some mutant lines and varieties evaluated under high temperature in the field conditions.

Genotypes	Panicle/m²	Full grain/panicle	Unfilled grain/panicle	1,000 grain weight	Yield (t/ha)
8551	250 c	83 c	22 cd	29.7 a	3.6 b
8552	289 a	95 a	19 d	28.9 b	4.2 a
8553	297 a	97 a	19 d	27.7 c	4.1 a
8554	255 bc	89 b	22 cd	28.5 b	3.4 b
8555	257 bc	87 b	21 d	27.5 c	3.2 bc
LP-7	264 b	88 b	23 cd	26.4 d	3.5 b
LP-8	266 b	92 ab	21 d	27.8 c	3.6 b
LP-9	267 b	88 b	27 c	27.0 d	3.5 b
LP-10	257 c	80 c	24 cd	28.0 c	3.1 c
LP-12	290 a	93 ab	21 d	27.5 c	4.2 a
LP-16	56.7 e	15 d	67 a	25.5 d	1.0 d
Amistad-82	220 d	78 c	36 b	27.4 c	3.0 c
ES x	10.21 *	4.91*	4.45*	0.42*	0.31*

*Means followed by a common letter(s) aren't significantly differed at 0.05 level by Tukey test.

Fig 1. Applied procedure to obtain advanced lines.

Table 2. Comparison of percentage value of pollen viability of four flower from each genotype. Stress treatmen (40° C for 1 hours) in growth chamber and the control (25° C for 3 hours) from indendent sample test (T Students) and the cell membrane thermostability in rice varieties. Stress treatment (40° C for 90 minutes) and control (25° C) from Multiple Comparisonsanalisis (LSD).

Genotypes	Pollen viability (%)			Cell membrane thermostability
	Control	Stress	T (sig.)	
8553	90.3	79.7	1.269(0.264)	85.6 a
8552	91.4	88.3	0.548 (0.603)	81.2 b
LP-12	89.5	78.6	4.9180 (0.08)	75.4 c
Amistad 82	91.3	58.9	14.766(0.00)	65.7 d
LP-16	82.3	36.2	36.014(0.00)	45.6 e
Std. Error				1.5556

*The mean difference is significant at the 0.05 level.

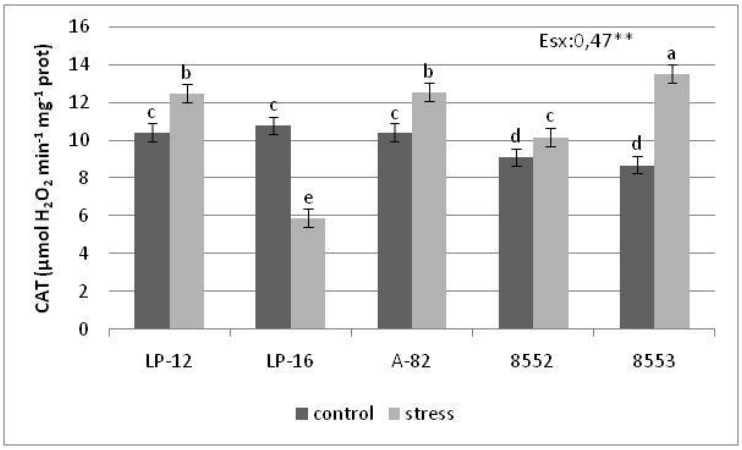

Fig 2. Specific activity of catalase (CAT) in leaves of rice mutants under stress temperature of 40°C for 3 hours (stress) and 25°C (control).

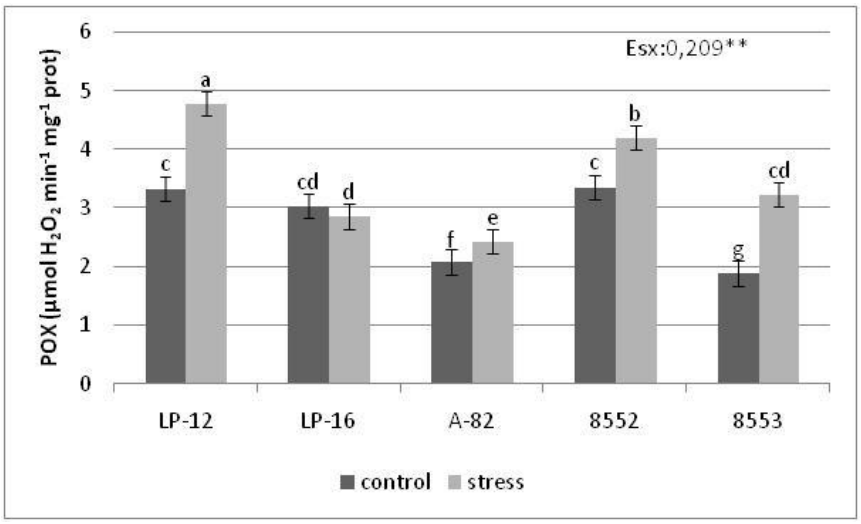

Fig 3. Peroxidase (POX) activity in leaves of rice mutants under stress temperature of 40°C for 3 hours (stress) and 25°C (control).

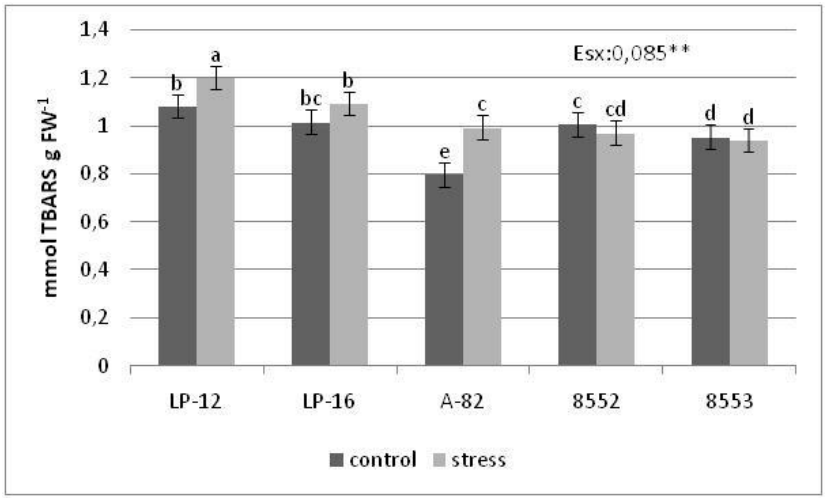

Fig 4. Malondialdehyde (MDA) in leaves of rice mutants under stress temperature of 40°C for 3 hours (stress) and 25°C (control).

CMT (%) = (1-(T1/T2)/(1-(C1/C2) X100
T1: First conductivity measurement after heat treatment,
T2: Second conductivity measurement (after autoclaving) of heat treatment explants,
C1: First conductivity measurement of control,
C2: Second conductivity measurement (after autoclaving) of control.

Pollen viability

At the time of flowering one individual panicle per plant from five plants were treated with heat at 40°C for one hour in Petri dishes.
The pollen grains were extracted and stained with cotton blue in lactophenol. The numbers of coloured and non-coloured pollen grains were identified with an optical microscope and the percentage of pollen viability was determined.

Biochemical evaluation

Seeds of rice lines (LP-12, LP-16, A-82, 8552, 8553) were placed in boxes with Red ferritic soil and organic matter (3:11v/v). After 30 days, they were placed in boxes under high temperature condition (40°C for 3 hours) controlplants were given 25°C in a germination cabinet. About 0.25 g of frozen leaves of the genotypes LP-12, LP-16, 8552 and 8553 per treatment were homogenized with $2.5x10^{-3}$ mol L^{-1} of tampon phosphate of potassium, 0.1 mol L^{-1} pH 7.8; EDTA $1.0x10^{-4}$ mol L^{-1}; Newt x-100 0.1% (v/v), PVPP 1.5 % (p/v) and 2.5 µg L^{-1} of inhibitors of proteases, aprotinin and leupeptin. The homogenate was centrifuged at 13,000 rpm for 20 minutes, and then the supernatant was used for further analyses of the lipid peroxidation and some enzymatic activity.

Peroxidase activity

Peroxidase (POX; EC 1.11.1.1) was measured by monitoring the formation of tetra guaiacol at 470 nm (ε =26.6 $mM^{-1}cm^{-1}$). The formation of "rusty" guaiacol was measured in a spectrophotometer UV/Visible (Genesys 6, Thermo Electron Corporation) by 5 second incremental absorbance at 420 nm at an interval of 30 seconds (Bergmeyer, 1974).

Catalase activity

Catalase (CAT; EC 1.11.1.6) was determined according to the method of Aebi (1984) in a reaction mixture containing 50 µl enzyme extract in 50mM sodium phosphate buffer (pH 7,0). The decomposition of the H_2O_2 was evaluated every 10 seconds over one minute and the quantity of H_2O_2 was considered for the absorbance to 240 nm (extinction coefficient of the H_2O_2 to 240 nm; 43,6 $M^{-1}cm^{-1}$) in a spectrophotometer UV/Visible (Genesys 6, Thermo Electron Corporation).

The Malondialdehydecontent

Malondialdehyde (MDA) as an indicator of lipid peroxidation was determined according to the method described by Hodges et al. (1999). The absorbance was measured between 532 and 600 nm in a spectrophotometer UV/Visible (Genesys 6, Thermo Electron Corporation). Correction was applied by subtracting the absorbance at 600 nm using extinction coefficient of 156 mM^{-1} cm^{-1} (Madhava Rao and Stresty, 2000).

Statistical analysis

Data for pollen viability, cell membrane conductivity and the biochemical evaluation were expressed as means of four plants in triplicate experiments and their means and the standard deviations were calculated. Data for field evaluation for each character was carried out as a simple variance analysis and the mean differences were calculated using the Tukey test.

Conclusions

Rice is a staple food and energy source for almost half the world's population. The climatic factors such as drought and high temperature negatively affect rice production. Hence, generating mutant cultivars tolerant to these parameters areof utmost importance. In this study, advanced mutant rice lines were evaluated, and their performances were compared with stress-related physiological and biochemical parameters in field conditions. Considering that the upper endurancelimit for rice is 35ºC, we made our analyses at 40ºC and identified three mutant lines, 8852, 8552 and LP-12, showing tolerance to high temperatures. The selected mutantsshowed noticeably better yield and yield component performance than the susceptible lines. Physiological parameters were identified as significant indicators for heat tolerance. Regarding the biochemical parameters; while the malondialdehyde content wasidentified as not a prominent indicator, cell membrane thermostability, the peroxidase and catalase activitieswere determined as important heat stress indicators due to their varying contentsand associations with field performances in tolerant and susceptible genotype. Evaluation of the physiological and biochemical indicators together allowed us to make better field analyses for high temperature and heat stress parameters. These indicators may also be used more broadly as a pre-screen for heat tolerance before field evaluation.

Acknowledgements

We thank the support of IAEA through the Coordinated Research Project 23029 "Climate proofing of food crops: genetic improvement for adaptation to high temperatures in drought prone areas and beyond".

References

Aebi H (1984) Catalase in vitro. Meth Enzym. 105:121–126.

Acevedo M, Castrillo W, Belmonte U (2006) Origen, evolución y diversidad del arroz. Agron Tropical. 56 (2):151–170.

Bajji M, Kinet JM, Lutts S (2001) The use of the electrolyte leakage method for assessing cell membrane stability as a water stress tolerance test in durum wheat. J Plant Growth Regul. 1:1–10.

Bergmeyer HU (ed) (1974) Peroxidase (En: HU Bergmeyer, ed, Methods of Enzymatic Analysis Academic Press Inc.) 2:685–690.

Blokina O, Virolainen E, Fagerstedt K (2003) Antioxidant oxidative damage and oxygen deprivation stress. Annu Rev Annal Bot. 91:179–194.

Campos PS, Quartin V, Ramalho JC, Nunes MA (2003) Electrolyte leakage and lipid degradation account for cold sensitivity in leaves of Coffea sp. Plants J Plant Physiol. 160:283 –292.

CecarelliS, Grando S, Maatougy M, Michael M, Slash M, Haghparast R, Rahmanian M, Taheri A, Al-Yassin A, Benkelkacem A, Labdi M, Mimoun H, Nachit M (2010) Plantbreedingandclimatechanges. J Agr iSci. 148:627–637.

Das S, Krishnan P, Nayak M, Ramakrishnan B (2014) High temperature stress effects on pollens of rice (*Oryza sativa* L.) genotypes. Environ Exp Bot. 101:36–46.

Dolatabadian A, and Saleh R (2009) Impact of exogenous ascorbic acid on antioxidant activity and some physiological traits of common bean subjected to salinity stress. Not Bot Horti Agrobot Cluj Napoca. 37:165–172.

FAO/IAEA (2017) Mutant Variety Database. www.mvd.iaea.org.

González MC, Perez N, Cristo E, Rodriguez M (2008) Cuban rice mutants obtained from protons radiations. Plan Mut Rep. 2(1):9–13.

González MC, Pérez y N, Cristo y Eç, Rodríguez M (2009) Development of salinity-tolerant rice varieties using Biotechnological and Nuclear Techniques. Induced Plant Mutation in the Genomic Era. FAOUN Rep.138–140.

González MC, Pérez y N, Cristo E (2009) GINES: Primer mutante de arroz obtenido a partir de la irradiación con protones. Cultivos Tropicales. 30:3–4.

Heath RL, Packer L (1986) Photoperoxidarion in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys. 125:189–198.

Hodges M, DeLong D, Forney JM, Prange CF (1999) Improving the thiobarbituric acid-reactive-substances assay for lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. Planta. 207:604–611.

Imlay JA (2003) Pathways of oxidative damage. Annu Rev Microbiol. 57:395–418.

Hasanuzzaman M, Nahar K, Fujita M (2013) Extreme temperatures, oxidative stress and antioxidant defense in plants. In: Vahdati K. Leslie C. (eds). Abiotic stress-plant responses and applications in agriculture. Tech Rijeka, pp. 169–205 (2013) Croatia.

Jagadish SVK, Muthurajan R, Oane R, Wheeler TR, Heuer S, Bennett J, Craufurd PQ (2010) Physiological and proteomic approaches to dissect reproductive stage heat tolerance in rice (*Oryza sativa* L.). J Exp Bot. 61:143–156.

Janska A, Marsik P, Zelenkova S, Ovesna J (2010) Cold stress and acclimation: What is important for metabolic adjustment. Plant Biol. 12:395–405.

Joseph B, and Jini D (2011) Development of salt stress-tolerant plants by gene manipulation of antioxidant enzymes. Asian J Agric Res. 5(1):17–27.

Kartashov AV, Raciyukina NL, Ivanov YV, Pashkovskii PP, Hevyakova N, Kuznetsov VV (2008). Role of antioxicant systems in wilci plant adaptation to salt stress. Russ J Plant Physiol. 55:463–468.

Kondamudi R, Swamy KN, Vishnuprasanth D, Rao YV, Rao PR, Sarla N, Subrahmanyam R, Voleti SR (2012) Heat stress in rice – physiological mechanisms and adaptation strategies. Crop Stress and its Management: Perspectives and Strategies, Springer Science, February. 2012.

Madhava Rao KV, and Sresty TVS (2000) Antioxidative parameters in the seedlings of pigeonpea (*Cajanus Cajan L.* Millspaugh) in response to Zn and Ni stresses. Plant Sci. 157:113–28.

Mittler R, Finka A, Goloubinoff P (2012) How do plants feel the heat? Trends Biochem Sci. 37:118–125.

Morejón RR, Díaz SH, Hernández JJ (2012) Comportamiento de tres variedades comerciales de arroz en áreas del complejo agroindustrial arrocero los palacios. Cultivos Tropicales. 33(1):46–49.

Morita S (2003) Effects of high air temperature on ripening in rice plants-analysis of ripening performance in growth chamber experiments. Jpn J Crop Sci. 69 (3):391–399.

Munns R, and Tester M (2008) Mechanisms of salinity tolerance. Ann Rev Plant Biol. 59:651–681.

Nakano Y, and Asada K (1981) Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiology. 22:867–880.
Oberley LW, and Spitz DR (ed) (1985) Assay of superoxide dismutase using nitroblue tetrazolium. In: Greenwald RA, handbook of methods for oxygen radical research. Boca Raton, FL: CRC Press pp. 217–227.
Shao H (2008) Higher plant antioxidants and redox signalling under environmental stresses. C R Biol. 331(3):433–441.
Sullivan CY, Norcio NV, Eastin JD (ed) (1977) Plant response to high temperature. "genetic diversity in plants", eds by A. Muhammed, R. Aksel, and R. C. Von Borstel, Plenum Publishing Corp. 301–317.
Tembo L, and Munyinda K (2015) Clustering common bean mutants based on heterotic groupings. AJOL. 3(1): 1–7.
Wang W, Vinocur B, Altman A (2003) Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Planta. 218(1): 1–14.
Watanabe T, and Kume T (2009) A general adaptation strategy for climate change impacts on paddy cultivation: special reference to the Japanese context. Paddy Water Environ. 9:313–320.
Yang J, and Zhang J (2006) Grain filling of cereals under soil drying. New Phytol. 169:223–236.
Yin X, Kroff MJ, Goudriann J (1996) Differential effects of day and night temperature on development to flowering in rice. Annal Bot. 6:203–213.
Yoshida Y (2006) Fundamentals of rice crops science. International Rice Research Institute, Los Banos, Philippines, August 2006.
Zakaria S, Matsuda T, Tajima S, Nitta Y (2004) Effect of high temperature at ripening stage on the reserve accumulation in seed in some rice cultivars. Plant Prod Sci. 5(2):160–168.
Ziska LH, and Manalo PA (1996) Increasing night temperature can reduce seed set and potential yield of tropical rice. Aust Plant Physiol. 23(6):791–794.

DOI: 10.21475/ajcs.21.15.09.sp-6

Australian Journal of Crop Science AJCS

Heat stress-induced changes in shoot and root characteristics of genotypes of tepary bean (*Phaseolus acutifolius* A. Gray), common bean (*Phaseolus vulgaris* L.) and their interspecific lines

Ligia Carmenza Muñoz[1*], Mariela Rivera[2], Jaime E. Muñoz[1], Fatma Sarsu[3] and Idupulapati M. Rao[2]**

[1]Universidad Nacional de Colombia- sede Palmira, Carrera 32, Chapinero, Palmira, Colombia
[2]Centro Internacional de Agricultura Tropical (CIAT), A.A 6713, Cali, Colombia
[3]Plant Breeding and Genetics Section, Joint FAO/IAEA Division, NAFA, Austria

****Present address: Plant Polymer Research Unit, National Centre for Agricultural Utilization Research, Agricultural Research Service, United States Department of Agriculture, 1815 North University Street, Peoria, IL 61604, USA**

***Corresponding author: lcmunozf@unal.edu.co**

Abstract

Heat stress is a major limitation to grain yield in common bean (*Phaseolus vulgaris* L.). Tepary bean (*Phaseolus acutifolius* A. Gray) is better adapted to heat stress than common bean. Ten tepary bean accessions, four common bean genotypes and four interspecific lines involving *P. vulgaris* and *P. acutufolius, P. coccineus* and *P. dumosus* were evaluated for tolerance to heat stress conditions induced under greenhouse conditions and these were compared to plants grown under ambient temperatures. The high temperature treatment was 29 ±5 °C during the day and was >24 °C (up to 27 °C) during the night, while the ambient temperature (AT) treatment was 25 ±5 °C during the day and 19± 2 °C at night. The genotypic differences were evaluated for morpho-physiological characteristics of shoot and root and also yield components. The Genotype and Genotype × Temperature interactions were significant for all shoot and root morpho-physiological characteristics evaluated. Higher temperature (HT) significantly affected leaf photosynthetic efficiency, total chlorophyll content, and stomatal conductance. The effect was positive or negative, depending on the genotypes. Tepary accessions showed reduced total chlorophyll content, while common bean genotypes and the interspecific lines were less affected. Tepary accessions also showed reduced stomatal conductance, but increased leaf photosynthetic efficiency under HT. Common bean genotypes increased stomatal conductance and decreased leaf photosynthetic efficiency. High temperature decreased total root length, specific root length and pod biomass compared to ambient conditions, but there was no marked effect on pollen viability of the tested genotypes. The superior adaptation of tepary germplasm accessions to high temperature is attributed to their ability to regulate stomatal opening and photosynthetic efficiency, together with a superior ability to remobilize photosynthates from older leaves to pods during physiological maturity.

Keywords: Abiotic stress; bean crop improvement; chlorophyll content; heat tolerance; photosynthetic efficiency; stomatal conductance.
Abbreviations: AT, ambient temperature; DAS, days after sowing; LA, leaf area; HT, higher temperature; PCA, Principal Component Analysis; QY, Efficiency of photosystem II; SPAD, Soil Plant Analysis Development.

Introduction

Current climate change estimates predict a rise in global temperatures between 1.4 °C and 3 °C by 2050 and region-specific variability in precipitation (IPCC, 2007). These dramatic climate scenarios over the short-term result in worrying predictions of crop yield reduction, especially in abiotic stress-sensitive crops such as common bean (Porch et al., 2013). Common bean, *Phaseolus vulgaris* L., is a key grain legume crop and a vital source of nutrition worldwide. However, abiotic and biotic constraints to its production result in an average global yields of 600 kg.ha^{-1} (Porch et al., 2013). Common bean originated from the highlands of Central America and the Andes (Gepts and Debouck, 1991). The optimal average daily temperature for reproductive development ranges from 20 °C to 25 °C. Temperatures > 30 °C during the day or > 20 °C during the night result in yield reduction (Rainey and Griffiths, 2005a). Heat sensitivity is a major limiting factor in the production of common bean, causing reduced yields, lower product quality and restricted geographical adaptation (Rainey and Griffiths, 2005b).

As an ecologically contrasting species, *Phaseolus acutifolius* A. Gray (tepary bean) is grown as a traditional crop of desert and semi-arid regions of Mexico and southwestern USA (Freeman, 1912; Nabhan and Felger, 1978). Tepary bean is cultivated successfully in places where high temperature and drought are common, making it a valuable crop for dryland environments. Compared to common bean, tepary bean possesses many traits that enable it to flourish in hot and dry regions. It is more heat tolerant at the tissue level and produces more leaves to compensate for reduced leaf size due to heat stress (Lin and Markhart, 1996). The substantially greater level of heat tolerance in tepary bean, compared with common bean, has been attributed to a lower level of sensitivity of the mitochondrial electron transport metabolism during photosynthesis (Lin and Markhart, 1990). Tepary bean also has a more extensive and thinner root system, better stomatal

control and more active para-heliotropism than common bean (Markhart., 1985; Bielenberg et al., 2003; Butare et al., 2012).
Tepary bean is part of the tertiary gene pool of common bean and is considered as a potential gene donor of heat-tolerance traits to common bean through interspecific hybridization (Muñoz et al., 2004; Blair et al., 2012). It is also considered as a valuable crop for dryland environments by itself or through inter-specific breeding (crosses between tepary bean and common bean, Mejia et al., 1994). An evaluation of tepary bean genome introgression showed that tepary DNA can be transferred to the interspecific progeny (Muñoz et al., 2004). But success is limited to a lower than expected percentage of genome contribution (Blair et al., 2012). The introgression of heat or drought tolerance from tepary into common bean might be feasible through breeding to generate elite lines that could tolerate up to 4 °C higher than the normal range of temperature tolerance (Muñoz et al., 2004, 2006).
The main objectives of this study were to: (i) determine phenotypic differences in heat stress-induced changes in shoot and root morpho-physiological characteristics of tepary bean and common bean and their interspecific lines; and (ii) identify heat tolerant genotypes that could serve as parents in breeding programmes that aim to improve heat tolerance in common bean.

Results

Effect of temperature on shoot and root morpho-physiological characteristics

Shoot traits
The total shoot biomass at mid-pod filling include: stem, leaf, dead leaf and the pod biomass. There was no difference of production of shoot biomass between high temperature (HT) and ambient temperature (AT) treatments. However, the average was higher under HT (13.1g/plant) compared to the AT (8.6g/plant) mostly because of the superior performance of both tepary accessions and interspecific lines. There were differences between the genotypes and the interaction between the Genotype x Temperature condition (P≤0.05) in the combined analysis. There were also differences between genotypes (P≤0.01) under AT but not under HT conditions.
Under HT and AT, the interspecific lines showed the highest production of shoot biomass, compared to tepary accessions and common bean lines. But the shoot biomass production was higher under HT (Table 2). Under AT, differences (P≤0.05) between the average values of shoot biomass were observed between the genotypes that showed the highest average: DAB 295 (common bean) G40022, G40159 (tepary) and ALB 91 and INB 827 (interspecific) and the genotypes: Calima (common bean) and G40001 (tepary) that produced the lowest average shoot biomass (Table 2).

Pod biomass and number of pods
There was no difference in pod biomass (g/plant) between HT and AT, but the average was higher under HT (6.1 g/plant) compared to AT (4.4 pods/plant) condition. There were only differences between the genotypes (P≤ 0.01) in the combined analysis and under AT conditions (P ≤ 0.01). The tepary accessions showed a higher pod biomass (7.9 g/plant) than the common bean (2.7 g/plant) or the interspecific lines (4.9 g/plant) under HT (Table 2). A significant difference (P ≤0.01) was observed between the G40001 accession versus common bean and the interspecific lines. Differences were also observed between common bean versus interspecific lines. Under AT, the tepary accessions showed a higher pod biomass (5.7 g/plant), compared to common bean (1.5 g/plant) and the interspecific lines (4.1 g/plant) (Table 2). Differences (P ≤0.01) were observed between the G40001 versus tepary accessions and the interspecific lines (P ≤0.01). A highly significant difference (P ≤0.01) was also observed between tepary versus common bean and common bean versus the interspecific lines, for the pod biomass under AT.
For the number of pods, there was no difference between HT and AT treatments, but the average was higher under HT conditions (12.3 pods/plant), compared to AT treatment (8.6 pods/plant). There were differences between the genotypes and Genotype x Temperature interactions (P≤ 0.01) in the combined analysis. Also, there were differences (P≤ 0.01) between genotypes under HT and AT. Under HT, the interspecific lines showed the highest number of pods/plant (14.3) followed by tepary (12.4) and common bean genotypes (9.4) (Table 2**)**. Under AT, tepary showed a higher number of pods/plant (10.7) compared to common bean (4.4) and the interspecific lines (7.3) (Table 2). There were average differences (P≤0.05) in pod number between the two tepary accessions (G40159 and G40022) that showed the highest number of pods /plant (17 to 18) and the total group of common beans and the interspecific lines (Table 2).

Dead leaf biomass
Under AT, there were differences in dead leaf biomass between the genotypes and the Genotype x Temperature interaction (P≤ 0.05) in the combined analysis. The average was higher (2.65 g/plant) under HT condition compared to AT (0.19 g/plant). There were differences in the production of dead leaves at the mid-pod filling growth stage, between the genotypes (P≤ 0.05) but only in the combined analysis. Under HT conditions the tepary accessions showed the highest production of dead leaves at mid-pod filling (4.58 g/plant), while the common bean and interspecific lines showed values lower than 1g/plant (Table 2). The three tepary bean accessions: G40022, G40001 and G40200 showed a remarkable high average production of dead leaves at mid-pod filling (15.8, 14.8 and 10.9 g/plant, respectively) (Table 2). The other tepary, common bean genotypes and the interspecific lines showed an average production of dead leaves at mid-pod filling lower than 1g/plant. Differences (P≤ 0.05) were observed between all groups of genotypes that were compared. Under AT all genotypes, showed a production of dead leaves at mid-pod filling, lower than 1g/plant (Table 2).

Root traits
With respect to root length and biomass, there was no difference between HT and AT treatments, but the average root length was higher under the AT (1,645cm/plant), compared to HT (1,318 cm/plant), whereas root biomass was higher under HT (1.02 g/plant) compared to AT (0.78 g/plant). For both traits, there were differences between genotypes (P ≤ 0.01) in the combined analysis and also under HT and AT (P≤ 0.01, data not shown). Under HT, the tepary accessions showed the lowest average root length (937 cm/plant) and biomass (0.67 g/plant)) compared to the common bean (1,762 cm/plant and 1.13g/plant, respectively) and the interspecific lines (1,828 cm/plant and 1.73 g/plant). Differences (P≤ 0.05) in root length were observed between the G40001 accession and common bean lines and also the interspecific lines, while the root biomass trait showed differences (P≤ 0.01) only between this accession and the interspecific lines. A significant difference (P ≤ 0.05 and P ≤ 0.01) was observed between the tepary accessions, the common bean and the interspecific lines for root length and biomass, respectively. The difference between the common bean and the interspecific lines was only significant (P≤ 0.05) for root biomass (data not shown). Under AT, the same trend was observed as under HT condition, tepary showed the lowest average root length and biomass (1,196 cm/plant and 0.53 g/plant, respectively) compared to the common bean (1,935cm/plant and 0.89g/plant) and interspecific lines (2,479 cm/plant and 1.28 g/plant). There were differences between all groups of genotypes compared for root biomass, while differences (P≤ 0.05) in root length were observed only

between G40001 accession versus the interspecific lines and tepary versus common bean and interspecific lines (P≤ 0.01). There were differences (P≤0.05) in the average diameter of roots between HT and AT in the combined analysis. But there was no difference between genotypes in any of the analyses (data not shown).

High temperature, reduced specific root length (relation between root length and root biomass of the plant) in all *Phaseolus* genotypes evaluated, indicating that roots are thinner under heat stress. Under HT and AT, the common bean lines showed higher specific root lengths, while tepary accessions showed intermediate and the interspecific lines showed the lowest values (Table 2). Under HT, tepary accessions showed low values for total root length and an intermediate specific root length (Table 2), indicating that the roots were relatively short and with average thickness. Common bean showed higher values of total root length and higher values of specific root length, indicating that the roots were relatively long and thin, while the interspecific lines showed intermediate values for both total root length and specific root length, indicating that these roots were relatively long and moderately thick. The same trend was observed under AT conditions.

Days to flowering

The average number of days to flowering (31 days) did not differ between HT and AT treatments (Table 3). There were differences (P ≤ 0.01) between genotypes in the combined analysis and also under HT and AT. HT induced early flowering in tepary, 26 days after sowing (DAS), compared to common bean, 40 DAS and the interspecific lines, 34 DAS (Table 3). Differences (P ≤ 0.01) were observed between the G40001 accession and common bean and also with the interspecific lines. A highly significant difference (P ≤ 0.01) was observed between: the tepary accessions, the common bean and the interspecific lines. The difference between the common bean and the interspecific lines was significant (P≤ 0.01). Under AT, the time to flowering was reduced for the common bean lines, while in tepary and the interspecific lines, the time to flowering was little affected (Table 3). A highly significant difference (P ≤ 0.01) was observed between the tepary accessions, the common bean and the interspecific lines. In relation to the pollen viability, there was no difference between HT and AT, for the 18 genotypes tested (data not shown).

Leaf area production

There were differences in leaf area (LA) production between genotypes and the Genotype x Temperature interaction (P ≤ 0.01) in the combined analysis. The average was higher under HT (721 cm^2) compared to AT (429 cm^2). There were differences between genotypes (P ≤ 0.01) under HT and AT. Under HT, tepary showed a lower value of LA (327 cm^2/plant), compared to common bean (1,198 cm^2/plant) and interspecific lines (1213 cm^2/plant, Table 3).

Photosynthetic efficiency

The combined analysis showed that temperature had a significant effect (P ≤ 0.05) on leaf chlorophyll content (SPAD readings), QY (quantum yield) efficiency of photosystem II, and leaf stomatal conductance. The SPAD reading was higher in AT (39.8) compared to HT (30.8). The efficiency of photosystem II variable showed higher values (0.66) under HT compared to AT (0.58). There were significant differences (P ≤ 0.01) in both variables among genotypes and for the Genotype x Temperature interaction. There was an interaction with leaf stomatal conductance (P ≤ 0.01). The analysis under HT showed differences between genotypes for leaf chlorophyll content SPAD readings and the efficiency of photosystem II, while under the AT, only SPAD readings and leaf stomatal conductance showed differences among the genotypes. Under HT condition, tepary showed a lower average value (26.3) in SPAD readings, compared to common bean (34.1) and the interspecific lines (37.2). Differences (P ≤ 0.05) were only observed between tepary and common bean and between tepary and the interspecific lines (Table 3).Under HT, the SPAD readings in tepary accessions decreased markedly: the values were reduced from 42.3 in AT to 26.3 under HT (Table 3). The average values of SPAD readings in the common and the interspecific lines were not affected significantly (Table 3). Highly significant differences (P ≤ 0.01) between the G40001 accession versus common bean lines, between tepary accessions versus common bean and between common bean and interspecific lines (P ≤0.05) were observed. Under the HT treatment, there was no difference in average QY efficiency of photosystem II, between tepary and the interspecific lines (0.68), while the common bean lines showed a lower value (0.57). Under AT, the common bean and the interspecific lines showed the same value (0.60), while tepary showed a lower value (0.57) (Table 3). With respect to the leaf stomatal conductance, tepary accessions showed a lower average (227) compared to common bean (312) and interspecific lines (323) under HT (Table 3). The tepary accessions and the interspecific lines, increased the value of the leaf stomatal conductance, while the common bean decreased it, under AT (Table 3).

Correlation among shoot and root morpho-physiological variables

Positive correlations (P≤0.01) were observed between shoot biomass and root biomass of plants under HT (r = 0.70) and AT (r = 0.68). Positive correlations were observed between shoot and pod biomass under both HT (r = 0.77) and AT (r = 0.76) and also between shoot biomass and LA under HT (r = 0.63) and AT (r = 0.55). Highly significant positive correlations (P≤0.01) were observed between pod biomass and pod number of plants under HT (r = 0.72) and AT (r = 0.85). Pod biomass was negatively correlated (P≤0.01) with number of days to flowering under HT (r = -0.65) and AT (r = -0.49). The tepary accessions that bloomed early had higher pod biomass under both conditions (Figures 1a, 1b). At HT, positive correlations were observed between pod biomass and the amount of dead leaves at the mid-pod filling stage (g/plant) (P≤0.01; r = 0.72) (Figures 2a, 2b), and also with the stem biomass (P≤0.05; r = 0.30) and root biomass (P≤0.05; r = 0.342). With respect to morpho-physiological variables, positive correlations were observed between pod biomass and SPAD readings (P≤0.01; r =0.37) under AT. For the root traits, positive correlations (P≤0.05) were observed between total root length and stem biomass of plants under HT (r = 0.33) and AT (P≤ 0.01, r = 0.43). Total root length was correlated with stem diameter under HT (P≤0.01; r = 0.47) and AT (r = 0.53), with leaf biomass under HT (P≤0.01; r = 0.40) and AT (P≤0.01, r = 0.45) and also with the amount of dead leaves at mid-pod filling (g/plant) under HT (P≤0.05; r = 0.32). Total root length was positively correlated with the number of nodules (data not shown) under HT (P≤0.01; r = 0.54), AT (P ≤0.01, r = 0.61), and root biomass (P≤0.01) under HT (r = 0.54) and AT (r = 0.61). Under HT, highly significant positive correlations (P≤0.01) were observed between root biomass and number of pods and number of nodules. Under AT, the root biomass showed the same correlations, except with number of pods which was not correlated (Figures 2a, 2b). Under HT, the variables 'length' and 'biomass of the roots' were positively correlated with SPAD readings and leaf stomatal conductance. Total root length was positively correlated with leaf area (LA) under HT (P ≤ 0.01; r = 0.42) and AT (P ≤ 0.01, r = 0.46), and a correlation between root biomass and LA was observed under HT (P ≤ 0.01, r =0.74) and AT (P ≤ 0.01, r = 0.77).

Multivariate analysis of shoot and root morpho-physiological variables

Principal component analysis (PCA) was performed to identify the major components that could explain much of the total variations observed in the data. The PCA showed that under HT and AT the first four components represented 89% and the 86%, respectively, of the total variance (Table 4). Under HT, the first component accounted for 44% of the variance, the second 27%, the third 9%, and the fourth 8%, while under AT, the first component accounted for 40% of the variance, the second 29%, the third11 %, and the fourth 6.0%. The dominance of these four components in the PCA suggests that they contained the main variables that discriminate the genotypes evaluated under HT and AT (Table 4). The quantitative traits that separate genotypes in the first component included LA and total root biomass under HT and AT. Under HT, two different root traits: length and superficial area, separates genotypes, while the number of nodules separated the genotypes under AT. The traits that contributed most to the discrimination in the second component are shoot biomass, pod number, and specific root length under HT and AT. In the third component, the separation of genotypes was mainly due to: efficiency of photosystem II, days to flowering under HT and dead leaf biomass at mid-pod filling under AT. In the fourth component the main traits were, biomass and number of nodules under HT, and the efficiency of photosystem II and SPAD readings under AT (Table 4)

Discussion

Heat stress induced changes in shoot attributes

High temperature stress increased pod biomass of *P. acutifolis*, *P. vulgaris* and interspecific lines, but most notably for the tepary accessions, especially the heat tolerant line G40001, confirming the heat stress tolerance of this species (Rainey and Griffiths, 2005; Chaves, 2015). This is an unexpected result but this superior performance under heat stress conditions is due to the improved genetic adaptation of both tepary accessions and interspecific lines that were used for evaluation in this study. Regarding the common bean lines, A774 and BAT 477 were reported as elite lines for tolerance to abiotic stress based on having a higher number of seeds per plant (Ojeda, 2015). As far as interspecific lines are concerned, the increase of pod biomass under HT stress may be explained by the genetic contribution of *P. acutifolius* in the pedigree of the lines evaluated. Under HT condition, only tepary bean accessions increased dead leaf biomass (accelerated senescence) at mid-pod filling. Positive correlations were observed between this variable and the pod biomass production (Table 2). This correlation was especially important for G40022, G40001 and G40200 accessions, and it was attributed to a greater plant efficiency and remobilization of photosynthates to seed production (Beebe et al., 2009, Polania et al., 2016; Rao et al., 2016). Dry matter partitioning to pods in snap beans has been observed to be efficient for the heat-tolerant cultivar Haisbushi compared to the heat-sensitive cultivar Kentucky Wonder. The photosynthates from the vegetative parts were mobilized to pods for cv. Haisbushi but not for cv. Kentucky Wonder (Omae et al., 2007). Number of pods/plants decreased under HT condition for tepary bean G40022 and G40277 accessions, which is in agreement with the results of Rainey and Griffiths (2005). However, unlike the results reported by these authors under 35°C day/32 °C night conditions, the G40005 and G40278 accessions studied here showed increased number of pods/plants under stress conditions. This difference is possibly due to the HT conditions used in our study (29 °C ± 5 day/ >23 °C night (up to 27 ºC). Several authors suggest that the effect of night HT is more critical than day HT. This was confirmed under controlled conditions with night time temperatures of 27 °C (Porch and Jahn, 2001; Rainey y Griffiths, 2005a; Omae et al., 2012). Temperatures above 30 °C during the day or above 20 °C during the night result in yield reduction (Rainey and Griffiths, 2005). In our conditions, the HT treatment was 29 ± 5 °C during the day and more than 24 °C (up to 27 °C) during the night. We therefore consider that the bean genotypes evaluated in our study were growing under HT stress conditions. Since *P. acutifolius* is better adapted to higher night temperatures, it performs better under HT during the night than the lower night temperatures in the ambient temperature conditions.

Leaf area increased considerably in common bean and interspecific lines but not in tepary accessions, under HT condition. One accessions (G40022) had the lowest LA and also the highest pod biomass (Table 3). This suggests a greater translocation of photosynthates to the pods to the detriment of leaf growth occurred with HT. A significant negative correlation, between pod biomass and days to flowering indicated that earliness contributed to better performance under HT and AT. The tepary bean accessions that flowered earlier had higher pod biomass under both conditions. The HT decreased the number of days to flowering in tepary, while in common bean HT seemed to increase it.

The period of flowering was extended in the heat-sensitive genotypes, which may be a plant survival mechanism. Also, the early flowering of tepary beans was associated with tolerance to high temperatures.

Heat stress induced changes in root attributes

Yield differences in beans are partly determined by variability in the root system. Larger and deeper root systems are associated with greater tolerance to drought, due to increased soil water extraction. A greater capacity to develop roots that go deep into the soil can provide a better adaptation to conditions of water stress (White and Castillo, 1992; Polania et al., 2009, 2016). There is a direct correlation between tolerance of drought and heat stresses, since during heat stress water availability can be a limitation caused by high temperature. In the present study, high temperature decreased total root length and specific root length in tepary accessions, common bean and interspecific lines. Although in tepary bean accessions, the total root length decreased considerably, compared to the other genotypes under HT conditions. Tepary beans produced more pod biomass and pods per plant, especially for the G40001 accession, suggesting that their tolerance to heat stress is due to a greater efficiency of their root system to utilize available resources. Chaves (2015) also reported a high yield for G40001 under high temperature stress conditions.

Heat stress induced changes in photosynthetic efficiency

The SPAD readings and photosynthetic efficiency were affected by the high temperature treatment in all genotypes evaluated. Tepary bean accessions had considerably reduced SPAD readings, while common bean lines and interspecific lines were relatively less affected. *P. acutifolius* also had reduced stomatal conductance under HT, while common bean increased it. These results agree with those of Djanaguiraman et al. (2011) and Chaves (2015) who showed that HT stress decreased chlorophyll content and leaf stomatal conductance in soybean and common bean, respectively. The photosynthetic efficiency increased in tepary with HT, while common beans tended to decrease it.

The superior adaptation of tepary can be ascribed in part to its ability to regulate stomatal opening and increase photosynthetic efficiency.

Multivariate analysis under heat stress

The PCA analysis of all traits evaluated showed that four components represented 88.8% of the total variance under HT conditions. Different traits that were evaluated here appeared to explain the variance in the PCA under HT, but not under AT. The traits in the first component were related to root traits

Table 1. Description of the germplasm investigated in the study: *P. acutifolius* accessions, *P. vulgaris* genotypes and interspecific lines of *P. vulgaris* with *P. acutifolius*, *P. coccineus* and *P. dumosus*.

Genotypes	Origin	Type of germplasm	Seed color
P. acutifolius A. Gray germplasm accessions			
G40001	Veracruz (Mexico)	Landrace	White
G40005	La paz (El salvador)	Landrace	White
G40022	Arizona (United States)	Landrace	Yellow
G40068	Arizona (United States)	Landrace	Yellow
G40084	Durango (Mexico)	Landrace	Cream brown
G40110	Campeche (Mexico)	Landrace	Black. cream
G40159	Sonora (Mexico)	Landrace	White
G40200	Guanacaste (Costa Rica)	Landrace	Cream. brown
G40277	Sonora (Mexico)	Landrace	White
G40278	Sonora (Mexico)	Landrace	White
P. vulgaris L. lines			
A 774	Brazil	Inbred line	Cream
BAT 477	Colombia	Inbred line	Cream
DAB 295	Colombia	Inbred line	Red
Calima	Colombia	Inbred line	Red
Interspecific lines			
INB 827 (*Pv* x *Pa*)	Colombia	Line	Red
SEF 60 (*Pv* x *Pa* x *Pc*)	Colombia	Line	Red
ALB 91 (*Pv* x (*Pv* x *Pc*)F_1	Colombia	Line	Red
SCM 140 (*Pv* x *Pa* x *Pd*)	Colombia	Line	Purple

Pa = Phaseolus acutifolius. Pv = P. vulgaris. Pc = P. coccineus L*., Pd = P. dumosus*

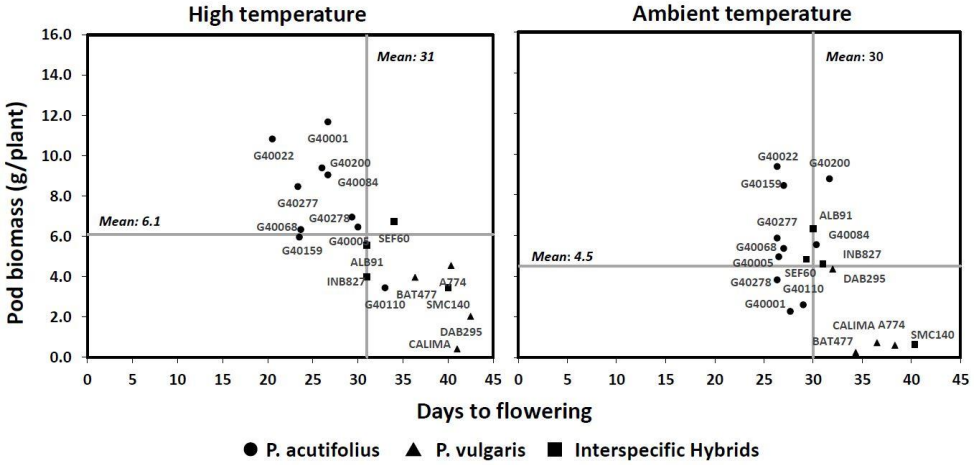

Fig 1. Relationship between pod biomass (g/plant) and days to flowering in P. acutifolius, P. vulgaris. species and the interspecific lines under a) High temperature and b) Ambient temperature conditions. The genotypes that bloomed early and had higher pod biomass were identified in the upper, left-hand quadrant for both conditions.

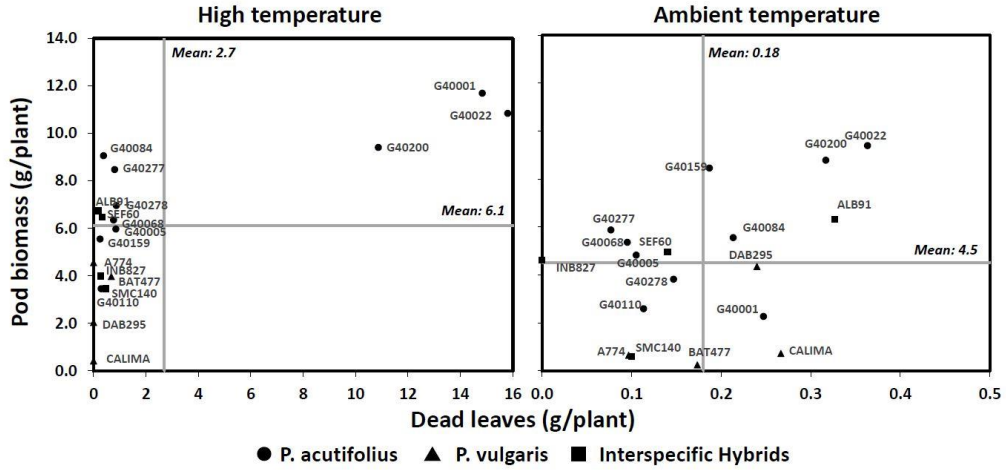

Fig 2. Relationship between pod biomass (g/plant) and dead leaves (g/plant) in *P. acutifolius*, *P. vulgaris* species and the interspecific lines under a) High temperature and b) Ambient temperature conditions. The genotypes that had higher pod biomass and show also higher amount of dead leaves were identified in the upper, right hand quadrant for both conditions.

Table 2. Comparison of the effects of high temperature (HT) with ambient temperature (AT) treatment on mean values of total shoot biomass, dead leaf weight at mid grain filling, pod biomass, pod number and specific root length per plant in lines tested.

	Shoot biomass (g/plant)		Dead leaves (g/plant)		Pod biomass (g/plant)		Number of pods/plants		Specific root length / plant (cm/plant)*	
Genotype	HT	AT	HT	AT	HT	AT	HT	AT	HT	AT
Tepary bean										
G40001	18.1	3.5 d	14.83 ab	0.14	11.7	2.3 def	17.4 ab	4.0 def	697.5	5,999.1
G40005	11.1	10.3 abc	0.33 c	0.09	6.5	5.0 dc	10.4 bc	9.5 bcde	1,313.8	1,473.7
G40022	14.7	12.4 ab	15.80 a	0.21	10.9	9.4 a	14.5 abc	17.4 a	1,486.3	1,762.9
G40068	8.8	6.7 bcd	0.76 abc	0.15	6.4	5.4 bcd	9.0 bc	9.7 bcd	625.2	3,491.1
G40084	13.1	10.7 abc	0.38 c	0.36	9.1	5.6 bcd	17.0 ab	10.4 bcd	1,560.3	1,556.5
G40110	10.4	5.9 cd	0.29 c	0.32	3.5	2.6 def	9.0 bc	4.4 cdef	1,494.3	3,202.4
G40159	8.9	12.7 ab	0.85 abc	0.25	6.0	8.5 ab	10.0bc	18.0 a	2,576.1	1,662.4
G40200	13.8	11.0 abc	10.90 abc	0.11	9.4	8.8 ab	11.7 bc	10.4 bcd	1,146.7	167.7
G40277	14.4	8.2 abcd	0.80 abc	0.19	8.5	5.9 bcd	12.4 abc	13.4 ab	1,042.7	2,440.5
G40278	10.1	5.3 cd	0.87 abc	0.08	7.0	3.8 cde	13.4 abc	10.7 bc	1,674.4	3,398.3
Mean	12.4	8.3	4.58	0.19	7.9	5.7	12.4	10.7	1,479.7	2,666.4
Common bean										
A774	21.7	5.7 cd	0.0 c	0.33	4.6	0.6 ef	18.0 ab	5.7 cdef	1,032.5	2,632.1
BAT477	17.3	5.9 cd	0.68 abc	0.09	4.0	0.2 f	16.7 ab	3.4ef	905.3	2,675.5
DAB295	6.4	13.7 a	0.0c	0.10	2.0	4.4 cd	2.0 c	6.5cdef	2,906.8	1,077.9
Calima	6.2	2.4 d	0.0 c	0.0	0.4	0.7 ef	3.0 c	2.0f	2,564.3	4,525.7
Mean	12.9	6.9	0.17	0.13	2.7	1.5	9.4	4.4	1,852.2	2,727.8
Interspecific lines										
INB827	12.8	12.5 ab	0.27 c	0.17	4.00	4.6cd	10.0 bc	10.4 bcd	1,121.7	1,711.1
SEF60	13.4	8.5 abcd	0.19 c	0.24	6.74	4.8 cd	9.0 bc	6.0 cdef	1,415.1	1,969.8
SMC140	17.9	13.3 a	0.48c	0.10	3.50	0.6 ef	25.0 a	2.7 f	850.7	2,239.1
ALB91	16.9	6.7 bcd	0.25c	0.27	5.60	6.3 abc	13.0 abc	10.0 bcd	951.1	1,966.4
Mean	15.2	10.1	0.30	0.20	4.9	4.1	14.3	7.3	1,084.6	1,971.6

*Means between a yield component and treatment not followed by the same letters are significantly different at P≤ 0.05 according to Duncan's multiple rang test. *Specific root length=root length /root biomass.

(length and surface area), the second ones to physiological variables (efficiency of photosystem and days to flowering) and the third ones to biomass and number of nodules. This indicates that these variables and others that were identified from the univariate analysis (pod biomass, dead leaves at mid grain filling and leaf area) could be used in bean breeding programmes as selection criteria to screen bean genotypes for tolerance to heat stress.

The mean agronomic data obtained from interspecific lines is mixed. For shoot biomass, dead leaves and pod biomass they showed a better response than common bean in high temperature conditions, indicating positive inheritance from other species (*P. acutifolius*, *P. coccineus* and *P. dumosus*). However, for other traits (number of pods, specific root length and root length) the performance was worse than that of common bean. This indicates that more breeding is required to introgress genes into common bean for yield improvement under heat stress conditions.

Material and methods

Plant materials

A total of 18 genotypes were tested: ten accessions of tepary bean (*Phaseolus acutifolius* A. Gray), four genotypes of common bean (*Phaseolus vulgaris* L.) and four interspecific lines from crosses of *P. vulgaris* with *P. acutifolius*, *P. coccineus* and *P. domosus* (Table 1). The common bean genotypes were developed to improve adaptation to different stresses: The A774 genotype was developed for improved adaptation to low soil fertility, BAT 477 was developed for improved adaptation to low soil phosphorus availability and drought, while DAB 295 was developed to tolerate drought and Calima was included as a commercial check. Four interspecific lines were included. SEF 60 was obtained from the cross (ALB74 x INB 841) F_1 x RCB593; ALB74 was obtained from the cross SER 16 x (SER 16 x G35346-3Q) F_1 with genes from *P. coccineus* (Butare et al., 2012). INB841 was derived from the cross (INB108 x INB605) (Mejia et al., 1994). INB 827 was obtained from the cross (INB 108 x INB 105) with genes from *P. acutifolius* (Mejia et al., 1994). SMC140 was obtained from the cross (INB 841 x SMC6) F_1 x (SXB 405 x MIB 780) F_1, where the INB 841 and the MIB 780 are interspecific lines of *P. vulgaris* x *P. acutifolius* and *P. vulgaris* x *P. dumosus*, respectively. ALB 91, an interspecific line obtained from the cross SER 16 x (SER 16 x G35346B) F_1 with genes from *P. coccineus* (Butare et al., 2012) (Table 1). The tepary bean germplasm accessions, and the common bean genotypes and the interspecific lines were obtained from the Genetic resources Unit and the Bean Programme of CIAT, respectively.

Experimental conditions

The experiments were conducted at the International Center for Tropical Agriculture (CIAT) in Palmira, Colombia, located at latitude 3° 29' N, longitude 76° 21' W and 965 altitude above sea level. Two experiments were conducted simultaneously to evaluate the tolerance of the various bean genotypes to high temperature. Both experiments were conducted using a split-plot design with three replications. Experiment 1 was carried out with high temperature treatment (HT) in a greenhouse and Experiment 2 was realized at ambient temperature (AT), outdoors in the field under rainout shelter. Seeds were sown in transparent plastic cylinders (120 cm long, 7.5 cm diameter) with a Mollisol soil from Palmira, Colombia (Polania et al., 2009). Soil cylinders were carefully packed with a 2:1 soil: sand mixture having a final bulk density of 1.4 g/cm^3. The seeds were germinated in paper towels and uniform seedlings were selected for transplanting to transparent plastic cylinders, each of which was inserted into PVC sleeve tubes. The plants of both experiments were inoculated at 10 days after sowing with *Rhizobium tropici* (strain CIAT 899). To induce high temperature

Table 3. Effect of high temperature (HT) compared to ambient temperature (AT) treatment on SPAD chlorophyll meter reading, photosynthetic efficiency, stomatal conductance, leaf area and days to flowering of 18 *Phaseolus* genotypes.

Genotype	SPAD chlorophyll meter reading		Photosynthetic efficiency (QY (Fv/FM))		Stomatal conductance ($mmol.m^{-2}.s^{-1}$)		Leaf area (cm^2)		Days to flowering	
	HT	AT	HT	AT	HT	AT	HT	AT	HT	AT
Tepary bean										
G40001	30.5 abc*	45.0 ab	0.66 ab	0.57	176.2	248.0 bcde	451.7 de	108.3 ef	27efgh	28 d
G40005	31.7 abc	39.5 abcd	0.70 a	0.56	239.2	443.4 abc	467.7 de	539.0 bcde	30defg	27 d
G40022	27.4 abcd	40.1 abcd	0.72 a	0.60	170.1	324.3 abcde	108.0 e	208.7 def	21 h	26 d
G40068	16.4 dc	28.3 e	0.58 ab	0.60	276.8	263.8 bcde	104.3 e	84.3 f	24 gh	27d
G40084	32.7 abc	41.4 abcd	0.70 a	0.58	329.0	397.7 abcd	367.3 de	401.7 cdef	27efgh	30bcd
G40110	31.5 abc	41.7 abcd	0.71 a	0.50	300.8	83.3 ed	760.7 cde	294.0 def	33 cdef	29cd
G40159	13.4 d	47.3 ab	0.68 ab	0.58	191.6	272.5 bcde	96.5 e	332.3 cdef	24 gh	27 d
G40200	18.4 bcd	48.2 a	0.70 a	0.59	192.8	224.9 cde	384.0 de	156.3 def	26 fgh	32bcd
G40277	24.4 abcd	43.1 abc	0.68 ab	0.61	184.4	163.7 e	369.7 de	140.0 ef	23 gh	26d
G40278	37.1 a	48.2 a	0.70 a	0.49	205.6	320.7 abcd	164.3 e	140.3ef	29 defg	26d
Mean	26.3	42.3	0.68	0.57	226.6	284.2	327.4	240.5	26	28
Common bean										
A774	35.7 ab	33.4 cde	0.51 bc	0.57	409.3	147.4 e	1,876.0 a	521.7 bcdef	36 abcd	34abcd
BAT477	37.6 a	31.8 cde	0.69 a	0.59	316.7	197.4 de	1,483.0 ab	596.3 bcd	40abc	38 ab
CALIMA	36.2 a	31.2 de	0.71 a	0.61	342.5	119.2 e	775.0 cde	200.5 def	41 ab	37 abc
DAB295	27.0 abcd	36.6 bcde	0.36 c	0.64	225.5	449.0 ab	660.0 de	1354.5 a	42.5 a	32 bcd
Mean	34.1	33.2	0.57	0.60	323.5	228.3	1198.5	668.3	40	35
Interspecific lines										
INB827	33.7 abc	43.0 abc	0.66 ab	0.61	362.5	243.9 bcde	905.3 b	996.5 bcd	31 defg	31 bcd
SEF60	38.8 a	41.0 abcd	0.68 ab	0.6	294.8	253.8 bcde	996.0 bcd	413.0 cdef	34 bcde	29 cd
SMC140	36.8 a	32.4 cde	0.71 a	0.58	325.5	342.4 abcde	1,535.7 ab	586.3 bcd	40 abc	40 a
ALB91	39.7 a	39.8 abcd	0.65 ab	0.61	265.9	491.5 a	1,416.0abc	742.0 bc	31 defg	30 bcd
Mean	37.2	39.1	0.68	0.60	312.1	332.9	1213.25	684.5	34	33

*Means between a yield component and treatment not followed by the same letters are significantly different at P≤ 0.05 according to Duncan's multiple

conditions and simulate the changes in temperature between day and night, conditions in the greenhouse were modified using heaters, the ventilation system and thermostats. The HT treatment was set at 29 °C ±5 during the day and >24 °C (up to 27 °C) during the night, with an average relative humidity of 65 %. In the field, average temperature was 25 °C ±5 during the day and ±19 °C at night, with an average relative humidity of 81 %, in both treatments. The soil was maintained at 80 % of field capacity and water was supplied to avoid drought stress. The data on relative humidity and temperature were monitored with thermo-hygrometers that registered the parameters every 15 min. The average, minimal and maximal temperatures were calculated per day.

Measurement of shoot and root morpho-physiological characteristics

Plants were harvested at 60 days of growth for Experiment 1 and at 70 days of growth for Experiment 2. Days to flowering (DF) were determined for each genotype in each experiment. Days to flowering was defined as the number of days after planting until 50% of the plants had at least one open flower. At mid-pod filling growth stage, the following non-destructive measurements were made. Leaf area was measured using a leaf area meter (model LI-3000, LI-COR, NE, USA). The leaf chlorophyll content of fully expanded leaves was measured using a non-destructive, hand-held chlorophyll meter (SPAD-502 chlorophyll meter Minolta camera Co., Ltd., Japan). The principle is based on the difference in light attenuation at wavelengths 430 and 750 mm. From the difference in light attenuation, a numerical SPAD (Soil Plant Analysis Development) unit, ranging from 0 to 80, is calculated by the microprocessor in the SPAD-502 chlorophyll meter. The efficiency of photosystem II (QY) in leaves adapted to light (Fv'/Fm') was also determined. The stomatal conductance ($mmol\ m^{-2}\ s^{-1}$) was measured with a portable leaf porometer (Deacagon SC-1) on a fully expanded young leaf of one plant within each replication. Measurements were made late in the morning (10:00-12:00 am) on clear and sunny days. The biomass of dead leaves (i.e. fallen, dry leaves) per plant at the mid-pod filling stage was measured. At the time of harvest, plants were cut and dry weights of: shoot biomass, stem, leaves, and pod biomass and pod number per plant were recorded. The roots of each soil cylinder were carefully washed free of soil. The washed roots were scanned as images using a desk scanner. From the scanned images, total root length ($cm\ plant^{-1}$) and proportion of fine roots or the proportion of roots (%) with a diameter less than 0.5 mm, were measured through image analysis using Win RHIZO (Reagent Instruments Inc., Quebec Canada). Root weight per plant was determined after the roots were dried in an oven at 60 °C for 48h.

Statistical analysis

A separate analysis was conducted for each experiment. The sources of variation within each experiment were replications and genotypes. All data were analyzed using the SAS (v. 9.2). Correlation coefficients were calculated by the PROC CORR. Values marked with * or ** are statistically significant at probability levels of 5% and 1%, respectively. The mean values were compared using the Duncan test. A complementary analysis of variance was performed to compare genotypes and/or groups of genotypes for the most important variables: the number of days to flowering, the SPAD chlorophyll meter readings, pod biomass, dead leaves at mid-grain filling, root biomass and root length. The tepary bean accession G40001was used as a heat tolerant check (Rao et al., 2013) to compare with: a) the other tepary accessions, b) the common bean genotypes and c) the interspecific lines.

Table 4. Eigen values and per cent of total variation and component matrix for the principal component axes - High temperature (HT) and ambient temperature (AT) conditions. Values in bold indicate the traits that were informative in genotype differentiation.

Principal components	CP1	CP2	CP3	CP4
High temperature (HT) Eigen value	7.09	4.29	1.49	1.33
Variance proportion	0.44	0.27	0.09	0.08
Cumulative proportion variance	0.44	0.71	0.80	0.89
Shoot biomass	0.075	0.442	0.177	-0.056
Pod biomass	- 0.268	0.256	0.219	-0.039
Dead leaf at mid-grain filling	- 0.291	0.172	0.204	0.144
Leaf area	0.318	0.179	0.253	-0.022
Pods number	| - 0.006	0.403	0.182	0.261
Days to flowering	0.285	-0.118	0.369	0.081
Roots length	0.325	-0.133	0.301	0.101
Roots volume	0.297	-0.202	0.243	0.189
Roots biomass	0.303	0.250	0.121	0.018
Supeficiel area of root	0.314	-0.172	0.274	0.149
Specific root length	-0.028	0.449	0.046	0.133
Nodules biomass	0.223	0.237	0.267	-0.458
Nodules number	0.242	0.204	0.310	-0.421
SPAD chlorophyll content	0.270	0.154	0.036	-0.190
Leaf stomatal conductance	0.292	-0.002	0.255	0.216
Efficiency of photosystem II	- 0.059	0.165	0.414	0.586
Ambient temperature (AT)				
Eigen value	6.35	4.59	1.73	1.02
Variance proportion	0.40	0.29	0.11	0.06
Cumulative proportion of variance	0.40	0.69	0.80	0.86
Shoot biomass	0.119	0.402	0.267	-0.053
Pod biomass	-0.102	0.423	0.094	-0.233
Dead leaf at mid- grain filling	-0.044	0.046	0.625	0.201
Leaf area	0.345	0.092	-0.179	0.210
Pod number	-0.111	0.390	0.197	-0.189
Days to flowering	0.229	-0.281	-0.035	-0.108
Root length	0.333	-0.118	0.287	0.047
Root volume	0.331	-0.180	0.263	-0.007
Root biomass	0.359	0.156	0.077	0.073
Superficial area roots	0.337	-0.152	0.279	0.020
Specific root length	-0.170	-0.326	-0.044	0.019
Nodules biomass	0.269	0.218	-0.307	-0.039
Nodules number	0.349	0.110	-0.173	0.173
Spad chlorophyll content	-0.177	0.259	0.094	0.349
Leaf stomatal conductance	0.132	0.279	-0.280	0.387
Efficiency of photosystem II	0.227	0.104	-0.075	-0.705

The tepary group of accessions were also compared with the common bean and the interspecific lines. Finally, the common bean genotypes were compared with the interspecific lines under HT (greenhouse) and AT (rainout shelter) conditions. A combined
analysis of two experiments was also conducted. The sources of variation were: temperature treatments, replication, genotype and genotype x temperature condition treatment interaction. Principal Component Analysis (PCA) was performed on the measured variables and was based on Pearson correlation matrix and Euclidean distances. Eigenvalues for all principal components (PCs) are shown. Eigenvectors generated by the PCA were used to identify parameters that best differentiated the genotypes in each experiment.

Conclusion

Tepary bean exhibits traits related to adaptation to high temperature. Beneficial morpho-physiological traits include a greater capacity to allocate dry matter to pods, fine roots and smaller leaves (for efficient water use and reduced stomatal conductance). Three tepary bean accessions that produced good pod biomass under heat stress were identified: G40001 (Veracruz, Mexico), G40022 (Arizona, United States) and G40200 (Guanacaste, Costa Rica). These accessions could serve as potential donors of genes to improve common bean through interspecific hybridizations and backcrossing. Alternatively, breeding of tepary beans for preferred consumer traits may be considered to safe guard food security in arid zones of the world.

Acknowledgments

We acknowledge partial support from the International Atomic Energy Agency (IAEA), Vienna, Austria for the project on "Heat effects in tepary beans and its relatives". We are grateful to the Bill and Melinda Gates Foundation (BMGF), and the CGIAR Research Program on Grain Legumes for financial support of research on improving drought resistance in common bean. We would also like to thank all donors who supported this work through their contributions to the CGIAR Fund and the National University of Colombia-Palmira.

References

Beebe S, Rao I, Blair M, Butare L (2009) Breeding for abiotic stress tolerance in common bean: present and future challenges. S J Breed Genet. 41:1-11.

Bielenberg D, Miller J, Berg V (2003) Paraheliotropism in two *Phaseolus* species: combined effects of photon flux density and pulvinus temperature, and consequences for leaf gas exchange. Environ Exp Botany. 49:95-105.

Blair M, Pantoja W, Muñoz L (2012) First use of microsatellite markers in a large collection of cultivated and wild accessions of tepary bean (*Phaseolus acutifolius* A. Gray). Theor Appl Genet. 125:1137–1147.

Butare L, Rao I, Lepoivre P, Polania J, Cajiao, Cuasquer C, Beebe S (2012) Phenotypic evaluation of interspecific recombinant inbred lines (RILs) of *Phaseolus* species for aluminium resistance and shoot and root growth response to aluminium-toxic acid soil. Euphytica 186 (3):715-730.

Chaves N-F (2015) Uso de recombinantes de *Phaseolus. vulgaris* L., *P. coccineus* L. y *P. acutifolius* A. Gray, para mejorar la tolerancia del frijol común a diferentes tipos de estrés abiótico. Tesis de Doctorado, Universidad Nacional de Colombia, Palmira, Colombia, Junio 2015.

Djanaguiraman M, Prasad P, Boyle V, Schapaugh D (2011) High temperature stress and soybean leaves: Leaf anatomy and photosynthesis. Crop Sci. 51:2125-2131.

Freeman G (1912) Southwestern beans and teparies. Bull Univ Ariz Agric Exp. Stn. 68:1-55.

Gepts P, Debouck D (1991) Origin, domestication, and evolution of common bean (*P. vulgaris* L.). In: Schoonhoven AV (ed) Common bean: Research for crop improvement, CAB Intern., Wallingford, UK, 7-53.

IPCC 2007: Climate change 2007: The physical science basis contribution of working group to the fourth assessment report of the IPCC. In Solomon, S, Qin D, Manning M, Chen Z, Marquis M, Averyt KB, Tignor M, Miller HL (eds) Cambridge University Press, Cambridge, United King dom, New York, N.Y,USA.1-18.

Lin T, Markhart A (1990) Temperature effects on mitochondrial respiration in *Phaseolus acutifolius* a. Gray and *P. vulgaris* L. Plant Physiol. 94:54-58.

Lin T, Markhart A (1996) *Phaseolus acutifolius* A. Gray is more heat tolerant than *P. vulgaris* in the absence of water stress. Crop Sci. 36:110-114.

Markhart A (1985) Comparative water relations of *Phaseolus vulgaris* L. and *Phaseolus acutifolius* A. Gray. Plant Physiol. 77:113-117.

Mejía J, Muñoz C, Jacobsen H, Roca W, Singh S (1994) Interspecific hybridization between common and tepary beans: Increased embryo growth, and efficiency of hybridization through recurrent and congruity backcrossing. Theor Appl Genet. 88:324–331.

Muñoz L, Blair M, Duque M, Thome J, Roca W (2004) Introgression in common bean x tepary bean interspecific congruity-backcross lines as measured by AFLP markers. Crop Sci. 44:637-645.

Muñoz L, Duque M, Debouck D, Blair M (2006) Taxonomy of tepary bean and wild relatives as determined by amplified fragment length polymorphism (AFLP) markers. Crop Sci. 46:1744-1754.

Nabhan G, Felger R (1978) Teparies in southwestern North America: a biogeographical and etnohistorial study of *Phaseolus acutifolius*. Econ Bot. 32:2-19.

Ojeda, L (2015) Reaccion de *Phaseolus acutifolius* A. Gray y relacionados a dos patogenos del suelo y a estrés abiotico. Tesis de maestria. Universidad Nacional de Colombia, Palmira, Colombia. P 48.

Omae H, Kumar A, Kashiwaba K, Shono M (2007) Influence of temperature shift after flowering on dry matter partitioning in two cultivars of snap bean (*Phaseolus vulgaris*) that differ in heat tolerance. Plant Prod Sci. 10:14-19.

Polanía J, Rao I, Beebe S, García R (2009) Desarrollo y distribución de raíces bajo estrés por sequía en frijol común (*Phaseolus vulgaris* L.) en un sistema de tubos con suelo. Agron Colomb. 27:25-32.

Polania J, Poschenrieder C, Beebe S, Rao I (2016) Effective use of water and increased dry matter partitioned to grain contribute to yield of common bean improved for drought resistance. Front Plant Sci. 7:660. doi:10.3389/fpls.2016.00660.

Porch T, Beaver J, Debouck D, Jackson S, Kelly J, Dempewolf H (2013) Use of wild relatives and closely related species to adapt common bean to climate change. Agronomy. 3: 433-461.

Rainey K, Griffiths P (2005a) Evaluation of *Phaseolus acutifolius* A. Gray plant introductions under high temperatures in a controlled environment. Genet Resour Crop Evol. 52: 117-120.

Rainey K, Griffiths P (2005b) Differential response of common bean genotypes to high temperature. J Am Soc Hort Sci. 130 (1):18-23.

Rao I, Beebe S, Polania J, Ricaute J, Cajiao C, Garcia R, Rivera M (2013) Can tepary bean be a model for improvement of drought resistance in common bean? Afr J Crop Sci. 21:265-281.

Rao I, Beebe S, Polania J, Grajales M, Cajiao C, Ricaurte J, Garcia R, Rivera M (2016) Evidence for genotypic differences among elite lines of common bean in their ability to remobilize photosynthate to increase yield under drought. J Agric Sci. 155:857-875.

White J, Castillo J (1992) Evaluation of diverse shoot genotypes on selected root genotypes of common bean under soil water deficits. Crop Sci. 32:762-765.

DOI: 10.21475/ajcs.21.15.09.sp-7

Australian Journal of Crop Science AJCS

Mutation breeding for heat and drought tolerance in tepary bean (*Phaseolus acutifolius* A. Gray)

Ligia Carmenza Muñoz[1]*, Daniel G. Debouck[2], Mariela Rivera[2], Jaime E. Muñoz[1], Deisy Alpala[1], Fatma Sarsu[3] and Idupulapati M. Rao[2,4]

[1]Universidad Nacional de Colombia- sede Palmira, Carrera 32, Chapinero, Palmira, Colombia
[2]Centro Internacional de Agricultura Tropical (CIAT), A.A 6713, Cali, Colombia
[3]International Atomic Energy Agency (IAEA), VIC, PO Box 100, 1400 Vienna, Austria
[4]Present address: Plant Polymer Research Unit, National Center for Agricultural Utilization Agricultural Research Service, United States Department of Agriculture, 1815 North University Street, Peoria, IL 61604, USA

***Corresponding author: lcmunozf@unal.edu.co**

Abstract

Tepary bean (*Phaseolus acutifolius* A. Gray) is more heat and drought tolerant than common bean (*P. vulgaris* L.). Four hundred mutant lines of two tepary accessions (G40068 and G40159) were generated by ethyl methane sulfonate (EMS) treatment. In preliminary studies of the M_5 mutant lines under abiotic stress, three mutant lines (CMT 38, CMT 109, CMT 187) were selected from six mutated lines based on morpho-physiological traits and superior yield and advanced to the M_6 generation. The M_6 mutant lines were uniform and genetically stable. These mutant lines and their original (M_0) parents were evaluated for heat and drought tolerance under greenhouse conditions. Their performance was evaluated for morpho-physiological attributes, seed yield and yield components. Under high temperature and drought conditions, the CMT 38 mutant (M_6 line) and its original tepary (M_0) accession (G40068) showed greater values of pod biomass, pod number and 100-seed biomass than the other lines tested. The CMT 109 and CMT 187 mutant lines and their G40159 original accession (M_0) also showed the highest value of seed number under high temperature and drought conditions. This suggests that the previous screening performed during the population advancement of these mutant lines, based on morphological traits like growth habit, was not detrimental to the yield variables evaluated here. Under combined heat and drought conditions, different parameters could be incorporated into tepary breeding programmes, as selection criteria to screen genotypes for tolerance to heat and drought stress. These parameters included: chlorophyll (SPAD) readings, seed biomass, 100-seed biomass and seed number because they explain the observed variance in the principal component analysis. Two additional traits (root biomass and stem diameter) were also identified as useful attributes, based on univariate analysis. The mutant lines evaluated here offer potential for further improvement of tepary bean to high temperature and drought.

Key Words: Abiotic stress; beans species; crop improvement; EMS mutagenesis; yield.
Abbreviations: EMS, ethyl methane sulfonate; M_6, Mutant line generation 6; PCA, principal component analysis; SPAD, Soil-Plant Analyses Development chlorophyll meter.

Introduction

Global warming is responsible not only for global temperature increase but also for region-specific increases or decreases in precipitation. This, in turn, has a negative impact on the production systems of crops that are vital for improving food and nutritional security of people in developing countries. Common bean, *Phaseolus vulgaris* L., is a valuable source of protein, starch and other nutrients. Drought affects 60% of the dry bean production area worldwide (Beebe et al., 2008). Temperatures > 30 °C during the day or > 20°C during the night result in significant yield reduction of common bean (Rainey and Griffiths, 2005).

Tepary bean, *Phaseolus acutifolius* A. Gray, is a traditional crop of desert and semi-arid regions of Mexico and southwestern USA (Freeman, 1912; Nabhan and Felger, 1978). Renewed interest in tepary is due to its possession of many traits that enable it to flourish in hot and dry regions: it is more heat tolerant at biological tissue levels than common bean and it produces more leaves to compensate for reduced leaf size due to heat stress (Lin and Markhardt, 1996). Tepary also has more extensive and a thinner root system, better stomatal control and more active para-heliotropism than common bean (Markhart, 1985; Bielenberg et al., 2003; Butare et al., 2012). It is part of the tertiary gene pool of common bean and considered as a potential useful donor parent of drought and heat tolerance traits for common bean improvement, through interspecific hybridization (Muñoz et al., 2004, Rao et al., 2013). Tepary could be a source of genes for the improvement of common bean through inter-specific crosses followed by backcrossing (Mejia et al., 1994). It could also serve as a valuable crop in itself, particularly for dryland environments where common bean is less adapted (Muñoz et al., 2006).

An evaluation of tepary gene introgression showed that tepary DNA markers can be transferred to the interspecific progeny (Muñoz et al., 2004). However, success is limited to a lower-than-expected percentage of genome contributed by tepary (Blair et al., 2012). Introgression of heat or drought tolerance from tepary into common bean might be feasible through breeding, to generate elite lines that can tolerate up to 4°C higher temperatures. However, most of the lines obtained

come from a limited number of crosses between common and tepary beans (Muñoz et al., 2004).

A diversity study of the tepary collection at the Genetic Resources Unit (GRU) of CIAT Colombia, using AFLP and microsatellite (SSR) markers showed that the genetic base of the cultivated tepary accessions is narrow (Muñoz et al., 2006; Blair et al., 2012). A similar conclusion was made after a study of variability of the seed storage proteins of wild and cultivated accessions of tepary (Schinkel and Gepts, 1988). A reason for this reduced genetic diversity might be the historic regression of tepary after the introduction of new watering technologies in Mesoamerica after 1492 (Nabhan and Felger, 1978; Nabhan, 1985; Debouck, 1992). Given this genetic extinction, future breeding rests on exploiting the significant diversity provided by wild teparies and related species (Muñoz et al., 2006), transformation (Dillen et al., 1997) or by inducing variation via mutagenesis (explored here). Traits that are particularly looked for in tepary are: uniform red seed colour, erect growth habit and grouped pod maturity (Pratt and Nabhan, 1988).

Mutagenesis has been used to broaden the genetic diversity of *Phaseolus* species (Ahloowalia et al., 2004; Blair et al., 2007; Gwata et al., 2016). The results of chemical mutagenesis of common bean with ethyl methane sulfonate (EMS) in morphological and physiological changes, as well as varietal development have been reported (Blair et al., 2007; Porch et al., 2009). With the objective of genetic improvement of tepary bean, a protocol was developed by Muñoz et al. (2013) for chemical mutation induction using EMS in two cultivated tepary accessions (G40068 and G40159).

From the research discussed above, three further questions arise: (i) Why is it important to induce mutations in tepary? (ii) Which novel traits could be achieved by mutation induction in tepary? and (iii) How would the mutant lines be used in a breeding programme for tepary and/or common beans? The main objectives of the present study were to: i) evaluate tepary bean M_6 mutant lines under conditions of high temperature and drought; and ii) identify heat and drought tolerant mutant lines that could serve as parents in breeding programmes that aim to improve heat and drought tolerance in common bean.

Results

Effect of high temperature (HT) on shoot and root morpho-physiological characteristics

Table 1 presents the results of the effect of HT on genotypic differences in yield components such as pod number, pod biomass, seed number, and 100-seed biomass and also the number and biomass of nodules (nodules variables). The genotype parameter showed an effect ($P \leq 0.01$) on seed number, pod number and nodule biomass under both conditions of temperature HT and CT (control temperature). Under HT conditions, the effect of genotype was significant for pod biomass, seed biomass and nodule number.

There was no difference between the mean values of pod numbers for the genotypes under HT and CT conditions, but the value was higher under CT (24.7 pods/plant) compared to HT (20.5 pods/plant). The mutant line CMT 38 showed a high mean value for pod number under HT and CT conditions. For pod biomass, a similar trend was observed: the mean value was higher under CT (17.2 g /plant) compared to HT (14.0 g /plant). The pod biomass of the CMT 38 mutant line was higher under both conditions of temperature and differences ($P \leq 0.05$) were observed between the CMT 38 mutant line and the other mutant lines or parental accessions G40068 and G40159 (Table 1).

The seed number/plant was also higher under CT (88.9 seeds/plant) compared to HT (69.3 seeds/plant). Under both conditions of temperature, the CMT 109 mutant line showed the highest seed number/plant. Differences ($P \leq 0.05$) were observed between this mutant line (CMT 109) and the other two mutant lines (CMT 38 and CMT 187) and differences were also observed between the mutant line CMT 109 and the tepary parental accession G40068 (Table 1).

The 100-seed biomass/plant was higher under HT (16.5) as compared to CT (15.7). Under HT, the value was higher for the tepary accession G40068 and mutant line CMT 38, and differences ($P \leq 0.05$) were observed between these genotypes and the others. Similar responses of these genotypes were observed under CT. The tepary accession G40068 and the mutant line CMT 38 showed the highest values of 100-seed biomass. The differences were also significant ($P \leq 0.05$) when compared to the other genotypes (Table 1).

Nodule formation (nodules/plant) was lower under HT (3.0) as compared to CT (12.5). The tepary accession G40068, showed a high mean value under HT. The mutant lines CMT 38 and CMT 109 and the tepary accession G40068 showed the highest values of nodule formation under CT (Table 1). The nodule biomass (g/plant) was lower under HT (0.006) compared to CT (0.039). Under HT conditions, the tepary accession G40068 showed the highest nodule biomass, while the mutant line CMT 109 and the tepary accession G40159 showed the highest mean nodule biomass) under CT. This value was significantly different ($P \leq 0.05$) to the other genotypes evaluated (Table 1).

The results of the leaf biomass, stem biomass and roots biomass under HT (data not shown) showed significant differences ($P \leq 0.001$) between genotypes. The mutant line CMT 38 (1.8 g/plant) and the G40068 (1.9 g/plant) tepary accession showed the lowest mean stem biomass compared to the CMT 187 (2.5 g/plant) and CMT 109 (2.2 g/plant) mutant lines and the G40159 (2.1 g/plant) tepary accession. Significant differences ($P \leq 0.05$) were observed between the two groups of genotypes. The highest mean values for root biomass were observed in the G40068 tepary accession (0.98 g/plant) and the CMT 187 mutant line (0.95 g/plant). Significant differences ($P \leq 0.05$) were observed between the means of these genotypes and the mean values of the mutant lines: CMT 38 (0.83 g/plant), CMT 109 (0.76 g/plant) and the G40159 tepary accession (0.76 g/plant). No differences were observed between genotype means for leaf biomass under HT (data not shown). No difference between genotypes was observed for stem diameter (data not shown). Under CT, significant differences ($P \leq 0.001$) between genotypes were observed for leaf biomass and stem biomass, but not for stem diameter and root biomass. The CMT 187 mutant line showed the highest means for leaf biomass and stem biomass and significant differences ($P \leq 0.05$) were observed between this mutant line and the other genotypes (data not shown). In relation to physiological variables, under HT (results not shown), significant differences ($P \leq 0.001$) were observed between the tepary parental (M_0) accessions and the mutant lines for the efficiency of the photosystem II (QY) and the stomatal conductance. The CMT 187 (46.6) and CMT 109 (58.8) mutant lines and the G40159 (58.1) tepary accession showed the lowest mean stomatal conductance values as compared to the CMT 38 (79.8) mutant line and the G40068 (66.7) tepary accession. Significant differences ($P \leq 0.05$) in stomatal conductance were only observed between CMT 187 and CMT38 mutant lines and G40068. No differences were observed between genotypes for the SPAD and leaf temperature variables.

Under CT, significant differences ($P \leq 0.001$) were observed between genotypes for the SPAD and the stomatal conductance tests. The CMT109 mutant line showed a SPAD mean higher (45.0, $P \leq 0.05$) than the CMT 38 mutant line (39.7). The G40159 tepary accession, the mutant line CMT 38 and the tepary M_0 accession G40068 showed higher means (121.9, 118.5 and 107.6) for stomatal conductance, respectively, as compared to the CMT 187 and CMT 109 mutant lines (95.9 and 75.9), respectively.

Effect of temperature under drought and irrigated soil conditions on shoot and root morpho-physiological characteristics

The results on the effect of HT and drought as compared to CT and irrigated conditions of soil on pod number, pod biomass, seed number, 100-seed biomass, nodule number and nodule biomass are shown in Tables 2 and 3. Soil conditions (drought or irrigated) showed significant effects (P≤0.01) on pod number /plant, seed number /plant, pod biomass /plant, 100-seed biomass/plant and nodule biomass /plant under both temperature conditions. The effect of soil condition was also significant (P≤0.01) for the nodule number/plant, under HT treatment.

Effect of HT under drought and irrigated conditions of soil

Under HT the pod number/plant was lower under drought (14.5) as compared to the irrigated treatment (26.6) for all genotypes (Table 2). Under drought conditions the mutant line CMT 38 showed the highest value as compared to other genotypes. Significant differences (P≤0.05) were observed between this mutant line, the mutant line CMT 109 and the tepary accession G40068 (Table 2). Under irrigated conditions, two mutant lines (CMT 38 and CMT 109) showed the highest values. Significant differences (P≤0.05) were observed between these mutant lines and the mutant line CMT 187 that showed the lowest pod number (23.4) (Table 2).

For pod biomass (g/plant), the value was also lower under drought (9.6) than the irrigated treatment (18.4) for all genotypes. The mutant line CMT 38 showed the highest value under drought and irrigated conditions. Under drought conditions, significant differences (P≤0.05) were observed between the mutant lines CMT 38 and CMT 109, which showed the lowest value. Under irrigated conditions, significant differences (P≤0.05) were observed between the mutant line CMT 38 and CMT 187 and the tepary accession G40159. The seed number/plant was lower under drought (45.9) compared to irrigated conditions (92.7) for all genotypes (Table 2). The tepary parental accession G40159 showed the highest value under drought conditions. Significant differences were observed between this accession, the mutant line CMT 38 and the tepary accession G40068. Under irrigated conditions, the mutant line CMT 109 showed the highest value. Significant differences (P≤0.05) were observed between this mutant line, the mutant line CMT 187 and the tepary accession G40068. There was a small difference in 100-seed biomass (g/plant), between drought (17.3) and irrigated conditions (16.1) for all genotypes. Under drought conditions, the tepary accession G40068 and the mutant line CMT 38 showed the highest value of 100-seed biomass. There were significant differences (P≤0.05) between these genotypes and the mutant lines (CMT 109, CMT 187) and the tepary accession G40159 (Table 2). Under irrigated conditions, the same genotypes also showed the highest 100-seed biomass values. Significant differences (P≤0.05) were observed between all genotypes. The nodule number/plant was very low under drought and irrigated conditions (1.01 and 5.10, respectively), for all genotypes evaluated. Under irrigated conditions, the G40068 tepary parental accession showed the highest number. There were significant differences (P≤0.05) between this tepary accession, the mutant line CMT 38 and the other genotypes (Table 2). Under drought conditions, all genotypes showed a lower level of nodule formation. With respect to nodule biomass (g/plant), only the G40068 tepary accession showed a higher mean (0.024) under irrigated conditions, and significant differences (P≤0.05) were observed between this accession and the other genotypes (Table 2).

Effect of CT under drought and irrigated conditions of soil

The results obtained with the five genotypes under normal (control) conditions of temperature (CT) in a greenhouse are shown in Table 3**.**

For each treatment (drought or irrigated), all variables were higher under CT as compared to HT. The pod number/plant was lower under drought (18.3) compared to irrigated conditions (31.2) under CT for all genotypes. The mutant line CMT 38 and the tepary accession G40068 showed the highest values under irrigated conditions, but there was no difference between the genotypes. Under drought conditions, no difference was observed between the genotypes, for this variable (Table 3). The pod biomass (g/plant) was also lower under drought (11.2) as compared to irrigated conditions (23.1) for all genotypes. Under irrigation, the mutant line CMT 38 and the tepary accession G40068 showed the highest values. There were significant differences (P≤0.05) between the mutant lines CMT 38, CMT 109 and CMT 187 and the tepary accession G40159. The mutant line CMT 38 also showed the highest value under drought conditions. There were significant differences (P≤0.05) between this mutant line and the other genotypes (Table 3). The seed number/plant was lower under drought as compared to irrigated conditions (56.8 vs 121) for all genotypes (Table 3). Under drought treatment, the mutant line CMT 109 and the tepary accession G40159 showed higher values. Significant differences were observed between these genotypes and the others (Table 3). Under irrigated conditions, the mutant line CMT 109 showed the highest value. There were significant differences (P≤0.05) between this mutant line, the mutant line CMT 38 and the tepary accession G40068. In relation to the 100-seed biomass (g/plant) variable, there was a modest difference between drought (16.2) and irrigated treatments (15.5) for all genotypes. Under drought and irrigated conditions, the mutant line CMT 38 and the G40068 showed the highest value for 100-seed biomass. Significant differences (P≤0.05) were observed between these two genotypes and the others under both conditions (Table 3). The number of nodules/plant increased considerably under CT and irrigated conditions as compared to results obtained under HT (Table 1). The value was 23.3 under irrigated conditions as compared to 1.62 under drought for all genotypes. The mutant lines CMT 38 and CMT 109 and the G40068 tepary accession showed the highest values (Table 3). The differences between all genotypes were not significant. Under irrigated conditions, the CMT 109 mutant line showed the highest nodule biomass, and there were significant differences (P≤0.05) between this mutant line and the other genotypes tested (Table 3). Under both temperature conditions and considering drought and irrigation treatments for all variables and genotypes, the values were lower under drought, except for the 100-seed biomass. In this case, the value was higher under drought (Table 2). Under HT and CT, significant differences (P≤0.05) were observed between the values obtained under drought and irrigation for all evaluated variables and genotypes. In relation to 100-seed biomass, under CT conditions, significant differences (P≤0.05) were observed between drought and irrigated conditions for the mutant lines and G40159 tepary accession. Under CT conditions, significant differences (P≤0.05) were observed only with the mutant lines (Table 3).

A significant strain effect (*Rhizobium tropici* or *Bradyrhizobium* spp.) was observed only for the number of nodules /plant (data not shown).

Multivariate analysis of shoot and root morpho-physiological variables

Effect of temperature

Principal component analysis was performed to identify the major components (i.e. principal components) that could explain much of the total variation observed in the data. The

Table 1. Mean values of pod number, pod biomass, seed number, 100 seed biomass, nodules number and nodule biomass for the M_6 mutant lines (CMT 38, CMT 109 and CMT 187) and their original M_0 tepary accessions (G40068 and G40159), grown in greenhouses under high temperature (HT) and controlled temperature (CT) conditions.

	Pods/plant		Pod biomass, g/plant		Seeds/plant		100 seeds biomass		Nodules/plant		Nodule biomass, g/plant	
	HT	CT	HT	CT	HT	CT	HT	CT	HT	CT	HT	CT
Genotype												
CMT 38	22.2 a	25.6 a	15.2 a	18.2 a	69.9bc	83.5c	18.0 a	18.0 a	3.6 a	15.3 a	0.006 ab	0.029 b
CMT 109	21.2 a	23.9 a	13.8 b	16.6 b	75.2 a	95.8 a	14.4 d	13.4 d	2.7 a	14.4 a	0.005 ab	0.054 a
CMT 187	18.9 a	24.7 a	13.4 b	16.9 b	68.3 c	91.1 b	15.9 b	15.2 b	2.8 ab	9.4 a	0.005 ab	0.029 b
G40068	20.1 a	24.9 a	14.0 b	17.3 b	60.2 d	78.0 d	18.9 a	17.3 a	5.1 a	14.1a	0.013 a	0.031 b
G40159	20.3 a	24.7 a	13.5 b	16.9 b	73.1ab	96.3 a	15.2 c	14.8 c	1.1 b	9.1a	0.003 a	0.054 a
Mean	20.5	24.8	14.0	17.2	69.3	88.9	16.5	15.7	3.0	12.5	0.006	0.0394

*Means between a yield component and treatment not followed by the same letters are significantly different at P≤ 0.05 according to Duncan's multiple rang test.

Table 2. Mean values of pod number, pod biomass, seed number, 100 seed biomass, nodules number and nodules biomass for the M_6 mutant lines (CMT 38, CMT 109 and CMT 187) and their original M_0 tepary accessions (G40068 and G40159), grown in a greenhouse under high temperature (HT) and irrigated and drought conditions.

	Pods/plant		Pod biomass, g/plant		Seeds /plant		100 seed biomass, g/plant		Nodules/plant		Nodule biomass, g/plant	
	Irrigated	Drought	Irrigated	Drought	Irrigated	Drought	Irrigated	Drought	Irrigated	Drought	Irrigated	Drought
Genotype												
CMT 38	28.8 a	15.6 a	20.3 a	10.1 a	95.5 ab	44.3 b	17.5 b	19.0 a	5.9 ab	1.3 a	0.0117 b	0.0017 a
CMT 109	28.5 a	13.9 b	18.5 ab	9.1 b	103.0 a	47.3 a	13.9 e	15.5 c	4.6 bc	0.9 a	0.0083 b	0.0018 a
CMT 187	23.4 b	14.5 ab	17.4 a	9.4 ab	88.8 bc	47.7 a	15.8 c	16.4 b	4.1 bc	1.5 a	0.0078 b	0.0017 a
G40068	26.5 ab	13.8 b	18.5 ab	9.6 ab	80.2 c	40.2 c	18.7 a	19.4 a	9.4 a	0.8 a	0.0233 a	0.0009 a
G40159	25.7 ab	14. 9 ab	17.4 b	9.7 ab	96.0 ab	50.2 a	14.7 d	16.1 b	1.6 c	0.6 a	0.0033 b	0.0008 a
Mean	26.6	14.5	18.4	9.6	92.7	45.9	16.1	17.3	5.1	1.02	0.0108	0.00138

*Means between a yield component and treatment not followed by the same letters are significantly different at P≤ 0.05 according to Duncan's multiple rang test.

Table 3. Mean values of pod number, pod biomass, seed number, 100 seed biomass, nodule number and nodules biomass and number of pods for the M_6 mutant lines (CMT 38, CMT 109 and CMT 187) and their original M_0 tepary accessions (G40068 and G40159), grown in a greenhouse under controlled temperature (CT) and drought and irrigated conditions.

	Pods/plant		Pod biomass, g/plant		Seeds /plant		100 seeds biomass		Nodules/plant		Nodule biomass g/plant	
	Irrigated	Drought	Irrigated	Drought	Irrigated	Drought	Irrigated	Drought	Irrigated	Drought	Irrigated	Drought
Genotype												
CMT38	32.4 a	18.7 a	24.4 a	11.9 a	113.6 b	53.4 c	17.7 a	18.5 a	28.9 a	1.6 a	0.053 b	0.007 a
CMT109	30.2 a	17.7 a	22.1 c	11.0 b	128.8 a	62.7 a	13.4 c	13.7 c	27.0 a	1.9 a	0.103 a	0.008 a
CMT187	30.9 a	18.4 a	22.6 bc	11.2 b	123.4 b	58.8 b	15.2 b	15.3 b	17.7 a	1.1 a	0.054 b	0.004 a
G40068	31.6 a	18.3 a	23.7 ab	10.9 b	108.9 b	47.1 d	17.5 a	18.7 a	25.9 a	2.3 a	0.056 b	0.005 a
G40159	31.0 a	18.4 a	22.6 bc	11.1 b	130.6 a	61.9 a	13.7 c	14.7 b	16.9 a	1.2 a	0.055 b	0.004 a
Mean	31.2	18.3	23.1	11.2	121.1	56.8	15.5	16.2	23.3	1.62	0.064	0.005

*Means between a yield component and treatment not followed by the same letters are significantly different at P≤ 0.05 according to Duncan's multiple rang test.

Table 4. Eigen values and per cent of total variation and component matrix for the principal component axes - high temperature (HT) and control temperature (CT)under greenhouse conditions.

Principal components	CP1	CP2	CP3	CP4
HT				
Eigen value	7.30	1.79	1.41	1.05
Variance proportion	0.46	0.11	0.09	0.07
Cumulative proportion variance	0.46	0.57	0.66	0.73
Shoot biomass (g/plant)	0.365	-0.395	0.027	0.019
Pods biomass (g/plant)	0.358	-0.160	0.079	-0.032
Stem biomass (g/plant)	0.296	-0.154	-0.179	0.131
Stem diameter (mm)	0.150	-0.024	0.025	0.368
Leaf biomass (g/plant)	0.322	-0.037	-0.038	0.126
Pod number	0.336	-0.067	0.003	-0.132
Seed total biomass (g/plant)	0.354	-0.008	0.086	-0.054
100-seed biomass	-0.103	0.361	0.323	-0.234
Seed number	0.352	-0.138	-0.029	0.032
Root biomass (g/plant)	0.221	0.224	-0.187	0.128
Nodule biomass	0.173	0.553	0.086	0.007
Nodule number	0.174	0.508	0.172	0.105
SPAD chlorophyll content	-0.176	-0.009	0.068	0.652
Leaf stomatal conductance	0.009	0.035	0.577	-0.101
Efficiency of photosystem II	0.108	-0.399	0.369	-0.317
Leaf temperature (°C)	0.002	0.204	-0.549	-0.435
CT				
Eigen value	8.48	1.26	1.19	1.08
Variance proportion	0.53	0.08	0.07	0.07
Cumulative proportion variance	0.53	0.61	0.68	0.75
Shoot biomass	0.337	-0.078	-0.061	-0.019
Pod biomass	0.329	-0.127	-0.031	-0.036
Stem biomass (g/plant)	0.299	0.060	-0.223	0.049
Stem diameter (mm)	0.188	0.034	-0.200	0.302
Leaf biomass (g/plant)	0.311	0.037	-0.049	0.003
Pods number	0.308	-0.103	-0.086	-0.065
Seed total biomass	0.326	0.183	0.001	-0.045
100-seed biomass	-0.056	-0.660	0.431	0.132
Seeds number	0.328	0.069	-0.164	-0.078
Roots biomass	0.253	-0.040	-0.120	-0.101
Nodules biomass	0.223	0.271	0.306	0.389
Nodules number	0.168	0.297	0.513	0.454
SPAD chlorophyll content	-0.259	0.217	-0.145	0.022
Leaf stomatal conductance	0.11	-0.152	0.392	0.357
Efficiency of photosystem II	0.046	0.493	0.255	-0.405
Leaf temperature (°C)	-0.135	-0.098	-0.267	0.463

Values in bold indicate the traits that were decisive in genotype differentiation.

PCA showed that under HT and CT, the first four components represented 73 and the 75 % of the total variance, respectively (Table 4). Under HT, the first component accounted for 46% of the variance, the second 11%, the third 9% and the fourth 7%, while under CT, the first component accounted for 53% of the variance, the second 8%, the third 7 %, and the fourth 7%. The dominance of these four components suggests that they contained the main variables that discriminate between the genotypes evaluated under HT and CT conditions (Table 4). The traits that separated genotypes in the first component included shoot biomass, pod biomass, total seed biomass and seed number under HT and CT. Under HT, only pod number/plant differed between genotypes. The traits that contributed most to the discrimination in the second component were: nodule biomass, nodule number under HT and 100-seed biomass and the efficiency of photosystem II under CT. In the third component, the separation of genotypes was mainly due to leaf stomatal conductance under HT and CT, leaf temperature under HT and nodule number under CT. In the fourth component, the main traits were: SPAD readings under HT and nodule number and leaf temperature under CT (Table 4).

Effect of HT and CT under drought and irrigated conditions

Effect of HT under drought and irrigated conditions

The PCA showed that, under drought and irrigation, the first five components represented 79% and 80 % of the total variance, respectively (Table 5). Under drought conditions, the first component accounted for 39% of the variance, the second 14 %, the third 10%, the fourth 9% and the fifth 7%; while under irrigated conditions, the first component accounted for 30% of the variance, the second 21%, the third 14 %, the fourth 9% and the fifth 6%. The dominance of these five components suggests that they contained the main variables that discriminate the genotypes evaluated under drought and irrigation (Table 5).

The traits that discriminated genotypes in the first component included shoot biomass, pod biomass, pod number and seed total biomass under drought and irrigated treatments. The traits that contributed most to the discrimination in the second component are 100-seed biomass under drought conditions and leaf biomass, and seed number and nodule biomass under irrigated conditions. In the third component, the differences between genotypes were mainly due to the root biomass under

Table 6. Eigen values and per cent of total variation and component matrix for the principal component axes - control temperature (CT) under drought and irrigated conditions in a greenhouse.

Principal components	CP1	CP2	CP3	CP4	CP5
CT -Drought condition					
Eigen value	6.67	2.10	1.75	1.41	1.18
Variance proportion	0.42	0.13	0.11	0.09	0.07
Cumulative proportion variance	0.42	0.55	0.66	0.75	0.82
Shoot biomass (g/plant)	0.368	0.179	-0.020	0.079	-0.059
Pods biomass (g/plant)	0.348	0.238	-0.070	0.143	-0.038
Stem biomass (g/plant)	0.312	-0.077	0.227	0.057	-0.093
Stem diameter (mm)	0.162	-0.062	-0.061	-0.506	-0.040
Leaves biomass (g/plant)	0.302	0.033	0.012	-0.223	-0.091
Pods number	0.343	0.120	-0.150	0.154	-0.040
Seed total biomass (g/plant)	0.332	0.288	-0.103	0.130	-0.023
100-seed biomass	-0.045	0.573	-0.314	0.168	0.057
Seeds number	0.319	-0.234	0.209	-0.021	-0.059
Roots biomass (g/plant)	0.136	0.170	0.210	-0.334	0.532
Nodules biomass	-0.189	0.334	0.459	0.181	0.195
Nodules number	-0.149	0.270	0.549	0.104	-0.190
SPAD chlorophyll content	0.172	-0.293	0.371	0.317	0.228
Leaf stomatal conductance	-0.033	0.316	0.228	-0.340	0.439
Efficiency of photosystem II	0.292	-0.042	0.229	-0.080	-0.175
Leaf temperature (°C)	0.053	-0.129	-0.021	0.465	0.579
CT-irrigated conditions					
Eigen value	4.85	3.43	1.76	1.64	1.01
Variance proportion	0.30	0.22	0.11	0.10	0.06
Cumulative proportion variance	0.30	0.52	0.63	0.73	0.79
Shoot biomass	0.439	-0.091	0.021	0.019	0.100
Pods biomass	0.365	-0.279	0.083	0.061	0.008
Stem biomass (g/plant)	0.280	0.346	-0.139	-0.063	0.029
Stem diameter (mm)	0.250	0.100	-0.107	-0.099	-0.517
Leaves biomass (g/plant)	0.330	0.166	-0.036	-0.050	0.349
Pods number	0.401	-0.182	0.045	0.011	0.049
Seed total biomass	0.307	-0.353	0.011	0.052	-0.113
100-seed biomass	-0.029	-0.492	0.027	-0.067	-0.179
Seeds number	0.319	0.284	-0.306	0.146	0.113
Roots biomass	-0.108	0.272	-0.114	0.413	-0.353
Nodules biomass	0.092	0.244	0.506	0.229	-0.014
Nodules number	0.021	0.022	0.701	0.028	-0.090
SPAD chlorophyll content	0.128	0.291	-0.196	-0.195	0.081
Leaf stomatal conductance	-0.135	-0.188	-0.115	0.387	0.331
Efficiency of photosystem II	-0.103	0.144	0.363	-0.421	0.508
Leaf temperature (°C)	0.005	-0.007	0.110	0.625	0.117

Values in bold indicate the traits that were decisive in genotype differentiation.

drought and irrigated conditions; and the nodule biomass and nodules number under drought conditions. Under irrigated conditions, differences were due to the efficiency of photosystem II and leaf temperature. In the fourth component, the main traits were: stem diameter under drought and irrigated conditions and leaf temperature under drought, stem diameter and nodule number under irrigated conditions. In the fifth component, the main traits were the SPAD readings and stem diameter under drought and irrigated conditions, respectively (Table 5).

Effect of CT under drought and irrigated conditions

The PCA showed that under drought and irrigated conditions, the first five components represented 82% and the 79% of the total variance, respectively (Table 6). Under drought conditions, the first component accounted for 42% of the variance, the second 13 %, the third 11%, the fourth 9% and the fifth 7%, while under irrigated conditions, the first component accounted for 30% of the variance, the second 22%, the third 11%, the fourth 10% and the fifth 6%. The dominance of these five components suggests that they contain the main variables that discriminate the genotypes evaluated under drought and irrigated conditions (Table 6). The traits that separate genotypes in the first component included shoot biomass, pod biomass, pod number and seed number under drought and irrigated conditions, and seed total biomass under drought conditions. The traits that contributed most to the discrimination in the second component were: 100-seed biomass under both drought and irrigation, stem biomass; and total seed biomass under irrigated conditions. In the third component, the separation of genotypes was mainly due to the nodule biomass and nodule number under drought and irrigated conditions. In the fourth component, the main traits were: leaf temperature under drought and irrigated conditions and the stem diameter under drought conditions. Under irrigated conditions, the main traits were root biomass, leaf stomatal conductance and the efficiency of photosystem II. In the fifth component the main traits were: root biomass and stem diameter under drought and irrigated conditions, respectively (Table 6).

Discussion

In all experiments the stress treatments (high temperature and drought) were effective as all genotypes performed less well under these stresses compared to control conditions. The treatments were also effective in discriminating between good performing and poor performing lines in stress treatments, with the mutant line CMT 38 showing superior characteristics in pods/plant and pod biomass/plant compared to its parental line G40068 and other mutant lines.

There are three main points for discussion: (i) Why is it important to induce mutations in tepary? (ii) Which novel traits are sought from mutagenesis in tepary bean? and (iii) How would the mutant lines be used in a breeding programme for tepary and /or common bean improvement? On the first point, although genetic variability among tepary wild accessions is high (Muñoz et al., 2006; Blair et al., 2012), these are in general more heat and drought tolerant, they also show agronomic disadvantages such as

indeterminate growth habit and very small seeds. The chemical mutagen (EMS) was used in this study to obtain variability in the cultivated accessions (Muñoz et al., 2013). The introduction of characteristics, such as an indeterminate erect growth habit, is necessary in the case of large-scale production, to facilitate mechanical harvesting and mechanical weed removal. This growth habit was also obtained in common beans, using breeding and screening, because it does not exist in traditional varieties. In terms of seed colour, it would probably be necessary to introduce a uniform red seed colour, the colour preferred by the consumers of Central America. On the second point, in the first generations of the mutant populations, lines with deleterious phenotypic variations were observed: dwarf plants, plants with apparent virosis, yellowing, or sterile plants. The selection of mutant lines, presenting desirable characteristics: plants with a determinate growth habit and/or a larger seed size, was carried out, through the generational development of the mutant lines (data not shown). In the present study, the CMT 38 and CMT 187 tepary mutant lines had larger seed size, as reflected by their higher values of 100-seed biomass, compared to the original accessions (G40068 and G40159) under CT conditions (Table 1). Gwata et al., 2016 showed that genotype does not affect seed size of three mutant tepary bean genotypes. The seed size was smaller, as compared to that reported for tepary in other studies. The analysis of a common bean variety and its 34 NaN_3-induced mutants (M_6 generation) showed that the seed yield and yield components differed among the 34 common bean mutants (Wang et al., 2010).

Heat and drought reduce yield and quality and restrict the geographic adaptation of common beans (Rainey and Griffiths, 2005; Beebe et al., 2008). The HT treatment applied to common bean genotypes reduced the yield components: seed number, pod number, mean seed weight and seeds/pod (Rainey and Griffiths, 2005b). In contrast, tepary accessions that produce substantial numbers of pods and seeds under very HT conditions or drought were reported (Rainey and Griffiths, 2005; Rao et al., 2013; Polania et al., 2016). The mutant lines evaluated here under HT and drought conditions, showed a yield higher or comparable to the original accessions G40068 and G40159. This indicates that screening based on morphological traits is useful and not detrimental to seed yield and yield components. G40068 and G40159 were outstanding in their adaptation to terminal drought stress. The superior performance of these accessions was associated with their ability to mobilize photosynthates from leaves and stems to developing grains. Tepary was superior to common bean in combining several desirable traits that contribute to adaptation to terminal drought stress (Rao et al., 2013). Under rainfed conditions, these two accessions yielded more than any elite line or accession of *P. vulgaris* under terminal drought, thus demonstrating the advantages that this species has over *P. vulgaris* under terminal drought stress (Rao et al., 2013, 2017).

The PCA analysis under heat and drought conditions of all traits evaluated showed that the four first components (CP_1, CP_2 CP_3 CP_4) represented 73% of the total variance under HT conditions (Table 4). The SPAD readings appeared to explain the variance in the PCA under HT but not under CT. Three variables: root biomass, stem diameter and biomass do not explain the variance in the analysis performed under HT or CT. But these variables appear to explain the variance in the PCA analysis, when the drought or irrigated conditions of the experiment were considered under HT and CT conditions (Tables 5 and 6). This indicates that these traits can be used to select better adapted genotypes under drought conditions. A greater capacity to develop roots that go deep into the soil can provide a better adaptation to conditions of water stress (White and Castillo, 1992; Polania et al., 2009, 2016). There is a direct correlation between drought and heat stresses, since during heat stress water availability can be at a deficit caused by the high temperature (Omae et al., 2012). It is necessary to identify specific morpho-physiological traits that contribute to improved resistance to combined stresses of heat and drought in beans, and that could be useful as selection criteria in breeding.

On the last point, how would the mutant lines be used in a breeding programme for tepary and/or common beans? These genetically stable mutant lines, which were selected for their phenotypic characters, and/or for their tolerance to HT and drought, could have two possible uses in a bean breeding programme. First, these mutant lines could be included in interspecific crosses, between *P. vulgaris* and *P. acutifolius,* to try to introgress these physiological characteristics to common bean. Second, they could be used for the improvement per se of the species *P. acutifolius*.

Materials and methods

Plant materials

We evaluated two accessions of tepary bean, *P. acutifolius* A. Gray (G40068 from Arizona, USA and G40159 from Sonora, Mexico, and three mutant M_6 lines (CMT 38, CMT 109 and CMT 187), which were uniform and genetically stable. The mutant line CMT 38 was obtained from the G40068 accession, while CMT 109 and CMT 187 mutant lines were obtained from the G40159 accession. . These mutant lines were selected based on two key traits: large seed size and/or a determinate growth habit and superior yield from previous experiments, where M_5 mutant lines were evaluated under drought and high temperature conditions in greenhouse tests (data not shown). The evaluated tepary mutant lines were obtained from a protocol established by Muñoz et al. (2013), using ethyl methane sulfonate (EMS).

Experimental conditions

The experiments were conducted at the International Center for Tropical Agriculture (CIAT) in Palmira, Colombia, located at latitude 3° 29' N, longitude 76° 21' W and 965m above sea level.

Two experiments were conducted simultaneously in two separate greenhouses, to evaluate the M_6 mutant lines and the two tepary parental (M_0) accessions (G40068 and G40159) to high temperature and drought stress conditions. Experiment 1 was carried out with a high temperature treatment (HT) in a greenhouse. Experiment 2 was carried out at normal (control temperature) conditions (CT) in another greenhouse. Both experiments included three replicates and were conducted using pots with a Mollisol soil from Palmira. The seeds were germinated in wet paper towels and uniform seedlings were selected for transplanting into pots. The plants of each accession and of the mutant tepary lines from the two experiments were inoculated at 10 days after sowing with *Rhizobium tropici* (strain CIAT 899) or *Bradyrhizobim* spp. (strain CIAT461) as is normal practice. To obtain high temperature

conditions and simulate the changes in temperature between day and night, conditions in the greenhouse were modified using heaters, ventilation and thermostats. The HT treatment was set at 29±5 ºC during the day and >24 ºC during the night, with an average relative humidity of 65%. The maximum day/night temperatures of the greenhouse for normal conditions (CT) were set at day/night of 30°C /20°C. Data on relative humidity and temperature were monitored with thermo-hygrometers that registered the parameters every 15 minutes. The mean and minimal/maximal temperatures were calculated per day.

Plants were grown in optimal conditions of soil moisture (80% field capacity) for 10 days and were then submitted to their respective treatment with soil moisture, either at 80% field capacity (irrigated) or 40% (drought). In both cases, the pots were weighed twice a week and water was added to bring back the required moisture level.

Measurement of shoot and root morpho-physiological characteristics

Plants were harvested between 80 to 86 days under drought and at 100 days under irrigated conditions. At the mid-pod filling growth stage, the following non-destructive measurements were made: leaf chlorophyll content of fully expanded leaves was measured using a non-destructive, hand-held chlorophyll meter (SPAD-502 chlorophyll metre, Minolta Camera Co., Ltd., Japan). The principle is based on the difference in light attenuation at wavelengths 430 and 750 mm. From the difference in light attenuation, a numerical SPAD (Soil-Plant Analysis Development) unit, ranging from 0 to 80, is calculated by the microprocessor in the SPAD-502 chlorophyll metre. The efficiency of photosystem II (QY) in leaves adapted to light (Fv'/Fm') was also determined. The stomatal conductance (mmol m^{-2} s^{-1}) was measured with a portable leaf porometer (Deacagon SC-1) on a fully expanded young leaf of one plant within each replication. Measurements were made late in the morning (10 am -12 noon) on clear and sunny days. The leaf temperature was measured with an infra-red thermometer (Telatemp AG-42D, Telatemp Co, US). At the time of harvest, plants were cut at soil level and dry weights of different shoot biomass components (stem, leaves, pod biomass and pod number, seed number, seed biomass per plant) were recorded. The roots of each pot were washed free of soil. Root weight per plant was determined after the roots were dried in an oven at 60 °C for 48h.

Statistical analysis

A separate analysis was conducted for each experiment. The sources of variation within each experiment were: replications and genotypes. All data were analyzed using SAS software (v. 9.2). Values marked with * or ** are statistically significant at probability levels of 5% and 1%, respectively. The mean values were compared with the Duncan test. A Principal Component Analysis (PCA) was performed on the measured variables and was based on Pearson correlation matrix and Euclidean distances. Eigen values for all principal components (PC) were shown. Eigen vectors generated by the PCA were used to identify parameters that best differentiated the genotypes in each experiment.

Conclusion

Mutation induction in G40068 and G40159 cultivated tepary accessions, increased the genetic variability in morpho-physiological characteristics of the species. In addition, the CMT 38, CMT 109 and CMT 187 mutant lines showed seed yield values per plant comparable to or higher than that of the original accessions, under heat and drought conditions. The identification of four key plant traits in the PCA analysis (SPAD readings, seed biomass, 100-seed biomass and seeds number) explained a major part of the variance under heat and drought conditions, and suggests that these traits and two others (root biomass and stem diameter, identified from the univariate analysis) could be incorporated into tepary breeding programmes as selection criteria to screen the tepary accessions and their mutant lines, for combined tolerance to heat and drought stresses.

Mutation breeding has potential to generate phenotypic and genotypic variations in tepary bean that can be exploited by plant breeders in the development of new cultivars with improved adaptation to heat and drought stress.

Acknowledgements

We acknowledge the partial support from the International Atomic Energy Agency (IAEA) under the CRP23029 for the project on "Heat effects in tepary beans and its relatives". We thank the late Mr. Orlando Toro at the Genetic Resources Unit of CIAT, for his technical support during the process of selection of the accessions and mutant lines of tepary bean.

References

Ahloowalia BS, Maluszynski K, Nichterlein K (2004) Global impact of mutation derived varieties. Euphytica 135:187-204.

Beebe S, Rao IM, Cajiao C, Grajales M (2008) Selection for drought resistances in common bean also improves yield in phosphorus limited and favorable environments. Crop Sci. 48:582-592.

Bielenberg DG, Miller JD, Berg VS (2003) Paraheliotropism in two *Phaseolus* species: combined effects of photon flux density and pulvinus temperature, and consequences for leaf gas exchange. Environ Exp Bot. 49: 95-105.

Blair MW, Porch T, Cichy K, Galeano CH, Laringuet P, Pankhurst CE, Broughton W (2007) Induced mutants in common beans (*Phaseolus vulgaris*) and their potential use in nutrition quality breeding and gene discovery. Isr J Plant Sci. 55:191-200.

Blair MW, Pantoja W, Muñoz, LC (2012) First use of microsatellite markers in a large collection of cultivated and wild accessions of tepary bean (*Phaseolus acutifolius* A. Gray). Theor Appl Genet. 125:1137–1147.

Butare L, Rao I, Lepoivre P, Polania J, Cajiao C, Cuasquer J, Beebe S (2012) Phenotypic evaluation of interspecific recombinant inbred lines (Rils) of *Phaseolus* species for aluminium resistance and shoot and root growth response to aluminium- toxic acid soil. Euphytica 186 (3) 715-730.

Debouck DG (1992) Frijoles (*Phaseolus* spp.). *In*: Hernández Bermejo E, León J (ed) Cultivos marginados: otra perspectiva de 1492. Food and Agriculture Organization of the United Nations, Rome, Italy. 45-60.

Dillen W, De Clercq J, Goossens A, van Montagu M, Angenon G (1997) *Agrobacterium*-mediated transformation of *Phaseolus acutifolius* A. Gray. Theor Appl Genet. 94: 151-158.

Freeman GF (1912) Southwestern beans and teparies. Univ Ariz Agric Exp Station Bull. 68: 573-619.

Gwata ET, Shimelis H, Matova M (2016) Potential of improving agronomical attributes in tropical legumes using two mutation breeding techniques in southern Africa. In: Petr K (ed) Alternative crops and cropping systems, IntechOpen Ltd, London, UK. 71-85.

Lin TY, Markhart AH (1996) *Phaseolus acutifolius* A. Gray is more heat tolerant than *P. vulgaris* in the absence of water stress. Crop Sci. 36 (1) 110-114.

Markhart III AH (1985) Comparative water relations of *Phaseolus vulgaris* L. and *Phaseolus acutifolius* A. Gray. Plant Physiol. 77: 113-117.

Mejía, J. A., C. Muñoz, H.J. Jacobsen, W.M. Roca, and S.P. Singh, 1994: Interspecific hybridization between common and tepary beans: Increased embryo growth, and efficiency of hybridization through recurrent and congruity backcrossing. Theor Appl Genet. 88: 324–331.

Muñoz LC, Blair WM, Duque MC, Tohme J, Roca W (2004) Introgression in common bean x tepary bean interspecific congruity-backcross lines as measured by AFLP markers. Crop Sci. 44: 637-645.

Muñoz LC, Duque MC, Debouck DG, Blair WM (2006) Taxonomy of tepary bean and wild relatives as determined by amplified length polymorphism (AFLP) markers. Crop Sci. 46: 1744-1754.

Muñoz LC, Toro O, Muñoz JE, Debouck DG (2013) Inducción de mutantes por vía química para el mejoramiento genético de *Phaseolus acutifolius* A. Gray. Presented at the VII congreso Colombiano de Botánica, Universidad del Tolima, Ibague, Colombia 6 - 10 Agosto 2013.

Nabhan GP (1985) Gathering the desert. The University of Arizona Press, Tucson, Arizona, USA. 209p.Nabhan GP, Felger RS (1978) Teparies in southwestern North America - A biogeographical and ethnohistorical study of *Phaseolus acutifolius*. Econ Bot. 32: 3-19.

Omae H, Kumar A, Shono M (2012) Adaptation to high temperature and water deficit in the common bean (*Phaseolus vulgaris* L.) during the reproductive period. J. Bot. doi:10.1155/2012/803413.

Polanía JA, Rao IM, Beebe S, García R (2009) Desarrollo y distribución de raíces bajo estrés por sequía en frijol común (*Phaseolus vulgaris* L.) en un sistema de tubos con suelo. Agron colomb. 27: 25-32.

Polania JA, Poschenrieder C, Beebe S, Rao IM (2016) Effective use of water and increased dry matter partitioned to grain contribute to yield of common bean improved for drought resistance. Front Plant Sci. 7: 660.

Porch T, Blair WM, Laringuet P, Galeano CH, Pankhurst CE, Broughton W (2009) Generation of a mutant population for tilling common bean genotype BAT 93. J Am Soc Hort Sci. 134 (3) 348-355.

Pratt RC, Nabhan GP (1988) Evolution and diversity of *Phaseolus acutifolius* genetic resources. In: Gepts P (ed) "Genetic resources of Phaseolus beans: their maintenance, domestication, evolution and utilization", Kluwer Academic Publ., Dordrecht, Holland. 409-440.

Rainey KM, Griffiths PD (2005) Evaluation of *Phaseolus acutifolius* A. Gray plant introductions under HTs in a control environment. Genet Resour Crop Evol. 52: 117-120.

Rainey KM, Griffiths PD (2005b) Differential response of common bean genotypes to high temperature. J Am Soc Hort Sci. 130: 18-23.

Rao IM, Beebe SE, Polania J, Ricaute J, Cajiao C, Garcia G, Rivera M (2013) Can tepary bean be a model for improvement of drought resistance in common bean? Afr J Crop Sci. 21: 265-281.

Rao IM, Beebe SE, Polania J, Grajales M, Cajiao C, Ricaurte J, Garcia R, Rivera (2017) Ability to remobilize photosynthate to increase yield under drought. J Agric Sci. 155:857-875.

Schinkel C, Gepts P (1988) *Phaseolin* diversity in the tepary bean, *Phaseolus acutifolius* A. Gray. Plant Breed. 101: 292-301.

Wang HH, Jeng TL, Chen ML, Chuang, Sung JM (2010) Molecular and morpho-agronomic characterization of NaN_3-induced common bean mutants. Crop Environ Bioinf. 7 (12) 221-231.

White JW, Castillo JA (1992) Evaluation of diverse shoot genotypes on selected root genotypes of common bean under soil water deficits. Crop Sci. 32: 762-765.

DOI: 10.21475/ajcs.21.15.09.sp-8

Australian Journal of Crop Science AJCS

Field performance of heat tolerant mutant rice lines generated from *Oryza sativa* and *Oryza glaberrima*

Paul Mbogo Kusolwa*[1], Yona Neema[1], Masanche Rajab[1], Ashura, Luzi- Kihupi[1], and Fatma Sarsu[2]

[1]Department of Crop Science and Production, Sokoine University of Agriculture P. O. Box 3005 Morogoro Tanzania
[2]Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, Plant Breeding and Genetics Section Vienna, Austria

***Corresponding author: kusolwap@gmail.com**

Abstract

This study evaluated mutant lines developed from two cultivated species of upland rice, *Oryza sativa,* and *Oryza glaberrima*, in field experiments conducted during the hot and dry seasons of 2014/2015 in Morogoro, Tanzania. The growth yield and yield components of 34 and 14 Gamma induced mutant upland rice lines developed from O. *sativa* (Kihogo red) and *O. glaberrima* were evaluated, respectively. The mutant lines were selected based on variable expression of heat shock protein genes (HSPs) in previously conducted heat tolerance studies. The minimum and maximum temperatures and rainfall during the field performance experiment were measured between 20 ^{o}C and 35 ^{o}C, and 32.7 mm and 155.5 mm, respectively. The data for 12 yield and yield component parameters such as days to early and 50% flowering, days to physical maturity, plant height, number of tillers, number of panicles, spikelets, filled grains, unfilled grains and 1,000 grain weights were collected and analysed using ANOVA and Principal Component Analysis. Significant differences ($P \leq 0.05$) were obtained among the mutant lines in terms of grain yield, spikelet sterility and other variables, which were further used as criteria for selection of heat and drought tolerant rice lines. Eight heat and drought tolerant mutant rice lines with high yields (over 3.5 ton/ha) and low spikelet sterility were selected for further advancement in breeding programmes.

Key Words: *Oryza sativa*, *Oryza glaberrima,* heat tolerance, mutant lines, growth, yield and yield components.
Abbreviations: Heat tolerant (HT), Heat Shock Proteins (HSPs), Kihogo Red (KR), International Atomic Energy Agency (IAEA)

Introduction

Rice is a staple food for more than half of the world's population, mostly living in developing countries, and is the leading cereal crop worldwide (Fageria et al., 2011). Climate changes due to global warming have negative and significant effects on rice production, particularly on its growth, development, and yield. Zhang et al. (2013) stated that extreme climate change associated with high temperature occurs more frequently and with longer duration in several rice production regions, worldwide. Increase in global temperature results in damage on growth and development of rice crops (Manneh et al., 2007).

Heat stress causes significant alterations in plant growth, development and physiological processes (Hasanuzzaman et al., 2013). High temperature has influence on almost all rice growing stages, especially, booting, flowering, and grain filling periods in many rice production regions; hence, plant growth, development, grain quality and yield are negatively affected (Ranga et al., 2011; Aghamolki et al., 2014). Zhang et al. (2013) reported that an increase in temperature of about 1 – 2 ° C above the optimum level during the reproductive stage results in shortening the grain filling period, with negative effects on yield and yield components in rice as well as in other cereal crops. As part of the strategies for adaptation and/or mitigation of yield reduction due to climate, concerted efforts to develop climate proof cultivars that stabilize yield have been set up. Heat tolerant (HT) mutant rice lines were developed through mutation induction using Gamma irradiation (Yona, 2015). Subsequent mutant generations were subjected to extreme heat stress and survived plants were selected for further field evaluation.

Tolerance to heat and drought stress in plants, including rice, is a complex phenomenon and controlled by multiple gene loci imparting a number of physiological and biochemical changes in plant cells (John, 2001). Plants exposed to stress parameters may survive by creating signals for changing their metabolism (John, 2001). The protection system may involve heat and drought resistance mechanisms that are associated with synthesis and accumulation of specific cell components known as heat shock proteins (HSPs) (John, 2001). The response of HSPs allows rice plants to become more tolerant to stress parameters including heat and drought (Chang et al., 2007). Several molecular plant breeding approaches have been proposed to identify the genes related to agronomic traits for rice improvement. Use of these approaches for evaluation of yield and yield component are on the verge of becoming important in terms of measuring response of a rice genotype to heat and drought stressed environments (Garge et al., 2012). The main objective of this study was to evaluate the growth, yield and yield components of the HT mutant rice lines based on their field performance tests.

Results

Evaluation of the HT mutant rice lines: their growth performances, yield and yield components

The variables considered for evaluation of the lines generated from parental rice genotypes, cv. Kihogo Red (*O. sativa*), CG 14 (*O. glaberrima*), WAB 56_50 (*O. sativa*) and WAB 56_104 (*O.*

sativa) were as follows; days to early flowering, days to 50 % flowering, days to 85 % physical maturity, plant height, number of tillers per plant, panicle number per plant, spikelets per panicle, filled grains per panicle, unfilled grains per panicle, 1,000 grains weight and grain yield. These variables were scored at different growth stages of the mutant lines during hot and dry field conditions. Vegetative, reproductive (booting and flowering) and maturity stages were the critical periods in this study. Collected data for lines derived from each genotype regarding the high temperature stress parameters are explained in the following sections.

Flowering rates (early and 50 %), physical maturity rates (85 %), plant height and panicle length

The mean variations of the mutant rice lines on days to early flowering, 50 % days to flowering, physical maturity (days to 85 %), plant height and panicle length are given in Table 1. The data related to days to early flowering are also presented in Figure 1A. There were significant differences ($P<0.05$) among the genotypes for days to early flowering and plant height parameters (Table 1, Figure 1A). However, the difference in days to 50 % flowering and days to 85 % physical maturity and panicle length were not significant (Table 1). Among the lines, both CG 14_16_1 and WAB 56_50_56_2 was observed to have a short duration as '74 days' from sowing date to the first flowering, while KR 38_1 line had a longer period,108 days from sowing to the first emerged flower (Table 1). A shorter duration of 81 days for 50 % flowering was observed for WAB 56_50_98_1 line; however, the longest duration was observed for KR 38_1 line, 125 days. In terms of physical maturity, both WAB 56_50_97_4 and WAB 56_50_127_5 lines required 98 days to reach physical maturity after sowing; however, the KR 38_1 line had a longer period of 149 days to attain 85 % physical maturity (Table 1).

Number of spikelets, tillers number, panicle number, filled grains and unfilled grains per plant

There were highly significant differences ($P<0.05$) among parameters for average number of spikelets per panicle, number of tillers per plant, panicle number per plant, filled and unfilled grains per panicle per plant among all mutant lines (Figure 1B, C, D and E). The highest number of spikelets per panicle was observed for CG 14_16_1 line (13 spikelets), while the lowest number was measured both in WAB 56-104 Control and CG 14_20_1 lines (nine spikelets) (Figure 1D). A high tiller number per plant was observed for WAB 56_104_150_2 line (33 tillers); however, the lowest number of tillers was identified in CG 14_16_1 line (six tillers) (Figure 1B). Similarly, the highest number of panicles was observed both for WAB 56-104 (control) and WAB 56_104_150_2 lines (27 panicles), but the lowest numbers were measured among several lines including CG 14_63_1 (Figure 1E). The highest number of filled grains per plant was observed in WAB 56_50_51_1 (759 grains) among mutant lines, while the least filled grains per plant was counted as 122 grains in WAB 56-104 control line (Figure 1C). Moreover, the lowest number of unfilled grains among all lines were measured as 74 in WAB 56_50_127_5. Data on number of tillers per plant, filled and unfilled grains in panicles per plant, number of panicles per plant and number of spikelets per panicle are presented in Figures 1B, C, D and E, respectively.

Grain yield, 1,000 grain weight, and spikelet sterility

The average data for the variations in grain yield (t/ha), 1,000 grains weight (g) and spikelet sterility (%) among mutant rice lines are given in Table 2. Significant differences were observed for grain yield and spikelet sterility; however, no significant difference was identified among the mutant lines in terms of 1,000 grain weight at $P<0.05$ level (Table 2).

The average data related to 1,000 grain weight, grain yield and sterility rate among the analysed mutant lines are presented in Figure 2A, B and C, respectively. The highest average grain yield was observed for CG 14_63_1 and CG 14_16_1 line as 6 and 5 t/ha, respectively; while the lowest grain yield was measured as 2 t/ha both in WAB 56_50_123_3 and WAB 56_50_127_5 lines (Figure 2B). The highest 1,000 grain weight was observed for WAB 56_104_36_1 line (33g), and the lowest was identified in KR 27_1 line (25g) (Figure 2A). The WAB 56_50_141_1 line however, showed the lowest sterility rate as 9% (Figure 2C).

Analyses of growth performance yield and yield components among the WAB 56_50 mutants and control lines

Some of the comparative values for the irradiated (mutants) and non-irradiated (control) WAB 56_50 rice lines are given in Figure 3. When the mutants generated from WAB 56_50 were compared with its control lines, the average values for parameters of days to early flowering, days to 50% flowering, plant height, panicle number per plant, spikelet per panicle, filled grains and unfilled grains per panicle, 1,000 grain weight and grain yield were observed to be higher in mutant rice lines. However, the number of tillers per plant was the same, and the average values of spikelet sterility (panicle sterility) and days to physical maturity were low in mutant rice lines as compared to the control (Figure 3).

Analysis of growth performance yield and yield components among the WAB 56_104 mutants and control rice lines

Some of the comparative data for the irradiated (mutants) and non-irradiated (control) WAB 56_104 rice lines are presented in Figure 4. When the mutants were compared with control lines, the average values for days to early flowering, days to physical
maturity, plant height, panicle number per plant, filled and unfilled grains per panicle, 1,000 grain weight and grain yield were measured higher in mutant rice lines as compared to the WAB 56-104 control (Figure 4). However, the data for spikelet sterility (panicle sterility) and days to physical maturity were high in non-mutant rice genotype as compared to HT mutant rice lines, except for the number of days to 50 % flowering and spikelets/panicle were observed as the same among HT mutant and control lines (Figure 4).

Analysis of growth performance yield and yield components among the CG 14 mutants and control rice lines

The comparative values for the irradiated mutants and non-irradiated control CG 14 rice lines are given in Figure 5. The average values for parameters of days to early flowering, days to 50 % flowering, days to physical maturity, plant height, spikelets/panicle, filled grains, bird loss (%) and grain yield were observed to be higher in CG derived mutant lines as compared to non-mutant control genotype. The unfilled grains/panicle and spikelet sterility parameters were low in mutant rice lines when compared with its control. However, the panicle number per plant and 1,000–grain weight parameters were observed to be the same both in mutant lines and control rice genotypes (Figure 5).

Analysis of growth performance yield and yield components among the Kihogo red (KR) mutants and control rice lines

The comparative values for the irradiated and non-irradiated Kihogo red (KR) rice lines are presented in Figure 6. The average values for the parameters of plant height, filled grains per panicle, 1,000 grain weight and grain yield in mutant rice lines were high, when compared with non-irradiated control genotype.

Table 1. The mean variations of the mutant rice lines on days to early flowering, 50% days to flowering, physical maturity (days to 85%), plant height and panicle length.

Rice line	Days to early flowering	50% days to flowering	Days to 85% Maturity	Plant height (cm)	Panicle length (cm)
KR 27_1	95de	104c	147d	130gh	23abcd
KR 38_1	108ef	125d	149d	131g	21abc
CG 14_16_1	74a	85a	108abc	104abcde	21abc
CG 14_20_1	77abc	82a	104abc	105abcde	23abcd
CG 14_58_1	81cd	89ab	109abc	113cdef	21abc
CG 14_61_3	79abcd	87ab	110abc	114ef	35bcd
CG 14_63_1	77abc	88ab	100ab	100ab	20ab
CG 14_63_2	77abc	82a	107abc	95a	22abcd
WAB 56_104_36_1	81cd	89ab	109abc	114ef	20ab
WAB 56_104_141_1	81bcd	97b	108abc	109bcdef	25abcd
WAB 56_104_141_2	78abcd	85a	106abc	110bcdef	21abc
WAB 56_104_141_3	75abc	83a	107abc	109bcdef	24abcd
WAB 56_104_150_2	77abc	84a	107abc	108bcdef	24abcd
WAB 56_50_51_1	76abc	80a	106abc	101abc	36d
WAB 56_50_56_1	80abcd	89ab	108abc	113def	20ab
WAB 56_50_56_2	74a	82a	111bc	99ab	21ab
WAB 56_50_74_1	77abc	85a	110abc	118f	25abcd
WAB 56_50_82_1	77abc	83a	111bc	107bcdef	21ab
WAB 56_50_85_2	79abcd	85a	105abc	106abcde	23abcd
WAB 56_50_85_3	76abc	86ab	111bc	114ef	22abc
WAB 56_50_97_2	76abc	84a	113c	106abcde	23abcd
WAB 56_50_97_3	77abc	84a	110abc	110bcdef	21ab
WAB 56_50_97_4	75ab	87ab	98a	103abcde	21ab
WAB 56_50_98_1	75ab	81a	111bc	104abcde	21ab
WAB 56_50_98_3	77abc	91ab	108abc	108bcdef	22abcd
WAB 56_50_123_1	75abc	84a	106abc	103abcde	22abcd
WAB 56_50_123_2	75ab	79a	104abc	104abcde	22abc
WAB 56_50_123_3	84d	86ab	111bc	108bcdef	20ab
WAB 56_50_127_3	78abc	82a	108abc	104abcde	22abcd
WAB 56_50_127_5	80bcd	85a	98a	108bcdef	21ab
WAB 56_50_135_1	75abc	81a	109abc	101abcd	21ab
WAB 56_50_141_1	80abcd	85a	105abc	112cdef	25abcd
WAB 56_50_141_2	79abcd	85a	109abc	104abcde	20a
WAB 56_50_152_3	81cd	86ab	108abc	103abcde	36cd
KR control	109f	122b	148d	127fg	109f
CG 14 Control	80bcd	91ab	106abc	113cdef	21abc
WAB 56-104 Control	78abcd	84a	107abc	110bcdef	23abcd
WAB 56-50 Control	76abc	83a	106abc	103abcde	22abc
Grand mean	78	85	107	107	23
S.E	3.106	6.04	6.131	6	7.3
CV	4.0	7.1	5.7	5.6	31.6

*Figures followed by the same letter (s) in columns are not significantly different at P< 0.05 according to DMRT.

In contrast, days to early flowering, days to 50 % flowering, panicle length, spikelets per panicle, unfilled grains per panicle, bird loss and spikelet sterility parameters were observed to be low in mutant rice lines when compared with the control lines. The number of tillers per plant, panicle number per plant and days to physical maturity values; however, were the same among irradiated mutants and non-irradiated control lines (Figure 6).

Principal Component Analysis

A Principal Component Analysis (PCA) was performed to compare all the genotypes with their controls and to identify the major traits which contribute to the variations observed for heat tolerance in the lines tested (Figure 8). The first component accounts for 47.76 % of the variation and the second 20.68%, The cumulative variance accounting for 68.46 % of the total variation. The PCA plot is given in Figure 8; and as it can be seen that most genotypes cluster are together. The outliers are: the KR lines (KR control and its mutant lines KR27_1 and KR38_1), WAB56_50_74_1, WAB56_104_36_1 and CG14-63-1. The PC1 axis separates the three KR genotypes from the rest of genotypes (mainly yield component traits), whereas WAB56_50_74_1, WAB56_104_36_1 and CG14_63_1 are separated from the More detailed comparisons of mutants with their respective parents are made in individual trait analyses (ANOVA). grown in several highly populated countries including China, India, Indonesia, Malaysia, Philippines, Taiwan and etc. Unlike *Oryza sativa* rice species, *Oryza glaberrima* is grown in limited areas in the world. However, this African rice species contains many unique and useful traits such as heat and drought tolerance, pest and disease resistance, weed competitiveness, and the ability to grow under low input conditions (Sarla and Mallikarjuna, 2005). Furthermore, *Oryza glaberrima* is a potential source of genes to enhance eating, cooking, and milling properties of rice grain.

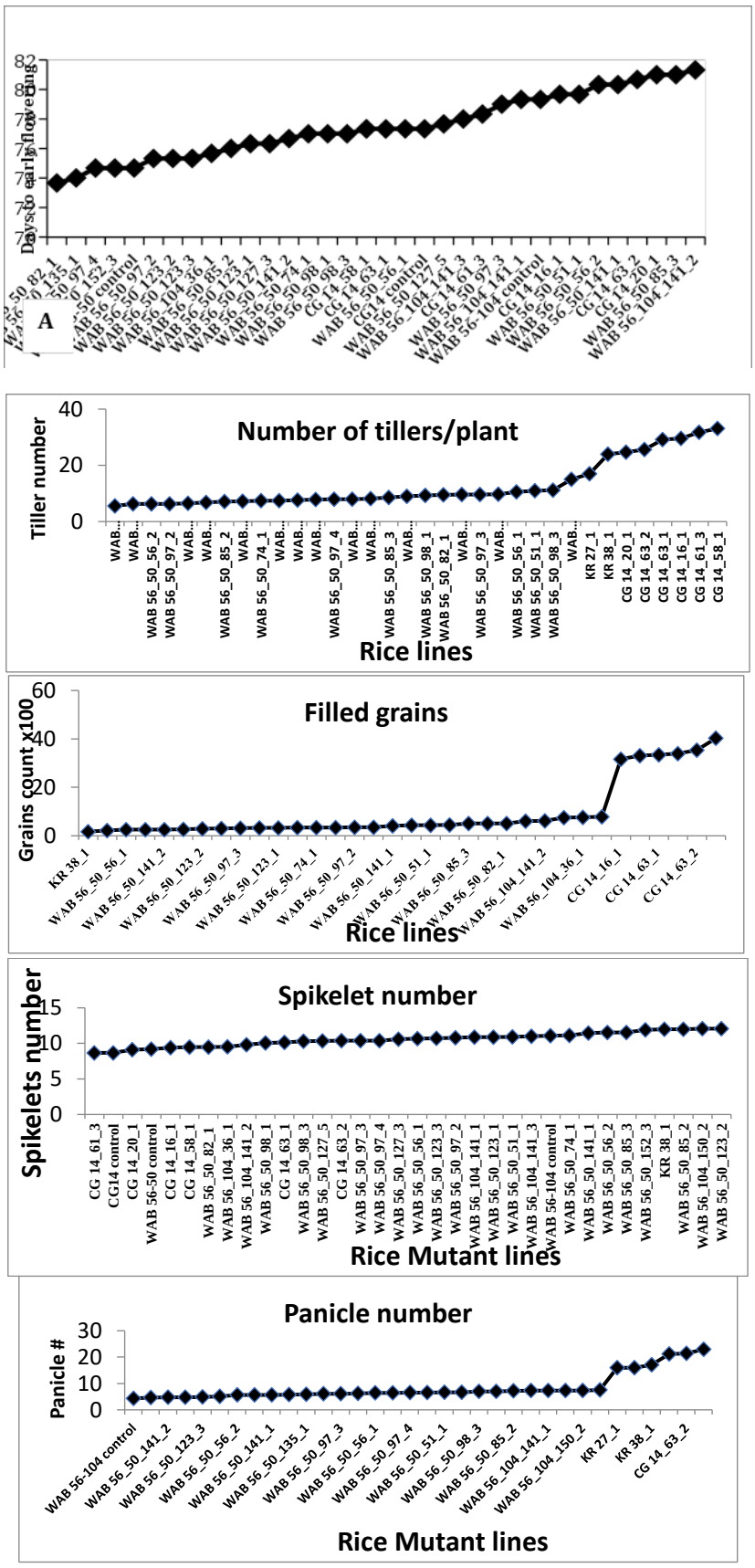

Fig 1. Identified data for some of the growth components of the evaluated mutant rice lines **(A)** Number of days to 50% flowering, **(B)** The average number of tillers, **(C)** filled grains, **(D)** spikelets, and **(E)** panicles of rice mutant lines ordered from the lowest to the highest.

Table 2. The mean summary for variations of mutant rice lines on grain yield (t/ha), 1,000 grains weight (g), spikelet sterility and bird loss percentage.

Rice line	Grain yield (t/ha)	1,000 grain wt. (g)	Spikeletsterility (%)
KR 27 1	3.4a	25a*	50.7a
KR 38_1	6.5a	31b	43.6a
CG 14_16_1	7e	28abc	21abc
CG 14_20_1	3abc	29abcd	26bc
CG 14_58_1	7e	28abc	18abc
CG 14_61_3	5cd	30abcd	14abc
CG 14_63_1	8e	29abcd	22abc
CG 14_63_2	6de	28abc	19abc
WAB 56_104_36_1	3abc	33d	14abc
WAB56_104_141_1	3abc	30abcd	13ab
WAB 56_104_141_2	4bc	30abcd	14abc
WAB 56_104_141_3	3ab	30abcd	18abc
WAB 56_104_150_2	2ab	29abc	11a
WAB 56_50_51_1	3abc	31bcd	14abc
WAB 56_50_56_1	2ab	30abcd	15abc
WAB 56_50_56_2	3ab	26a	17abc
WAB 56_50_74_1	3ab	31cd	13ab
WAB 56_50_82_1	3ab	30abcd	16abc
WAB 56_50_85_2	3abc	29abc	19abc
WAB 56_50_85_3	3abc	29abcd	17abc
WAB 56_50_97_2	3ab	30abcd	20abc
WAB 56_50_97_3	2ab	28abc	17abc
WAB 56_50_97_4	3ab	29abcd	23abc
WAB 56_50_98_1	2ab	27abc	28c
WAB 56_50_98_3	3ab	30bcd	19abc
WAB 56_50_123_1	3ab	31bcd	20abc
WAB 56_50_123_2	3ab	31bcd	19abc
WAB 56_50_123_3	2a	30abcd	15abc
WAB 56_50_127_3	2ab	29abcd	21abc
WAB 56_50_127_5	2a	27abc	22abc
WAB 56_50_135_1	2ab	28abc	20abc
WAB 56_50_141_1	3abc	31bcd	9a
WAB 56_50_141_2	2ab	29abcd	26bc
WAB 56_50_152_3	2ab	27ab	11a
KR control	3.7a	27a	48.9a
CG14 Control	6de	29abcd	17abc
WAB 56_104 Control	2ab	31cd	12ab
WAB 56_50 Control	3ab	28abc	19abc
Grand mean	3	29	18
S.E	0.92	1.95	7.12
CV	28.4	6.6	40.3

*Values followed by the same letter (s) in columns are not significantly different at P< 0.05 according to DMRT.

Oryza glaberrima and *Oryza sativa* breeding programmes resulted in several positively inbreed lines including traits such as adaptation to difficult West African conditions with high yield, resistance to drought, and pest and diseases (Sarla and Mallikarjuna, 2005). The main hindrance in the use of *Oryza glaberrima* as a germplasm is its incompatibility in pollination with *Oryza sativa*; however, this obstacle has been overcome by generating fertile progeny from *Oryza sativa* × *Oryza glaberrima* crosses. Therefore, new inbreed lines presented a broader range of germplasm for rice breeders (Dingkuhn et al., 1998). Of the 34 mutant lines used in this study, 28 were generated from *Oryza sativa* (two from KR, five from WAB 56_104 and 21 from WAB 56_50 genotypes) and six from *Oryza glaberrima* (from CR 14 genotype) (Table 3). Comparison of the 12 yield and yield component parameters of these mutant lines revealed that *Oryza glaberrima* derived CR 14 genotyped mutant lines showed better performance than the *Oryza sativa* derived KR genotyped mutant lines, and almost the same performance with WAB 56_104 and WAB 56_50 genotyped mutant lines in terms of days to early and 50 % flowering, days to physical maturity, plant height, filled grains, 1,000–grain weight and grain yield parameters (Figure 3, 4, 5 and 6). These mutant lines from *Oryza glaberrima* present a unique opportunity to African breeders as novel germplasm for future breeding programmes. Interestingly, the PCA plot (Figure 8) did not separate the *O. oryza* genotypes from the *O. glaberimaa* genotypes (they clustered together), however, the tall KR (*O. Sativa*) genotypes were separated (Fig. 8).

Discussion

Differences between *Oryza sativa* and *Oryza glaberrima*

Two main rice species, *Oryza glaberrima* and *Oryza sativa*, and their four different genotypes, cv. Kihogo Red, WAB 56_50, WAB 56_104 (*Oryza sativa*) and CG 14 (*Oryza glaberrima*) were used as plant materials in the study.

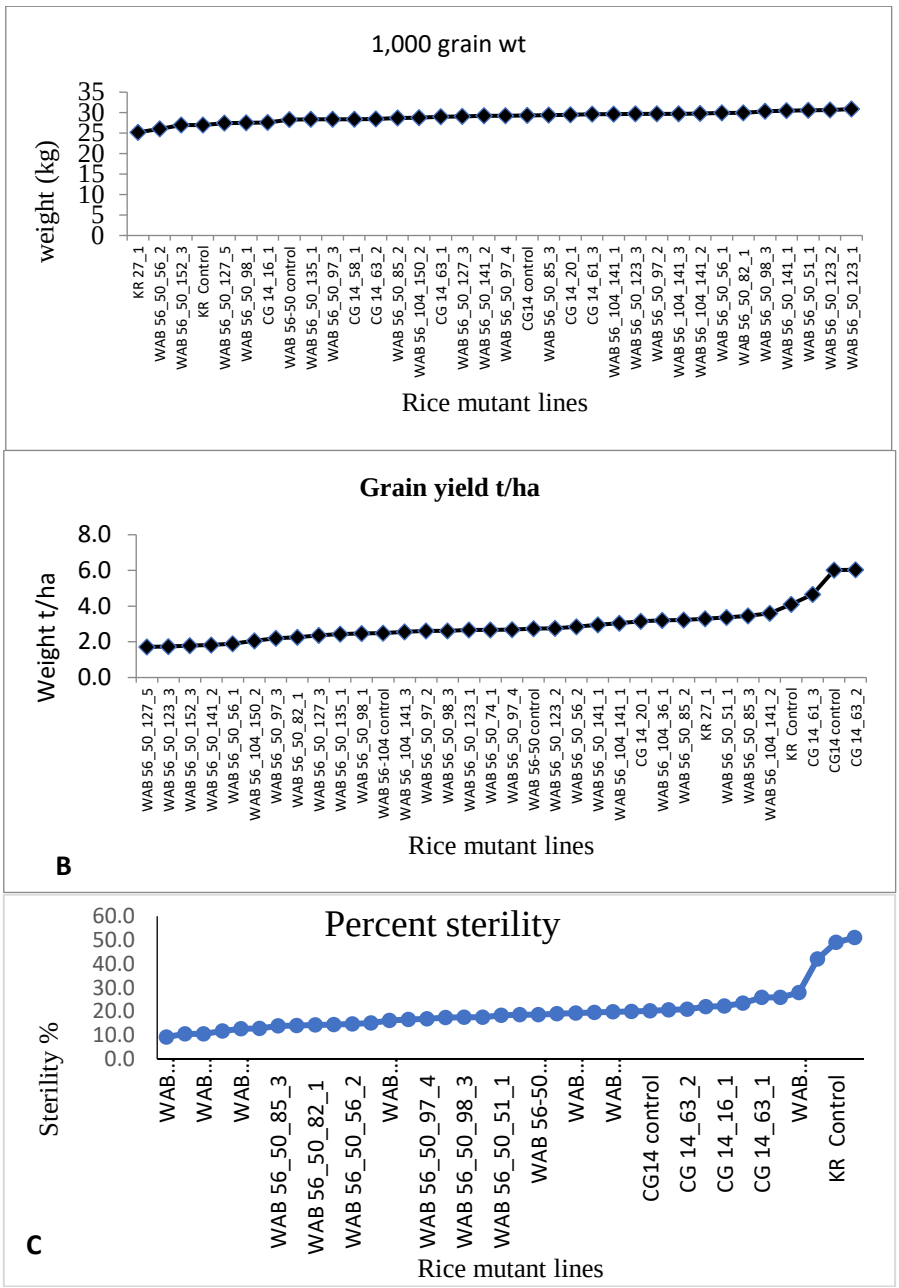

Fig 2. Yield and yield components of the evaluated mutant rice lines (A) 1,000 grain weight (g), (B) grain yield (t/ha), and (C) spikelet sterility of rice mutant lines.

Effects of induced mutations on growth performance of the HT mutant rice lines

The evaluation of the effects of induction mutation on growth performance of the HT mutant rice lines was accomplished by assessing the parameters of plant height, panicle length, days to early flowering, days to 50% flowering and days to 85% physical maturity. Days to early flowering and plant height parameters revealed significant differences; however, no significant difference was observed for panicle length, days to 50 % flowering and days to 85 % physical maturity. The induced mutations may have positive or negative effects on plant growth characteristics. The results indicate that the mutant lines had better field performances than their parental lines in terms of growth.

The mutant rice lines flowered earlier than the control lines indicating that they are adapted to short growing periods and this adaptation may assist in overcoming high temperature stress. Thus, the flowering and fertilization processes will not be affected by high temperature (Matsui et al., 2001). During the reproductive stage, high temperatures negatively affect flowering, pollination, grain filling and pollen production leading to spikelet sterility and low grain yield (Matsui et al., 2001; Ranga et al., 2011; Aghamolki et al., 2014). Jiang-lin et al. (2011) and Norvie et al. (2014) stated that the plant growth parameters of heat-susceptible-rice-genotypes can be negatively affected by high temperature at all growth stages as compared to HT rice. High temperature stress (above optimum levels) is also expected to affect development and all growth stages of the heat susceptible genotypes, and to decrease grain quality and yield (Shah et al., 2011). Therefore, use of the HT rice may help to improve rice production.

Table 3. HT mutant upland rice lines with modified expression of HSPs.

Sample ID	Mutant rice line	Species
1	KR 27_1	*O sativa*
2	KR 38_1	*O sativa*
3	CG 14_16_1	*O. glaberima*
4	CG 14_20_1	*O. glaberima*
5	CG 14_58_1	*O. glaberima*
6	CG 14_61_3	*O. glaberima*
7	CG 14_63_1	*O. glaberima*
8	CG 14_63_2	*O. glaberima*
9	WAB 56_104_36_1	*O sativa*
10	WAB 56_104_141_1	*O sativa*
11	WAB 56_104_141_2	*O sativa*
12	WAB 56_104_141_3	*O sativa*
13	WAB 56_104_150_2	*O sativa*
14	WAB 56_50_51_1	*O sativa*
15	WAB 56_50_56_1	*O sativa*
16	WAB 56_50_56_2	*O sativa*
17	WAB 56_50_74_1	*O sativa*
18	WAB 56_50_82_1	*O sativa*
19	WAB 56_50_85_2	*O sativa*
20	WAB 56_50_85_3	*O sativa*
21	WAB 56_50_97_2	*O sativa*
22	WAB 56_50_97_3	*O sativa*
23	WAB 56_50_97_4	*O sativa*
24	WAB 56_50_98_1	*O sativa*
25	WAB 56_50_98_3	*O sativa*
26	WAB 56_50_123_1	*O sativa*
27	WAB 56_50_123_2	*O sativa*
28	WAB 56_50_123_3	*O sativa*
29	WAB 56_50_127_3	*O sativa*
30	WAB 56_50_127_5	*O sativa*
31	WAB 56_50_135_1	*O sativa*
32	WAB 56_50_141_1	*O sativa*
33	WAB 56_50_141_2	*O sativa*
34	WAB 56_50_152_3	*O sativa*
35	KR Control	*O sativa*
36	CG 14 Control	*O. glaberima*
37	WAB 56_104 Control	*O sativa*
38	WAB 56_56 Control	*O sativa*

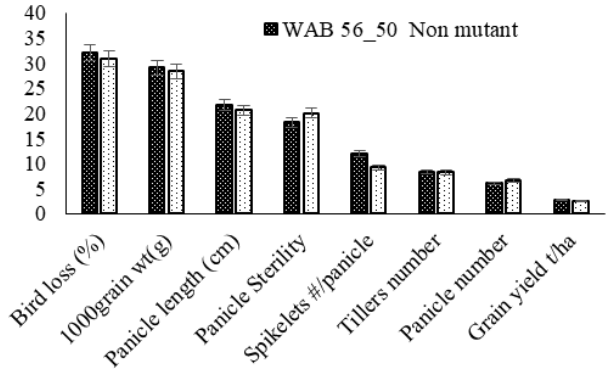

Fig 3. Comparison of growth yield and yield component values of mutant lines with their non-mutant control rice line both generated from the WAB 56_50 genotype (the Y axis is the proportion of significant mean performance of each variable derived from analysis of variance).

Effects of induced mutations on number of tillers, panicle number, number of spikelets, filled grains and grain yield in mutant rice lines

The current study revealed that there were significant differences (at P<0.05 level) among the parameters: number of spikelets per panicle, number of tillers/plant, panicle number/plant, filled and unfilled grains/panicle/plant among lines (Table 2).

The results of HT mutant rice lines indicated that such mutations were associated with increased number of tillers, number of panicles, number of spikelets and number of filled grains/plant. The *Oryza glaberrima* lines seemed to perform better than *Oryza sativa*; however, *Oryza glaberrima* derived genotypes used in mutation had undesirable logging characteristics beside their higher yields. Therefore, we discarded them in the selection process.

These findings were in agreement with Cheema and Atta (2003), where an increase in the number of reproductive tillers/plants also resulted in an increase in panicle number/plant and spikelets/plant, and thus grain yield. Furthermore, De Datta (1975) findings confirmed that tillering was a major factor determining rice yield. Surek and Beser (2003) reported that there was a positive relation between grain yield/plant and yield components; total spikelets/panicle, filled grains/panicle and 1,000 grain weight. An increase in these yield components also increased grain yield of the mutant rice lines as well as their productivity under heat and drought stresses. Surek and Beser (2003) also suggested that individual yield components might contribute valuable information in rice breeding for yield. In general, an increase in yield components also resulted in increase for grain yield among all the HT mutants as compared to control rice lines.

The effects of induced mutations on grains yield, 1,000 grain weight and percentage spikelet sterility in mutant upland rice lines

The results showed that there were significant differences among all rice lines in terms of grain yield and spikelet sterility; however, no significant difference was observed among lines for 1,000 grain weight at P<0.05 level (Table 2). Surek and Beser (2003) stated that effects of mutations may contribute alterations in tye number of tillers/plant, number of spikelets/panicle, number of panicles/plant and filled grains; hence, they were directly associated with higher grain yield. Similarly, our study revealed that the grain yield was higher in all HT mutant rice lines than the parental control genotypes. These outputs indicate that heat tolerant mutant rice lines are better able to tolerate heat stress and they show higher performance in terms of production capacity. The spikelet sterility rates identified for both *Oryza sativa* and *Oryza glaberrima* driven mutants were observed to be very low among all evaluated HT mutant rice lines as compared to controls (Table 3). The main reasons to obtain these results are their ability to sustain plant growth even under heat and drought stress and to produce more filled grains than unfilled grains. Hence, the selected mutant rice lines are highly tolerant to heat and drought stress. Indeed, Porter and Semenov (2005) and Mahmood et al. (2010) reported that both grain number and grain yield in many temperate cereal crops, and as well as in rice, seemed to be affected by heat stress, and the decline in grain yield was directly proportional with increasing temperatures during flowering and grain filling stage among heat susceptible rice genotypes. The high temperature (35^{o}C) during panicle development, heading and flowering stages also cause a high percentage of spikelet sterility in heat susceptible rice varieties (Shah et al., 2011; Wopereis et al., 2008; Sheehy et al., 2005).

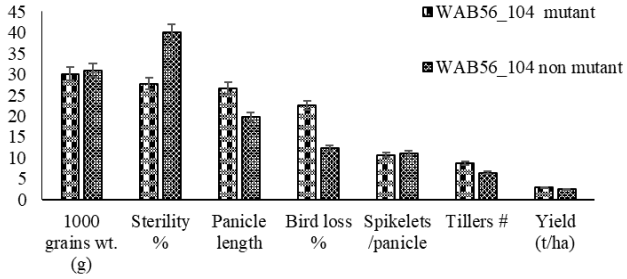

Figure 4. Comparison of yield and yield component values of mutant lines with their non-mutant control rice line both generated from WAB 56_104 genotype (the Y axis is the proportion of significant mean performance of each variable derived from analysis of variance).

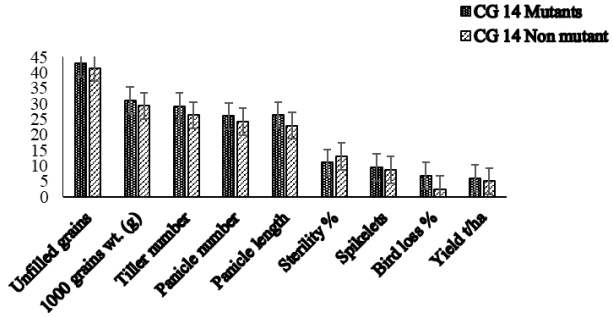

Figure 5. Comparison of yield and yield component values of mutant rice lines with their non-mutant control rice lines both generated from CG 14 rice genotype (the Y axis is the proportion of significant mean performance of each variable derived from analysis of variance).

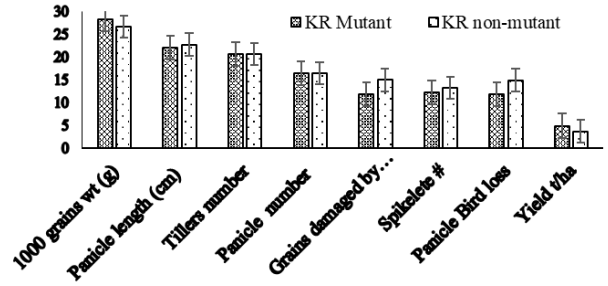

Figure 6. Comparison of yield and yield component values of mutant rice lines with their non-mutant control line both generated from KR rice genotype (the Y axis is the proportion of significant mean performance of each variable derived from analysis of variance).

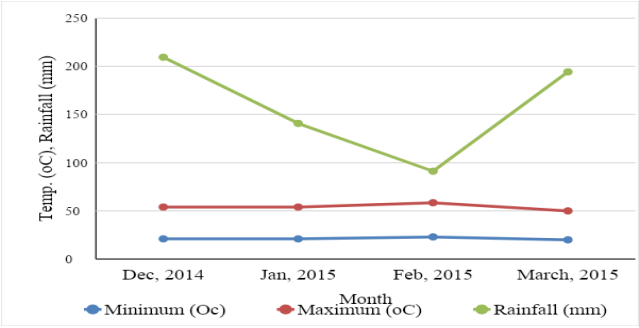

Figure 7. Climate trends of four months in SUA, Morogoro 2014/2015. Source; Tanzania Meteorological Agency, Morogoro-Weather Station, 2014/2015.

High temperature during booting and flowering stages lead to abnormal pollen development (Jiang-lin et al., 2011) and increase pollen sterility (Matsui et al., 2001), respectively. Thus, it leads to a serious panicle infertility and decrease grain yield in heat susceptible rice varieties. The current results indicated that there were no significant differences in 1,000 grain weight at 5% statistical interval among all lines including *Oryza sativa* and *Oryza glaberrima* driven mutant rice lines. Even though the mutant lines generated from CG 14 genotype of *Oryza glaberrima* presented similar data; however, there were significant differences among the mutant lines generated from WAB 56_50, WAB 56_104 and Kihogo red (KR) genotypes of *Oryza sativa*. These results indicated that grain sizes were not affected by the high temperature stress among all mutant rice lines.

The selected HT induced mutants from both *Oryza sativa* and *Oryza glaberrima* rice species also showed promising performances in the field trials. Therefore, induced mutations have a high potential to protecting the yield under heat stress conditions.

Material and Methods

Plant material

Two main rice species, *Oryza glaberrima* and *Oryza sativa*, and their four different genotypes, cv. Kihogo Red, WAB 56_50, WAB 56_104 (*Oryza sativa*) and CG 14 (*Oryza glaberrima*) were used as plant materials in the study. Uniform sized seeds (about 500 gr corresponding to 20,000 seeds for each genotype) with 12% moisture content of *Oryza sativa* Kihogo red, WAB 56_50 and WAB 56_104 genotypes were irradiated with 150 Gy dose, and *Oryza glaberimma* CG 14 genotype was also irradiated with 250 Gy dose (a higher dose is required for *O. glaberimma* as seeds were harder, lower water content than *O. sativa*) at FAO/IAEA Plant Breeding and Genetics Laboratories at Seibersdorf and shipped back to Tanzania. After the irradiation process, the seeds were immediately planted for generation of M_1 plants. The M_1 generation was grown in the experimental field of the Sokoine University Farming area in Morogoro. Following the M_1 stage, single spikes were harvested from each plant for M_2 generation, and selection of the best lines from M_1 to M_2 was carried out based on individual plants. Among the 20,000 seedlings, a total of 160 M_3 mutant lines from each genotype, which were previously subjected to the heat stress screening in growth chamber in seedling stage at 45° C, were identified. The surviving individual plants were selected following a complete growth recovery in the screen house after the heat shock; maintained for seed production and were considered as putative tolerant lines at the seedling stage.

Experimental design and field studies

The selected M_4 lines were studied in a randomized block design (RCBD) with three replications to evaluate the performance of 34 heat tolerant mutant upland rice lines. The best lines were compared with their parents in order to select the best improved mutants. Seeds of best mutants were collected individually from each generation. The standard rice agriculture practices were followed through all production stages.

Field experiments were conducted in 2014/2015 period starting from December 2014 to February 2015 during 'vuli', which was the short rainfall and hot season before the long rainfall season called 'Masika'. The experiment was designed at the rice experimental plots of the Department of Crop Science and Production of Sokoine University of Agriculture, Morogoro, Tanzania, located at latitude 6° 50' 55" S latitude and 37° 39' 22" E longitude. The daily average maximum and minimum temperatures and rainfalls were recorded about three

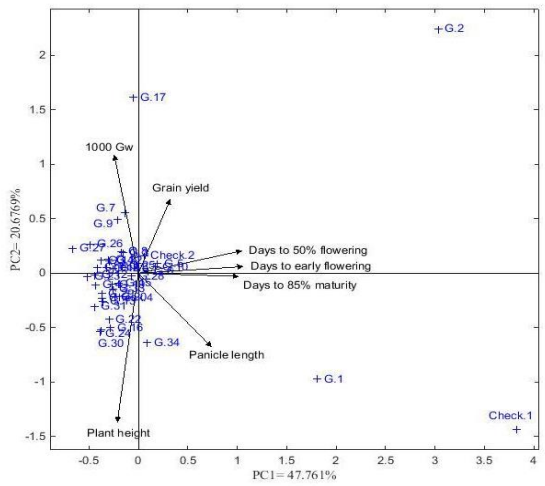

Figure 8. PCA plot of PC1 and PC2 showing the direction of main influencing traits (1,000 grain weight, grain yield, days to 50% flowering, days to early flowering, days to 85% maturity, panicle length and plant height) on all genotypes tested

months. These values were between 20°C and 35.5°C temperature, and 32.7 mm and 155.5 mm rainfall in December 2014, January, February and March 2015, respectively. A total rainfall and average minimum and maximum temperature for the above-mentioned month periods was presented in Figure 7.

Performance of 34 heat tolerant mutant upland rice lines were evaluated under natural and uncontrolled heat and scarce rainfall stress parameters in field conditions (Table 3). These lines were planted as single rows in 15 hill plots (20 x 20 cm) and each mutant rice line was treated as one plot (0.2 m x 3 m).

Data collection

Data for growth, yield and yield components were measured for the following parameters as described; number of reproductive tillers per plant at the maximum tilling stage, days to early and 50% flowering (days at which the first flowers appeared and the day when at least half of the rice plants exerted a fully opened panicle per plot), days to 85% physical maturity (counted from the day of sowing until more than 85% of rice grain turned from green to brown), plant height (cm) from at least five plants per line (measured at maturity stage, from the collar of the plant to the longest leaf and averages calculated), panicle number (the number of panicles per plant per plot), number of spikelets per panicle in each treatment, panicle length, number of spikelets per plant and spikelet sterility percentage (obtained through a relationship between the number of unfilled grains and total number of grains per panicle). The supplementary tables were also provided regarding ANOVA results of each variables and for each genotype of mutant lines identified (Supplementary Tables 1-15). The plots were manually harvested at physiological maturity using sickles when 90% of the panicles turned brown. The panicles were sun dried for a period of 3 – 4 days to reduce moisture content to approximately 12 – 13%. Yield data were determined based on weight of 1,000 grains, and grain yield per plot were measured using a seed counter, and then weighed using a sensitive digital scale. Grain yield per plot was recorded by weighing the filled grain per plant in per plot, and then were converted to grain yields (t/ha). Apart from several yield and yield components, bird loss was also recorded as an additional parameter.

Data analysis

Analysis of variance (ANOVA) was carried out on all traits (see Tables 1 and 2). In addition, a principal component analysis (PCA) was performed (Figure 8) on the measured variables, based on Pearson correlation matrix and Euclidean distances. Eigenvectors generated by the PCA were used to identify parameters that best differentiated the genotypes in each experiment.

Conclusion

Rice is a staple food and energy source for over half of the world's population. Climate changes, particularly, high temperature may have negative impacts on rice production via resulting in deleterious effects on almost all rice growth stages. Hence, developing high temperature tolerant cultivars are utmost importance. The HT rice mutant lines were previously developed through mutation induction using Gamma irradiation. The current study; however, evaluated these lines in the field conditions. Several mutant lines representing two main rice species; *Oryza sativa* (grown worldwide), and *Oryza glaberrima*, which was grown in limited areas but contained many unique and useful traits including heat and drought tolerance, were analysed. Significant data among the comparison of the mutants and parental lines were obtained in terms of growth performance, yield and yield component parameters. Regarding the growth performance, days to early flowering and plant height parameters showed significant differences. Regarding the yield and yield component parameters; however, grain yield, spikelet sterility, number of spikelets per panicle, number of tillers/plants, panicle number/plant, filled and unfilled grains/panicle/plant revealed significant differences. Hence, these variables were proven to be used as criteria for selecting heat and drought tolerant rice lines. The selected HT induced mutations from both *Oryza sativa* and *Oryza glaberrima* rice species showed promising performances in field experiments. Eight heat and drought tolerant mutant rice lines showing high yields (over 3.5 ton/ha) were selected for further advancement in breeding programmes. Therefore, the induced mutation technique has a potent effect on generating rice lines to protect and even increase yield under high temperature, heat and drought stress conditions.

Acknowledgements

We are grateful to the International Atomic Energy Agency (IAEA), Joint FAO/IAE Division for financial and technical support this investigation.

References

Aghamolki MTK, Yusop MK, Oad FC, ZakikhaniHawa H. Jaafar ZS, Kharidah SM, Hanafi MM (2014) Response of yield and morphological characteristic of rice cultivars to heat stress at different growth stages. International Journal of Biological, Biomolecular, Agricultural, Food and Biotechnological Engineering 8(2):98–100.

Chang PFL, Jinn TL, Huang WK, Chen Y, Chang HM, Wang CW (2007) Induction of a cDNA clone from rice encoding a class II small heat shock protein by heat stress, mechanical injury, and salicylic acid. Plant Sci. 172:64–75.

Cheema AA, Atta MB (2003) Radio sensitivity studies in basmati rice. Pak J Bot. 35(2):197–207.

De Datta SK (1975) Upland rice around the world. In major research in upland rice, IRRI, Los Banos, (1975), Philippines.

Dingkuhn M, Jones MP, Johnson DE, Sow A (1998) Growth and yield potential of oryza sativa and oryza glaberrima

upland rice cultivars and their interspecific progenies. Field Crops Res. 57(1):57–69.
Fageria NK, Moreira A, Coelho AM (2011) Yield and yield components of upland rice as influenced by nitrogen sources. J Plant Nutr. 34:361–370.
Garge D, Sareen S, Dalal S, Tiwari R, Singh R (2012) Heat shock protein-based SNP marker for terminal heat stress in wheat (Triticum aestivum L.). Aust J of Crop Sci. 6(11):1516–1521.
Hasanuzzaman M, Nahar K, Fujita M (ed) (2013) Extreme temperatures, oxidative stress and antioxidant defence in plants. In: (Edited Vahdati K. & Leslie C.) abiotic stress-plant responses and applications in agriculture. In tech; Rijeka, pp 169–205, (2013), Croatia.
Jiang-lin L, Hong-yu Z, Xue-lian S, Ping-an Z, Ying-jin H (2011) Identification on heat tolerance in backcross recombinant lines and screening of backcross intogression lines with heat tolerance at milky stage in rice. Rice Sci. 18(4):1–8.
John JB (2001) Identification of genetic diversity and mutations in higher plant acquired thermo tolerance. Physiol Plant. 112(2):167–170.
Mahmood S, Wahid A, Javed F, Basra SMA (2010) Heat stress effects on forage quality characteristics of maize (Zea mays) cultivars. Int J Agric Biol. 12:701–706.
Manneh B, Kiepe P, Sie M, Ndjiondjop M, Drame NK, Traore K, Rodenburg J, Somado EA, Narteh L, Youm O, Diagne A, Futakuchi K (2007) Exploiting partnerships in research and development to help african rice farmers cope with climate variability. ICRISAT. Africa rice center (WARDA). Cotonou 4 (1), (2007), Benin.
Matsui T, Omasa K, Horie T (2001) The difference in sterility due to high temperature during the flowering period among Japonica rice varieties. Plant Prod Sci. 4:90–93.
Norvie LM, Leslie AFL, Luvina BM, Corazon CC (2014) Germplasm innovation of heat tolerance in rice for irrigated lowland conditions in the philippines. Rice Sci. 21(3):162−169.
Porter JR, and Semenov MA (2005) Crop responses to climatic variation. Philos Trans R Soc B Biol Sci. 360:2021–2035.
Ranga SVK, Jagadishb QM, Zhoua PQ, Craufurdc S, Heuer B (2011) Effect of high temperature and water stress on pollen germination and spikelet fertility in rice. Environ Exp Bot. 70:58–65.
Sarla N, and Mallikarjuna SBP (2005) Oryza glaberrima: a source for the improvement of Oryza sativa. Curr Sci. 89(6):955–963.
Shah F, Huang J, Cui K, Nie L, Shah T, Chen C, Wang K (2011) Impact of high-temperature stress on rice plant and its traits related to tolerance. J Agric Sci. 149(5):545–556.
Sheehy JE, Elmido A, Centeno G, Pablico P (2005) Searching for new plants for climate change. J Agric Meteo. 60:463–468.
Surek H, Beser N (2003) Selection for grain yield and yield components in early generations for temperate rice. Philipp J Crop Sci. 28(3):3–15.
Wopereis MCS, Defoer T, Idinoba P, Diack S, Dugue M J (2008) Participatory learning and action research for integrated rice management (IRM) in inland valleys of sub-saharan africa: Technical manual. WARDA training series. Africa rice centre pp 128 (2008), Benin.
Yona N (2015) Genetic characterization of heat tolerant (HT) upland mutant rice selected from rice genotypes. MSc Dissertation, Sokoine University of Agriculture, pp 111 (2015), Tanzania.
Zhang Y, Tang Q, Peng S, Zou Y, Chen S, Shi W, Qin J, Laza CR (2013) Effects of high night temperature on yield and agronomic traits of irrigated rice under field chamber system condition. Aust J of Crop Sci. 7(1):7–13.

DOI: 10.21475/ajcs.21.15.09.sp-9

Australian Journal of Crop Science AJCS

Correlation of yield with early seedling performance and physio-biochemical traits in Basmati rice mutants subjected to heat stress

Muhammad Ashraf[1*], Zial-ul-Qamar[1], Amjad Hameed[1], and Fatma Sarsu[2]

[1]Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan
[2]International Atomic Energy Agency (IAEA), Vienna, Austria

[*]Corresponding author: ashraflyp@yahoo.com

Abstract

The present study aims at deciphering the response of Basmati rice mutants to high temperature stress. The work aims to find an early screening method by correlating seedling and physiological response to yields. After rigorous screening in controlled environment (growth chamber) and field conditions over a period of four years 16 mutants' lines were selected: HTT-18, HTT-29, HTT-31, HTT-39, HTT-51, HTT-53, HTT-74, HTT-81, HTT-92, HTT-97, HTT-98, HTT-104, HTT-114, HTT-119, HTT-132 and HTT-138. These have been tested against standards: Super Basmati and IR-64. Field trials were conducted at three locations while early seedling-stage traits and their biochemical analyses were studied in growth chamber experiments. Data of seedling traits were used to establish correlations with paddy yield under hot field conditions. The temperatures were high at two sites: Multan and Bahawalpur ranging 24-46 °C and 25-45 °C respectively), and relatively lower at NIAB field station (26-45 °C. The paddy yield was significantly correlated with early seedling-stage traits such as shoot length (0.79**), shoot fresh and dry weight (0.48* and 0.49*), and cell membrane thermo-stability (0.60**). Additionally, significant higher activities of antioxidants (SOD and APX) and lower stress indicators (MDA, Esterase and TOS) were observed in the heat tolerant mutants. These mutants were classified for their heat tolerance by principle component analysis (PCA) using yield-correlated early seedling-stage and other physio-biochemical parameters. As a result, the heat tolerance classification of mutants based on PCA coincided with the yield of mutants grown under hot field conditions. The present study suggests that these seedling parameters may be used as surrogates for field performance and used in the selection of thermo-tolerant Basmati rice. Our next objective is to screen these thermo-tolerant lines for multiple weather adversities resistance by applying the strategy being reported here.

Key Words : High temperature, Basmati rice, seedling trait correlations with yield, antioxidants, paddy yield.
Abbreviations: APX_Ascorbate Peroxidase, SOD_Superoxide Dismutase, CMTS Cell Membrane Thermo-Stability, POD Peroxidase, TPC Total Phenolic Content, TSP Total Soluble Protein and CAT Catalase, HTT Heat Temperature Tolerant, PCA Principle Component Analysis, NIAB Nuclear Institute for Agriculture and Biology.

Introduction

The global mean temperature is rising every year and it is predicted that by 2100 it will be 3.7°C higher than present (IPCC, 2013). The current global climate predictions are expected to adversely affect rice production by up to 25-32% (Wassmann et al., 2009; Ray et al., 2015; Van Oort and Zwart, 2018). Increased daytime temperatures of more than 34°C will depress rice yield by 8% (Bahuguna et al., 2015; Shi et al., 2014). In 2003, about 5.2 million tons of paddy rice was lost due to a heat wave with temperatures above 38 °C for more than 20 days (Xia and Qi, 2004; Yang et al., 2004). Rice is differentially sensitive to temperature stress at seedling to grain filling stages , and 83, 53 and 11% losses in rice yield have been reported when heat stress was imposed at panicle exertion, early grain filling and late grain filling stages (Ali et al., 2018) whereas Kumar et al. (2015) reported losses in yield (9-55%) in rice genotypes when subjected to heat stress from anthesis to maturity. A significant influence of high temperature has been observed on seedling traits including a decline in shoot dry mass, relative growth rate and net assimilation rate (Prasanth et al., 2012). Sailaja et al. (2015) reported negative impacts of cell membrane injury on yield in rice. High temperature resulted in electrolyte leakage and ultra-structural modification of the cell membrane system (Zhang et al., 2009; Liu et al., 2013). Heat stress induced changes in membrane fluidity and the production of free radicals resulting in the loss of membrane integrity and ion leakage in different crop plants (Wahid et al., 2007; Bita and Gerats, 2013; Ali et al. 2013; Sailaja et al. 2015). The severity of reactive oxygen species (ROS) induced damage depends on the antioxidant status of the plant (Mohammed and Tarpley, 2009) and under environmental stress, the increased production of active oxygen species reduces the protective activity of antioxidants (Shah et al., 2011; Shalata and Neuman, 2001). Thus, along with several other factors, oxidative stress damage caused by high temperature disrupts growth and development of rice plants (Mohammed and Tarpley, 2011). Many studies demonstrate that elevated temperature injury is caused by excessive production of reactive oxygen radicals and consequent low activities of antioxidant enzymes and membrane damage in plants (Zhang et al., 2006; Zhu et al., 2005). Plants with the ability to scavenge and/or control the level of cellular ROS may be able to withstand heat stress (Almeselmani et al., 2006; Bita and Gerats, 2013), and naturally higher levels of antioxidant enzymes in a plant may be considered as an aid to combat high temperature stress (Bahuguna et al., 2016; Ramesh et al. 2017). Cao et al. (2009) suggested that the relatively higher yields in heat tolerant rice genotypes under high temperature are associated with high levels of activities of ATPase and antioxidant enzymes.

Plant breeders strive extensively to find simple, quick and reproducible screening methods to identify heat-resistant plants from segregating populations and germplasm stocks. Even if paddy yields are accepted as the most suitable trait for identification of rice under high-temperature conditions, the screening for thermo-tolerant rice in the field conditions is labor intensive, time consuming and takes up valuable land.

Therefore, pre-screening under controlled conditions with rapid, efficient and reproducible approaches is required, e.g. at early developmental stages. Higher temperature significantly reduced the rice yield under field conditions (Ohe et al., 2007; Shah et al., 2014; Jumiatun et al., 2016; Chaturvedi et al., 2017; Yang et al., 2017). Screening for heat tolerance in the field presents a challenge due to interactions with other environmental factors and certain genotypes are required that are adapted to that environment, thus precluding the screening of exotic germplasm. Nevertheless, given these constraints, a wide range of traits are available that may allow successful selection in the field (Hall, 2011). However, attempts had been made in different crop plants to identify thermo-tolerant genotypes using yield-correlated early-stage traits (Demirel et al., 2016). Heat tolerance to seedlings is critical for adequate crop establishment. The aim of the study was to investigate correlations between early morpho-physiological stage traits and paddy field yield.

Results

Seedling stage screening in a growth chamber

The average maximum temperature for the whole growing season (June-November) is presented in fig. 1. The average temperature at NIAB, Faisalabad was 36.3°C, 36.9°C at Multan and 37.5°C at Bahawalpur while minimum temperature at NIAB, Faisalabad was 27.3°C as compared to Multan (28.9°C) and Bahawalpur (24.9°C). The mean relative humidity at NIAB, Faisalabad was 62.9%, 55.5% at Multan and 53.8% at Bahawalpur. The total rainfall recorded during the growing season at NIAB, Faisalabad was 585.2 mm, 170.2 mm at Multan and 77.0 mm at Bahawalpur. The cell membrane thermo-stability (CMTS) presented in Fig 2 at normal temperature (28±2°C) ranged from 84.2 - 93.0% with a mean of 88.4% among the mutants. The controls had high values: Super Basmati (92.7%) and IR-64 (93.0%). However, CMTS decreased 20-42% with a mean of 27% at high temperature stress (45±2°C). At high temperature, the CMTS ranged from 50.6 - 74.2% with a mean of 64.2% among the mutants as compared to Super Basmati (55.7%) and IR-64 (42.0%).

The shoot length stress tolerance index (SLSTI) varied among the mutants (Table 1) and the maximum value (87.2%) was noted in Heat Temperature Tolerant (HTT) mutant HTT-18 whereas mutant HTT-29 maintained the minimum value of 73.9% as compared to Super Basmati (74.9%) and IR-64 (75.8%). For RLSTI, the values ranged from 80.9% (HTT-53) to 108.9% (HTT-98) as compared to Super Basmati (86.5%) and IR-64 (84.2%). The maximum values of STI for shoot fresh and dry weights (76.4 and 80.4%, respectively) were observed in HTT-132 and HTT-97 while the minimum STIs of 72.6 and 73.9%, respectively were noted in HTT-31 and HTT-29 as compared to Super Basmati (75.2 and 74.9%, respectively) and IR-64 (75.0 and 75.8% respectively). For root fresh and dry weights, the maximum STIs for root fresh and dry weights were noted 87.3 and 64.6%, respectively in HTT-97 and HTT-114 whereas HTT-31 and HTT-51 maintained the minimum values of 82.5 and 54.8%, respectively as compared to Super Basmati (85.8 and 57.8%, respectively) and IR-64 (87.0 and 60.5%), respectively. The maximum CMTS was observed in HTT-18 (80.1%) whereas the least was noted in HTT-132 (58.5%) as compared to Super Basmati (60.1%) and IR-64 (44.7%).

Stress tolerance indices (Table 2) showed that mutant HTT-18 had the highest score (8.72) for shoot length while the minimum score (7.39) was noted in HTT-29 as compared to Super Basmati (7.49) and IR-64 (7.58). The maximum score (18.15) for root length was observed in mutant HTT-98 whereas the minimum score (13.48) was obtained by HTT-53 as compared to Super Basmati (14.42) and IR-64 (14.04). Mutant HTT-98 obtained the highest score of 6.27 for shoot fresh weight whilst HTT-31 had a score of 6.05 as compared to Super Basmati (6.27) and IR-64 (6.25). For shoot dry weight, the maximum score was noted in HTT-97 (8.04) whereas HTT-29 had the minimum score of 7.39 as compared to Super Basmati (7.49) and IR-64 (7.58). HTT-97 showed the highest score (6.72) for root fresh weight and the minimum was 6.35 in HTT-31 as compared to Super Basmati (6.60) and IR-64 (6.69). The maximum (4.97) and the minimum (4.22) scores for root dry weight were noted in mutants HTT-114 and HTT-51, respectively as compared to Super Basmati (4.44) and IR-64 (4.65). The highest score (24.02) for CMTS was noted in HTT-18 while the minimum was 17.56 in HTT-132 as compared to Super Basmati (18.03) and IR-64 (13.40). On a cumulative basis of the scores, mutant HTT-98 obtained the highest score of 75.12 while the least was 71.10 obtained from HTT-97 and these were higher than the standards, Super Basmati (64.74) and IR-64 (60.20). Based on seedling growth traits, none of the mutants were categorized as sensitive to heat stress (as expected, as the mutants under study were selected after years of testing (2012-14) and reported as having better responses to heat stress, (Zafar et al., 2017). Ten mutants HTT-98, HTT-18, HTT-51, HTT-29, HTT-97, HTT-39, HTT-92, HTT-119, HTT-81, and HTT-31 exhibiting cumulative scores of 73 and above showed tolerance to heat stress as compared to Super Basmati (68.06) exhibiting moderately tolerant and IR-64 (62.82) showing sensitive behavior to high temperatures.

The correlation analysis (Table 3) among different seedling growth parameters and yield under high temperature stress indicated highly significant positive correlations of shoot length with CMTS, shoot fresh weight with shoot dry weight, and root fresh weight with root dry weight, however, CMTS had negative associations with root fresh and dry weights. Paddy yield exhibited highly significant correlations with shoot length, shoot fresh and dry weights, and CMTS, however, root dry weight showed negative correlation with paddy yield.

The principal component analysis (PCA) was performed to reveal the pattern and clustering of data matrix for determination and identification of selection criteria. The results explained the genetic diversity among the rice mutants (Table 4). From the PCA, first three principal components with eigenvalue > 1 were selected, and these components accounted for more than 90% of the cumulative variance. The remaining components were eliminated and considered less significant. Based on the component loadings after varimax rotation, the first two components were extracted, and the other components were eliminated. These two principal components accounted for 82.8% of the total variance of the original data and the communalities showed that all the variables had been described to an acceptable level as communalities ranged from 0.722 to 0.959. The first component gave information on variation in shoot length, root dry weight, CMTS and paddy yield which described more than 53.1% of the variance. In this component, shoot length, root dry weight and CMTS were observed as more important for the improvement of rice paddy yield. The second component described about 30% variation, which originated mainly from shoot fresh and dry weight. To classify the mutants for their heat tolerance, the values of yield-correlated traits like shoot length, shoot fresh and dry weight, root dry weight and CMTS were used in the PCA. The first two vectors (PC1 and PC2) accounted for 82.8% of total variation. The mutants were classified into four groups based on PC1 and PC2 values (Fig 3). The mutants with +PC1 and +PC2 were graded as tolerant, with +PC1 and −PC2 as moderately tolerant, with −PC1 and +PC2 as moderately susceptible, and with −PC1 and –PC2 as susceptible. According to this classification, the mutants HTT-53, HTT-92, HTT-97 and HTT-98 were graded as tolerant, and HTT-18, HTT-29, HTT-31, HTT-39, HTT-74 and HTT-

81 as moderately tolerant mutants. However, Super Basmati and IR-64 exhibited moderately susceptible to susceptible response.

Biochemical analysis

The enzymatic and non-enzymatic antioxidants along with other important biochemical attributes were analyzed to understand the mechanism of heat tolerance in rice mutants.

Stress biomarkers

The oxidative damage during peroxidation of membrane lipids is often estimated by the quantification of malondialdehyde (MDA) production. The MDA contents among the mutants (Fig 4.A) generally increased under heat stress as compared to control conditions, however, some of the mutants maintained the MDA contents under the both conditions (HTT-31 and HTT-138) or decreased under heat stress (HTT-18, HTT-97 and HTT-104). The increased MDA level indicated that the production of reactive oxygen species (ROS) was greater under heat stress than control conditions. The highest stress-induced increase in MDA contents was observed in HTT-119. A relatively higher MDA content was also observed in heat sensitive mutants while the level was low in heat tolerant mutants under normal conditions. It indicates that MDA content can be simply used for potential heat stress tolerance in normal conditions. The total oxidant status (TOS) decreased under heat stress, however, some of the mutants showed higher status of TOS as compared to their respective control conditions (Fig 4. J). The highest TOS was observed in HTT-18 under heat stress and also it showed the maximum value of TOS among the mutants under heat stress whereas the highest reduction of TOS was observed in mutant HTT-138. Based on relative tolerance responses, five mutants HTT-104, HTT-97 HTT-98, HTT-31 and HTT-18 showed promise against heat stress as compared to Super Basmati.

Enzymatic antioxidants

For scavenging ROS in plants, two types of antioxidant systems (enzymatic and non-enzymatic) were deployed. The peroxidase (POD) and catalase (CAT) are the key enzymes along with superoxide dismutase (SOD) and others. The POD activity (Fig 4.B) reduced as a result of heat stress in all the mutants with varying degree of decrease. The highest POD activity under heat stress was observed in HTT-39 with least decrease (12%) as compared to control conditions whereas the highest stress-induced increase in POD activity was exhibited by HTT-98 and HTT-104. The CAT activity (Fig 4.C) generally decreased under heat stress as compared to control conditions, however, in some of the mutants, the CAT activity increased with maximum value in HTT-18 followed by HTT-29 and HTT-98. The SOD activity (Fig 4.D) increased slightly under heat stress as compared to normal conditions, however, some of the mutants showed lower levels of SOD activity as compared to their respective controls. The highest SOD activity under heat stress was observed in HTT-98 whereas the lowest SOD activity was observed in HTT-114. Heat stress enhanced the ascorbate peroxidase (APX) activity among the mutants (Fig 4.F); however, some of the mutants (HTT-18, HTT-29, HTT-53 and HTT-81) showed less APX activity as compared to their respective control conditions. The highest APX activity under heat stress was observed in HTT-51.

Non-enzymatic antioxidants

The total phenolic content (TPC) generally decreased under heat stress (Fig 4.E), however, mutants HTT-18 and HTT-39 exhibited higher TPC contents, and mutants HTT-29 and HTT-119 maintained the TPC contents under stress and normal conditions. The highest stress-induced incline in TPC contents was observed in HTT-132.

Hydrolytic enzymes

Esterase activity decreased very slightly under heat stress (Fig 4.G), however, most of the mutants exhibited higher esterase activity compared to their respective control conditions. The highest stress-induced incline in esterase activity was detected in HTT-51 and increase in HTT-114.The protease activity generally decreased under heat stress (Fig 4.H). The maximum protease activity under heat stress was observed in HTT-138, however, HTT-81 exhibited the least decrease as compared to other mutants under heat stress. **Biochemical attributes**

The total soluble protein (TSP) content decreased in all the mutants except in mutants HTT-29 and HTT-31 where TSP contents increased under heat stress (Fig 4. I). The highest decrease in TSP contents under heat stress was observed in HTT-132 whereas the least decrease was noted in HTT-119.

Principal component analysis based on biochemical indices

For classification of the mutants for their heat tolerance, the values of yield-correlated traits such as POD, TPC, CAT, MDA, TSP, and TOS were used in the PCA (Table 5). The first four vectors (PC1-PC4) accounted for 85.5% of total variation. The mutants were classified into four groups based on first and second factor values (Fig 5). The mutants with +PC1 and +PC2 were graded as tolerant, with +PC1 and −PC2 as moderately tolerant, with −PC1 and +PC2 as moderately susceptible, and with −PC1 and –PC2 as susceptible. According to this classification, the mutants HTT-92, HTT-114 and HTT-119 were graded as tolerant, and HTT-18, HTT-29, HTT-74 and HTT-81 as moderately tolerant mutants.

Field experiments

The mutants along with standards were studied under field conditions with varying temperatures at NIAB Faisalabad, Multan and Bahawalpur. Although the temperatures at all the locations were on the high side, those at Multan and Bahawalpur were higher than at NIAB overall growth stages (from vegetative to maturity). The analysis of variance showed highly significant differences among genotypes for paddy yield and other related agronomic traits at all locations except for productive tillers plant^{-1} at Bahawalpur (Table 6). There was a significant difference among different locations for paddy yield. The mutants showed significant differences in paddy yield. Paddy yield at NIAB was adversely correlated with other locations (Fig 6) suggesting that high potential yield at NIAB did not necessarily result in improved yield under stressful locations. Overall, 38% paddy yield reduced at Multan and 31% at Bahawalpur as compared to NIAB. The mean paddy yields ranged from 2,583 to 4,567 kg ha^{-1} at NIAB, 1,357 to 3,163 kg ha^{-1} at Bahawalpur and 2,241 to 2,683 kg ha^{-1} at Multan. All the mutants produced lesser paddy yields at Bahawalpur and Multan as compared to NIAB. Among the mutants, the paddy yield declined 10-70% at Multan whereas at Bahawalpur the decline was 4-57%. The high-yielding mutants HTT-18 produced the highest paddy yield (4,567 kg ha^{-1}) at NIAB followed by HTT-74 (4,556 kg ha^{-1}) and HTT-29 (4,059 kg ha^{-1}) as compared to standards Super Basmati (3408 kg ha^{-1}) and IR-64 (3228 kg ha^{-1}). The mutant HTT-74 showed higher reduction in paddy yield (70%) followed by HTT-29 (49%) and HTT-18 (38%) at Multan as compared to Super Basmati (46%) and IR-6 (37%) whereas at Bahawalpur, the mutant HTT-74 showed 35% reduction in paddy yield followed by HTT-18 (31%) and HTT-29 (29%) as compared to Super Basmati (23%) and IR-64 (54%).

Discussion

High temperature stress is one of the major factors affecting plant growth and development (Howarth, 2005; Sailaja et al., 2014; Tayade et al., 2018). The crucial limitation for breeding high-yielding heat tolerant rice cultivars is the lack of reliable screening techniques. A multi-trait approach may be needed owing to the nature of heat tolerance as a complex phenotypic trait under the control of multiple physiological and genetic factors (Wahid et al., 2007; Collins et al., 2008; Ainsworth and Ort, 2010; Sailaja et al., 2015; Tayade et al., 2018). Screening must reflect yield performance of genotypes grown under hot field conditions. Screening at early developmental stages under controlled environmental conditions is preferred due to its advantages of applicability in the off-season and in a short time, testing potential in non-adapted germplasm, saving land space, labor and inputs. The identification of yield-related early seedling/physiological traits at an early seedling-stage has been of great interest to physiologists and breeders (Demirel et al., 2016; Sailaja et al., 2015). Thus, we aimed at to develop an early stage screening technique by investigating yield-related early seedling-stage/physiological traits (shoot and root lengths, their fresh and dry weights and cell membrane thermo-stability), as well as biochemical attributes (enzymatic and non-enzymatic antioxidants, stress biomarkers and hydrolytic enzymes etc.). Principle component analysis was performed to identify productive thermo-tolerant mutants.

The present study revealed that seedling traits were significantly influenced by high temperature. In general, high temperature treatment reduced shoot/root lengths and their respective fresh and dry weights, and the cell membrane thermo-stability (CMTS). The adverse effects of high temperature on seedling growth traits and CMTS in rice has been observed by other researchers (Prasanth et al., 2012; Zhou et al., 2012; Ali et al., 2013; Sailaja et al., 2014; Zafar et al., 2017). All seedling growth traits significantly varied among the mutants under both control and stress conditions. However, only root dry weight (RDW) under high temperature was adversely correlated with paddy yield grown under hot field conditions. The study indicated that high temperature decreased root dry weight. Similarly, early seedling growth traits in rice had been negatively affected in response to high temperature (Zhou et al., 2012), therefore, for pre-screening of rice with high yield in hot field conditions, shoot length, and its fresh and dry weight and CMTS may be evaluated together with other yield-associated traits applying multi-level screening approach.

Based on data sets of the previous studies conducted for Indica seedling traits in 46 genotypes including 39 mutants, the mutants were characterized as moderately tolerant to tolerant (Zafar et al., 2017). Among these, 16 mutants that produced higher paddy yield (2-31% higher than standard) were further tested for seedling growth traits as well as for their yield performance under field conditions at three locations. The relative heat tolerance of the mutants was assessed based on various seedling growth/physiological traits and field conditions. The data from seedling growth traits and yield data obtained from the hot field conditions (see section on weather) were used for correlation analysis. According to the correlation analysis, shoot length and its fresh and dry weights, root dry weight and CMTS were correlated with yield performance under hot field conditions. Afterwards, the mutants were classified for their thermo-tolerance by PCA using all yield-correlated traits. PCA is considered as a useful statistical tool for screening multivariate data which are highly correlated with each other (Johnson, 1998). Use of PCA to classify rice genotypes for heat tolerance has been recommended by several researchers (Kakani et al., 2005; Liu et al., 2006). The PCA results showed considerable similarity to yield performance of the mutants under hot field conditions with the performance of seedling growth traits. The first component represented the significance of this PC for heat related traits such as shoot length and CMTS. The mutants having vigorous shoots and higher CMTS may be considered as selection criteria under hot field condition. The mutants HTT-18, HTT-29, HTT-31, HTT-39, HTT-51, HTT-74 and HTT-81 exhibited larger shoots as well as better yield under hot conditions. Based on PCA results, mutants HTT-53, HTT-92, HTT-97 and HTT-98 were classified as tolerant, HTT-18, HTT-29, HTT-31, HTT-39, HTT-51, HTT-74 and HTT-81 as moderately tolerant, HTT-104, HTT-119 and Super Basmati as moderately susceptible. The remaining three mutants HTT-114, HTT-132 and HTT-138 along with standard IR-64 were classified as susceptible. According to the productivity under hot field conditions, the mutants HTT-18, HTT-29, HTT-31, HTT-39, HTT-51, HTT-74 and HTT-81 were considered as heat tolerant. Interestingly, the classification of mutants based on PCA using shoot length, and its fresh and dry weight and CMTS coincided neatly with the heat tolerance level of mutants grown in the hot field conditions. The important characters coming together in different PCs tended to remain together, which may be kept into consideration during utilization of these characters in thermo-tolerance breeding programmes to bring about rapid improvement for yield and other associated traits. The leaf related traits are very important in thermo-tolerance because the major part of the starch in rice grains at harvest is the photosynthetic product of the leaves (source), which is translocated from the leaves directly to the growing grains (Venkateswarlu and Visperas, 1987).

Field screening for heat stress is difficult due to variation in environmental conditions like humidity and temperature. Alternatively, screening for heat stress tolerance may be done on the basis of various biochemical parameters such as malondialdehyde (MDA), SOD, CAT, POD, APX, TSP content and protease (Kang and Saltveit, 2002; Maestri et al., 2002; Iqbal et al., 2010; Hameed et al., 2012; Ramesh et al., 2017). Heat stress affects the biochemistry of the plants and the activities of antioxidant enzymes increased significantly in the heat tolerant varieties (Cao et al., 2009). High temperature stress caused up-regulation of several heat responsive genes which code for numerous heat shock proteins (Charng et al., 2007). In the present study, the level of TSP fell under heat stress in most of the mutants. TSP content was also reported to be decreased under heat stress in wheat (Hameed et al., 2012). An effective heat tolerance mechanism at sensitive stages of plant growth can be adopted by protecting the structural proteins, membranes and different enzymes from damage by heat shock. HSPs and some other stabilizing factors might play important role in these processes related to heat tolerance (Maestri et al., 2002).

The PCA results showed considerable similarity to yield performance of the mutants under hot field conditions with the antioxidant activities. The first component represented the significance of this PC for heat related traits such as TPC and TOS. The higher activities of protective enzymes in the antioxidant system in plants might be one of the physiological mechanisms for heat tolerance in rice (Ramesh et al., 2017). The mutants exhibiting higher TPC and TOS may be considered in selection under hot field condition. The mutants HTT-18, HTT-29, HTT-39, HTT-92 and HTT-114 exhibited higher levels of TPC or TOS as well as better yield under hot conditions. Based on PCA results, mutants HTT-18, HTT-29, HTT-74, HTT-81, HTT-92, HTT-114 and HTT-119 were classified as tolerant to moderately tolerant and rest of the mutants were classified as susceptible to moderately susceptible.

Table 1. Range (maximum and minimum) of different stress tolerance indices (STIs) in Basmati mutants with respect to their responses to high temperature stress in the growth chamber

Mutant	Stress Tolerance Index (%)						
	SL	RL	SFW	SDW	RFW	RDW	CMTS
HTT-18	87.2^{A}	95.1^{B}	74.8^{ABCD}	74.9^{CDE}	86.5^{BCDE}	59.0^{DE}	80.1^{A}
HTT-29	73.9^{G}	95.7^{B}	75.3^{ABCD}	73.9^{A}	85.9^{EF}	55.7^{GH}	78.6^{AB}
HTT-31	75.8^{FG}	82.8^{GHI}	72.6^{E}	75.8^{BCD}	82.5^{H}	55.8^{GH}	79.1^{AB}
HTT-51	74.7^{G}	94.7^{BC}	75.8^{ABC}	74.8^{CDE}	86.9^{ABC}	54.8^{H}	78.4^{AB}
HTT-53	75.9^{FG}	80.9^{I}	75.7^{ABC}	75.8^{BCD}	86.2^{DEF}	57.7^{EF}	75.0^{CD}
HTT-97	80.4^{CD}	95.1^{B}	76.2^{AB}	80.3^{A}	87.3^{A}	57.3^{EFG}	72.3^{DEF}
HTT-98	76.0^{FG}	108.8^{A}	75.2^{ABCD}	76.1^{BC}	86.1^{DEF}	64.0^{AB}	79.9^{A}
HTT-114	76.7^{EFG}	93.6^{BC}	76.1^{AB}	76.8^{B}	87.1^{AB}	64.6^{A}	64.5^{G}
HTT-132	75.9^{FG}	107.6^{A}	76.4^{A}	75.9^{BCD}	86.1^{DEF}	63.4^{AB}	58.5^{I}
Super Basmati	74.9^{G}	86.6^{E}	75.2^{ABCD}	74.9^{CDE}	85.8^{F}	57.8^{EF}	60.1^{HI}
IR-64	75.8^{FG}	84.2^{FG}	75.0^{ABCD}	75.8^{BCD}	87.0^{ABC}	60.5^{CD}	45.2^{J}

SL: Shoot length, RL: Root length, SFW: Shoot fresh weight, SDW: Shoot dry weight, RFW: Root fresh weight, RDW: Root dry weight, CMTS: Cell membrane thermo-stability

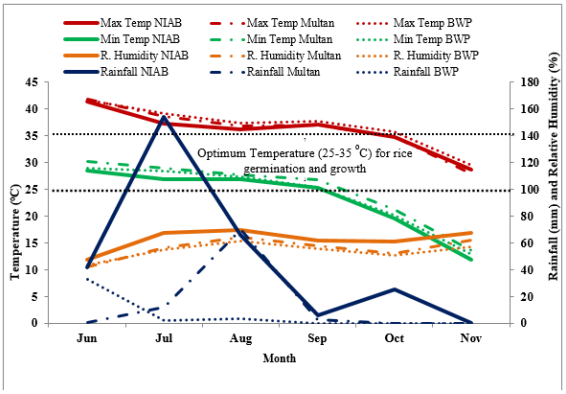

Fig 1. Mean temperature, rainfall and relative humidity for whole rice growing season at NIAB, Multan and Bahawalpur during 2016.

Table 2. Range (maximum and minimum) of scores obtained on the basis of different stress tolerance indices for different seedling growth traits in Basmati mutants

Mutant	Score							Total Score
	SL	RL	SFW	SDW	RFW	RDW	CMTS	
HTT-18	8.72^{A}	15.85^{B}	6.23^{ABCD}	7.49^{CDE}	8.65^{BCDEF}	5.90^{DE}	24.03^{A}	76.9
HTT-29	7.39^{G}	15.94^{B}	6.28^{ABCD}	7.39^{E}	8.59^{G}	5.57^{GH}	23.58^{AB}	74.7
HTT-31	7.58^{FG}	13.79^{EFG}	6.05^{E}	7.58^{BCD}	8.25^{I}	5.58^{FGH}	23.74^{AB}	72.6
HTT-51	7.47^{G}	15.78^{B}	6.31^{AB}	7.48^{CDE}	8.69^{ABC}	5.48^{H}	23.53^{AB}	74.7
HTT-53	7.59^{FG}	13.49^{G}	6.31^{AB}	7.58^{BCD}	8.62^{DEFG}	5.77^{EFG}	22.49^{CD}	71.8
HTT-97	8.04^{CD}	15.85^{B}	6.35^{AB}	8.03^{A}	8.73^{A}	5.73^{EFG}	21.68^{DEF}	74.4
HTT-98	7.60^{FG}	18.13^{A}	6.27^{ABCD}	7.61^{BC}	8.61^{EFG}	6.40^{AB}	23.96^{A}	78.6
HTT-114	7.67^{EFG}	15.60^{BC}	6.34^{AB}	7.68^{B}	8.71^{AB}	6.46^{A}	19.36^{G}	71.8
HTT-132	7.59^{FG}	17.93^{A}	6.36^{A}	7.59^{BCD}	8.61^{FG}	6.34^{AB}	17.56^{I}	72.0
Super Basmati	7.49^{G}	14.43^{DE}	6.27^{ABCD}	7.49^{CDE}	8.58^{G}	5.78^{EF}	18.02^{HI}	68.1
IR-64	7.58^{FG}	13.10^{G}	6.25^{ABCD}	7.58^{BCD}	8.70^{ABC}	6.05^{CD}	13.55^{J}	62.8

SL: Shoot length, RL: Root length, SFW: Shoot fresh weight, SDW: Shoot dry weight, RFW: Root fresh weight, RDW: Root dry weight, CMTS: Cell membrane thermo-stability.

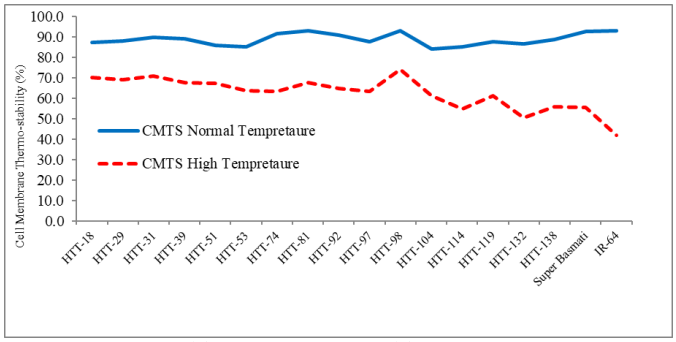

Fig 2. Cell Membrane Thermo-stability (CMTS) (%) in Basmati rice mutants.

Table 3. Correlations among different stress tolerance indices of various seedling growth traits in Basmati rice mutants

Parameter	Seedling growth traits						
	Shoot Length	Root Length	Shoot Fresh Weight	Shoot Dry Weight	Root Fresh Weight	Root Dry Weight	Cell Membrane Thermo-stability
Root length	0.291						
Shoot Fresh Weight	0.424	0.128					
Shoot Dry Weight	0.412	0.122	0.973**				
Root Fresh Weight	-0.210	-0.202	0.247	0.182			
Root Dry Weight	-0.392	0.008	0.051	0.025	0.652**		
Cell Membrane Thermo-stability	0.536*	0.307	0.074	0.067	-0.639*	-0.585*	
Paddy Yield	0.794**	0.357	0.477*	0.487*	-0.383	-0.560*	0.595**

*: Significant at 5-10%.

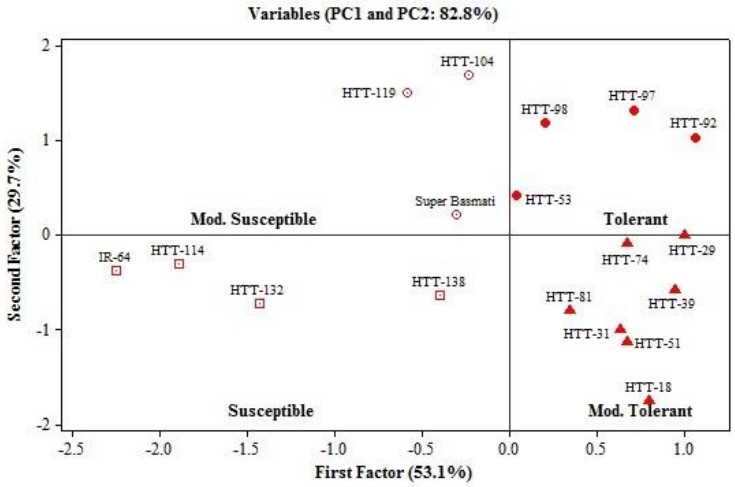

Fig 3. Classification of Basmati rice mutants based on yield-correlated early-stage seedling traits through PCA. Mutants that had +PC1 and +PC2, +PC1 and −PC2, −PC1 and +PC2, −PC1 and −PC2 scores were classified as tolerant, moderately tolerant, moderately susceptible, and susceptible, respectively.

Table 4. Principal component Analysis in Basmati rice mutants based on seedling growth traits and yield.

Variable	Principal Component					
	PC1	PC2	PC3	PC4	PC5	PC6
Eigenvalue	3.186	1.779	0.467	0.384	0.157	0.026
% of variance explained	53.1	29.7	7.8	6.4	2.6	0.4
% of cumulative variance explained	53.1	82.8	90.5	96.9	99.6	100.0
Trait	Factor loadings after varimax rotation				Communality	
	F1		F2			
Shoot Length	0.718		-0.464		0.732	
Shoot Fresh Weight	0.050		-0.978		0.959	
Shoot Dry Weight	0.057		-0.974		0.952	
Root Dry Weight	-0.836		-0.149		0.722	
Cell Membrane Thermo-stability	0.856		0.005		0.733	
Paddy Yield	0.795		-0486		0.869	

Table 5. Principal component analysis in Basmati rice mutants based on biochemical parameters and yield.

Variable	Principal Component							
	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8
Eigenvalue	2.730	1.645	1.401	1.062	0.542	0.306	0.190	0.125
% of variance explained	34.1	20.6	17.5	13.3	06.8	03.8	02.4	0.16
% of cumulative variance explained	34.1	54.7	72.2	85.5	92.2	96.1	98.4	1.000
Trait	Factor loadings after varimax rotation				Communality			
	F1	F2	F3	F4				
POD	0.038	-0.858	0.345	0.866	0.966			
TPC	0.881	-0.186	-0.090	0.823	0.823			
CAT	0.401	0.098	-0.796	0.804	0.804			
MDA	0.178	0.054	0.834	0.740	0.740			
TSP	0.006	-0.105	-0.972	0.964	0.964			
TOS	0.874	0.135	0.249	0.852	0.852			
Yield	0.163	-0.890	-0.262	0.050	0.890			

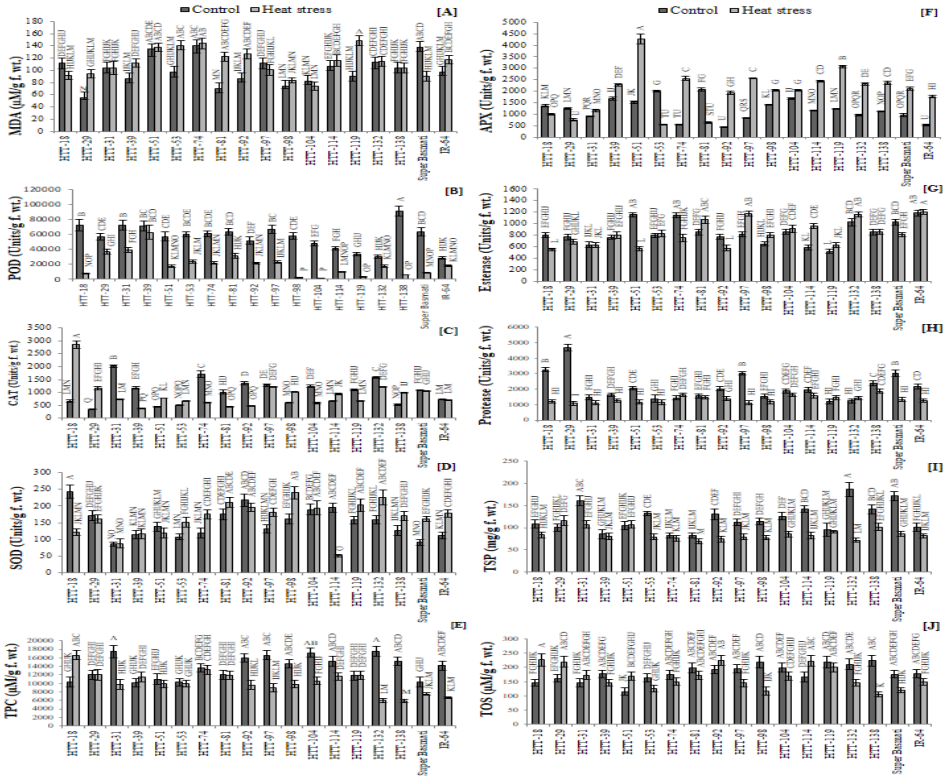

Fig 4. Effect of heat stress on (A) MDA, (B) POD, (C), CAT, (D) SOD, (E) TPC, (F) APX, (G) esterase (H) protease (I) TSP and (J) TOS in Basmati rice mutants.

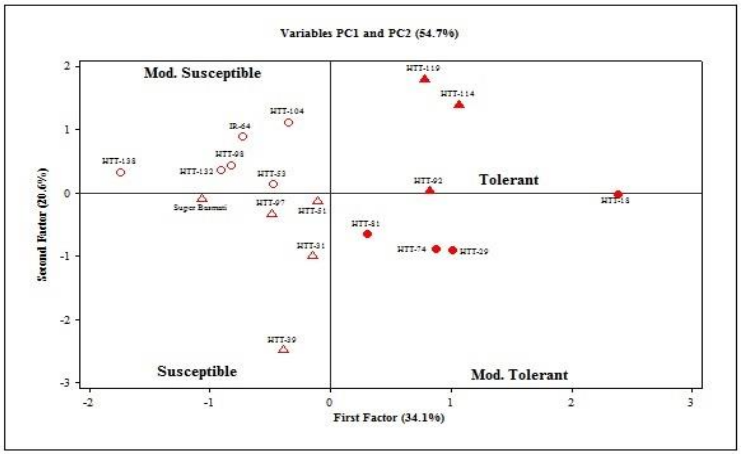

Fig. 5. Classification of Basmati rice mutants based on biochemical parameters. Mutants that had +PC1 and +PC2, +PC1 and −PC2, −PC1 and +PC2,−PC1 and −PC2 scores were classified as tolerant, moderately tolerant, moderately susceptible, and susceptible, respectively.

According to the productivity under hot field conditions, the mutants HTT-18, HTT-29, HTT-39, HTT-92 and HTT-114 were considered as heat tolerant. Interestingly, the classification of mutants based on PCA using POD, TPC, CAT, MDA, TSP and TOS coincided with the heat tolerance level of mutants grown in the hot field conditions. The important characters coming together in different PCs had the tendency to remain together, which might be kept into consideration during selection for thermo-tolerance.

Materials and Methods

Screening for heat tolerance in the growth chamber

A total of 161 Basmati rice mutant lines and advanced mutants of Basmati-370 along with standards Super Basmati and IR-64 varieties were screened to study their physiological responses at seedling stage against high temperature stress (45 ±2 °C) for 12 h along with controls (28±2 °C) in the growth chamber during the years 2012-14 at NIAB, Faisalabad, Pakistan. The seeds were sown in the plastic pots filled with autoclaved standard compost and in two sets and placed in the growth chamber running at normal temperature (28±2 °C). Both sets were placed in the dark till the initiation of germination (3-4 days). After germination, 12 h photoperiod was maintained and one set of six days old seedlings after germination was shifted to another growth chamber for high temperature stress running at 45±2 °C for 12 h. After high temperature exposure, the seedlings were allowed to recover for three days by placing them under normal temperature conditions (28±2 °C) at 12 h photoperiod.

Table 6. Analysis of variance for different agro-morphological traits in Basmati mutants at NIAB, Multan and Bahawalpur during 2016.

Source of variation	Degree of freedom	Mean square								
		Days to Flower	Plant Height	Productive Tiller Plant^{-1}	Panicle Length	Spikelet Main Panicle^{-1}	Panicle Fertility	Biomass	Paddy Yield	Harvest Index
Replicate										
NIAB	2	1.685	0.079	0.305	0.087	5.766	1.633	13727.7	40477.384	2.586
Multan	2	0.241	2.848	0.745	0.574	73.803	4.496	12452.157	2396.487	0.099
Bahawalpur	2	2.296	0.127	26.271	0.662	60.539	0.145	35706.1	36941.7	1.409
Genotype										
NIAB	17	104.9**	600.9**	7.8**	6.1**	1181.9**	32.6**	7976353.6**	820759.2**	41.134**
Multan	17	151.6**	450.7**	8.3**	10.6**	1158.6**	508.5**	24518568.6**	866006.4**	94.728**
Bahawalpur	17	166.1**	701.9**	44.4	6.2**	613.1**	280.6**	4313296.5**	1047760.7**	69.7**
Error										
NIAB	34	1.215	0.373	0.180	0.124	22.227	0.000	41754.535	9761.109	0.766
Multan	34	1.319	0.758	2.355	0.665	111.603	6.008	18121.863	7504.564	1.186
Bahawalpur	34	0.963	0.982	30.143	1.053	72.925	2.540	14158.592	13974.615	1.275

**&*: Significant at p<0.01 and 0.01, respectively

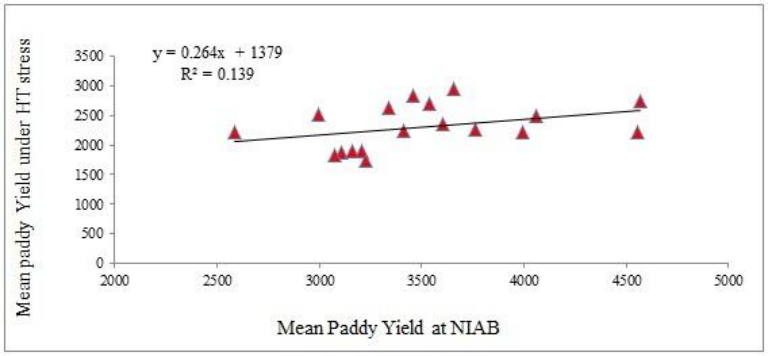

Fig 6. Relationship between paddy yield at NIAB and mean paddy yield under other locations in Basmati rice

Based on the performance of 161 Basmati rice mutants/lines against high temperature stress during the years 2012-14 trials in the growth chamber, 16 mutants were finally selected exhibiting promise to high temperature stress. During 2015, after seedling emergence of the selected 16 mutants along with Super Basmati and IR-64, 12 h photoperiod was maintained for two weeks following a completely randomized design with three replications. One set of seedlings was subjected to heat stress for 6 h daily for 6 days in growth chamber running at 45±2 °C while, the other set was kept at normal temperature that served as control. After high temperature exposure, the seedlings were allowed to recover for three days by placing under normal temperature (28±2 °C) in the growth chamber and data on five random seedlings per replicate were recorded on shoot and root lengths, seedling fresh and dry weights, cell membrane thermo-stability (CMTS). Fresh weights were recorded immediately after harvesting to avoid evaporation. For dry weight estimations, pre-weighted seedlings were kept at 72±2 °C till complete drying with no further decrease in weight. For biochemical analysis, twenty seedlings (bulk sample) were used. The cell membrane thermo-stability (CMTS) was calculated as follows.

$$\text{Percent Injury (PI)} = [1-(1-T_1/T_2)/(1-C_1/C_2)]x100$$

where T_1: 1st conductivity measurement of heat stressed leaf segments (45 °C).

T_2: 2nd conductivity measurement (after autoclaving) of heat stressed leaf segments.

C_1: 1st conductivity measurement of control plant leaf segments (28 °C).

C_2: 2nd conductivity measurement (after autoclaving) of control plant leaf segments.

CMTS = 100- Percent Injury

The stress tolerance indices (STIs) for the above-mentioned traits were calculated as follows.

$$\text{STI} = \frac{\text{Value under stress}}{\text{Value at control}} x100$$

The scoring allocations of the mutants were carried out based on cumulative STIs of the seedling traits. Based on cumulative scores of the traits, the scoring was made, and the mutants were ranked as 1, 2, and 3, and grouped as tolerant, moderately tolerant and sensitive respectively.

Biochemical Analysis

For estimation of different stress biomarkers, enzymatic and non-enzymatic antioxidants, hydrolytic enzymes and other biochemical attributes, the methodologies are given below.

Stress biomarkers

The level of lipid peroxidation in the leaf tissue measured in terms of malondialdehyde (MDA) content was determined by the thiobarbituric acid (TBA) reaction using method of Heath and Packer, (1968) with minor modifications as described by Dhindsa et al. (1981). The MDA content was calculated by using extinction coefficient of 155 $mM^{-1}cm^{-1}$. The esterase activity was measured by Fast Blue BB method. The absorbance of colour compound produced was measured at 590 nm using spectrophotometer (U-2800, 122-003 Hitachi, Japan). Total oxidant status (TOS) was determined by using a novel automated Erel's (2005) formulated method. The absorption of colour compound produced was measured at 560 nm by using spectrophotometer (HITACHI U-2800).

Enzymatic antioxidants

For estimation of enzymes, bulk samples of fresh leaves of twenty-five seedlings (0.15 g) were ground in cold extraction buffer specific for different enzymes. Samples were centrifuged at 15,000×*g* for 20 min at 4°C. The supernatant was separated and used for the determination of different enzyme activities and other biochemical assays.

The enzyme activities were expressed on a fresh weight basis. The aactivity of peroxidase (POD) was measured using the method of Chance and Maehly (1955) with some modification. One unit of POD activity was defined as an absorbance change of 0.01 min^{-1}. The ccatalase (CAT) was estimated using the method described by Beer and Sizer (1952). An absorbance changes of 0.01 min^{-1} was defined as 1 U of CAT activity. For superoxide dismutase (SOD) activity, leaf extracts and analysis are as described by Dixit et al. (2001). The activity of SOD was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) following the method of Giannopolitis (1977). One unit of SOD activity was defined as the amount of enzyme that caused 50% inhibition of photochemical reduction of NBT. The ascorbate Peroxidase Activity (APX) was measured using the method of Dixit et al. (2001). The oxidation rate of ascorbic acid was estimated by following the decrease in absorbance at 290 nm after every 30 seconds (Chen and Asada, 1989).

Non-enzymatic antioxidants

Total phenolic content (TPC) was estimated as total phenolics assay by micro colorimetric method (Ainsworth and Gillespie, 2007) which utilizes Folin-Ciocalteau (F-C) reagent. Phenolic content (gallic acid equivalents) of samples was determined using linear regression equation.

Hydrolytic enzymes

The protease activity was dictated by the casein digestion assay described by Drapeau et al. (1974). By this method one unit is that amount of enzyme, which releases acid soluble fragments equivalent to 0.001 A280 per minute at 37°C and pH 7.8. Enzyme activity was expressed on a fresh weight basis. Esterase activity was measured by the Fast-Blue BB method. The absorbance of color compound produced was measured at 590 nm using spectrophotometer (U-2800, 122-003 Hitachi, Japan).

Biochemical attributes

The estimation of total soluble proteins content (TSP) used the method of (Bradford, 1976) using spectrophotometer (HITACHI, U2800).

Evaluation under field conditions

Based on the performance of 161 Basmati rice mutants/lines at the seedling stage against high temperature stress during the years 2012-15 trials in the growth chamber, 16 mutants were finally selected exhibiting promise to high temperature stress. These mutants were further evaluated in the field following randomized complete block design with three replications at three locations (NIAB, Multan and Bahawalpur) during 2016 and the data were recorded on paddy yield and other agro-morphological traits (days to 50% flower, plant height, productive tiller per plant, panicle length, spikelets on main panicle, panicle fertility, biomass, paddy yield and harvest index).

Weather data

The weather conditions at NIAB, Multan and Bahawalpur (temperature, relative humidity and rainfall) for the whole growing season including vegetative and reproductive growth periods during the season 2016 are presented in Fig 1. Both the sites (Multan and Bahawalpur) had higher temperatures than NIAB at vegetative as well as reproductive stage and were relatively hotter than NIAB which affected the productivity of mutants at these locations. The average maximum temperature for the whole growing season ranged from 26.0-45.0°C with an average of 36.3°C at NIAB, Faisalabad, 24.0-46.0°C with an average of 36.9°C at Multan and 25.0-45.0°C with an average of 37.5°Cat Bahawalpur (June-November) while minimum temperature at NIAB, Faisalabad ranged 22.0-31.5°C with an average of 27.3°C as compared to 22.0-33.0°C with an average of 28.9°C at Multan and 11.0-31.0°C with an average of 24.9°C at Bahawalpur. The relative humidity ranged 33.5-86.0% with an average of 62.9% at NIAB, Faisalabad, and 23.0-79.0% with an average of 55.5% at Multan and 29.0-76.0% with an average of 53.8% at Bahawalpur. The total rainfall recorded during the growing season at NIAB, Faisalabad was 585.2 mm, 170.2 mm at Multan and 77.0 mm at Bahawalpur.

During vegetative stage (June-August), the average maximum temperature range was 31.0-45.0°C with an average of 37.9°C at NIAB, Faisalabad, 30.0-46.0°C with an average of 38.9°Cat Multan and 35.0-45.0°C with an average of 39.2°C at Bahawalpur while minimum temperature at NIAB, Faisalabad ranged 22.0-31.5°C with an average of 27.3 °C as compared to 22.0-33.0 °C with an average of 28.9 °C at Multan and 23.0-31.0 °C with an average of 28.2°C at Bahawalpur. The relative humidity ranged 33.5-86.0% with an average of 63.1% at NIAB, Faisalabad, 23.0-79.0% with an average of 55.1% at Multan and 29.0-76.0% with an average of 54.0% at Bahawalpur. The total rainfall recorded during this stage at NIAB, Faisalabad was 261.8 mm, 82.1 mm at Multan and 38.5 mm at Bahawalpur. At the flowering/grain-filling stage (September-November), the average maximum temperature range was 26.0-39.0°C with an average of 34.6°C at NIAB, Faisalabad, 24.0-39.0°C with an average of 34.6°C at Multan and 25.0-40.0°C with an average of 35.5°C at Bahawalpur while minimum temperature at NIAB, Faisalabad ranged 9.7-26.5°C with an average of 20.5 °C as compared to 11.0-29.0 °C with an average of 22.2 °C at Multan and 11.0-27.0°C with an average of 21.0°C at Bahawalpur. The relative humidity ranged 47.0-80.0% with an average of 62.6% at NIAB, Faisalabad, 33.0-71.0% with an average of 56.0% at Multan and 34.0-66.0% with an average of 53.6% at Bahawalpur. The total rainfall recorded during this stage at NIAB, Faisalabad was 30.8 mm, 3.0 mm at Multan and 0.0 mm at Bahawalpur.

Statistical Analysis

The analysis of variance (ANOVA) and correlations were computed using MSTATC statistical programme to determine differences among the mutants for different agronomic and seedling growth traits. The significance of correlation between yield and other agronomic/seedling growth traits was determined at 0.01 and 0.05 levels of probability. Principal component analysis (PCA) was performed using mean values to find traits accounting for phenotypic variation as well as to classify the mutants for heat tolerance using computer software "Minitab 14" for Windows. For PCA, procedures of Chatfield and Collin (1980), Mahloch (1974), Mazlum (1994), and Mazlum et al. (1999) were followed. The component loadings (correlation coefficients) and the variances (eigenvalues) regarding the components were computed for all the characters at the first step following a correlation matrix as all the traits had equal importance with different scales. The proportion of the total variance explained by each principal component was additive, with each new component contributing less than the preceding one to the explained variance. According to Brejda et al. (2000), data were considered in each component with eigenvalue >1 which determined at least 10% of the variation. The higher eigenvalues were considered as best representatives of system

attributes in principal components. Subsequently, the components were selected whose eigenvalue (λ) was >1, and varimax rotations were performed until all the communalities were ~0.7. The values of only yield-correlated seedling growth traits (shoot length, root length, shoot dry weight, root fresh and dry weights, and cell membrane thermo-stability) were included in the PCA. The eigenvalues generated by PCA were used to grade mutants for their heat tolerance. The first two PC scores (PC1 and PC2), accounted for maximum variability of the parameters tested, were used to classify the mutants. The mutants that had +PC1 and +PC2 scores were classified as tolerant, those with +PC1 and −PC2 scores as moderately tolerant, those with −PC1 and +PC2 scores as moderately susceptible, and those with −PC1 and −PC2 scores as susceptible following Kakani et al. (2005).

Conclusion

Several studies revealed that high temperature damage to plants was caused by the excessive production of reactive oxygen radicals and consequent low activities of antioxidant enzymes and the cell membrane damage (Zhang et al., 2006 and Zhu et al., 2005) and ultimately yield. The decrease in antioxidant activity under stressed conditions resulted in higher levels of ROS that might contribute to cell injury (Fadzillah et al., 1996). The present studies revealed that screening for seedling growth related traits (shoot length, shoot fresh and dry weight and cell membrane thermo-stability), and trends of biochemical parameters at the seedling stage might support the selection for thermo-tolerance in rice. The results of the growth-related traits and biochemical parameters coincided favourably with yield data under hot field conditions. In this study, the levels and activities of MDA, SOD, APX and esterase increased/maintained over control indicating higher production of ROS under high temperature stress.

Acknowledgements

The authors are thankful to the International Atomic Energy Agency (IAEA) for providing technical and financial support through Coordinated Research Project (CRP) Pak-16589 "Breeding for high yielding long rain rice with adaptation to high temperatures" under an IAEA-CRP (D-23029) "Climate Proofing of Food Crops: Genetic Improvement for Adaptation to High temperatures in drought prone areas and beyond "

References

Ainsworth EA, Gillespie KM (2007) Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin-Ciocalteu reagent. Nature Protocols. 2: 875-877.

Ainsworth E A, Ort D R (2010) How Do We Improve Crop Production in a Warming World? Plant Physiol. 154: 526-530.

Ali F, Waters DLE, Ovenden B, Bundock P, Raymond CA, Rose TJ (2018) Australian rice varieties vary in grain yield response to heat stress during reproductive and grain filling stages. J Agro Crop Sci. 1-9.

Ali MK, Azhar A, Galani S (2013) Response of rice (*Oryza sativa* L.) under elevated temperature at early growth stage: Physiological markers. Russian J Agric Socio-Econ Sci. 20: 11-19.

Almeselmani M, Deshmukh PS, Sairam RK, Kushwaha SR, Singh TP (2006) Protective role of antioxidant enzymes under high temperature stress. Plant Sci. 171: 382-388.

Bahuguna RN, Jha J, Pal M, Shah D, Lawas LMF, Khetarpal S, Jagdesh KSV. (2015) Physiological and biochemical characterization of NERICA-L-44: a novel source of heat tolerance at the vegetative and reproductive stages in rice. Physiol Planta. 154: 543-559.

Bahuguna RN, Chaturvedi AK, Pal M (2016) Physiological traits for improving high temperature stress tolerance in rice. Ind J Plant Physiol. 21(4): 420-427.

Beers RF, Sizer IW (1952) A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J Biol Chem. 195: 133-140.

Bita C, Gerats T (2013) Plant tolerance to high temperature in a changing environment: scientific fundamentals and production of heat stress-tolerant crops. Front Plant Sci. 4(article 273): 1-18.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 72: 248-254.

Brejda JJ, Moorman TB, Smith JL, Karlen DL, Allan DL, Dao, TH (2000) Distribution and variability of surface soil properties at a regional scale. Soil Sci Soc Amer J. 64(3): 974-982.

Cao YY, Duan H, Yang LN, Wang ZQ, Liu LJ, Yang JC (2009) Effect of high temperature during heading and early filling on grain yield and physiological characteristics in Indica rice. Acta Agron Sin. 35: 512-521.

Chance B, Maehly A (1955) Assay of catalases and peroxidases. Methods Enzymol. 2: 764-775.

Charng YY, Liu HC, Liu NY, Chi WT, Wang CN, Chang SH (2007) A heat-inducible transcription factor, HsfA2, is required for extension of acquired thermo tolerance in Arabidopsis. Plant Physiol.143: 251-262.

Chatified C, Collin AJ (1980) Introduction to multivariate analysis. Chapman and Hall in association with Methuen. Inc. 733, Third Avenue, New York NY. 10017.

Chaturvedi AK, Bahuguna RN, Shah D, Pal M, Jagadish SVK (2017) High temperature stress during flowering and grain filling offsets beneficial impact of elevated CO_2 on assimilate partitioning and sink strength in rice. Sci Rep. 7: 1-13.

Chen GX, Asada K (1990) Hydroxyurea and p-aminophenol are the suicide inhibitors of ascorbate peroxidase. J Biol Chem. 265: 2775-2781.

Collins NC, Tardieu F, Tuberosa R (2008) Quantitative trait loci and crop performance under abiotic stress: Where do we stand? Plant Physiol. 147: 469-486.

Demirel U, Copur O, Gur AA (2016) Early-stage screening for heat tolerance in cotton. Plant Breed. 135: 80-89.

Dhindsa RS, Plumb-Dhindsa P, Thorpe TA (1981) Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. J Exp Bot. 32: 93-101.

Dixit V, Pandey V, Shyam R (2001) Differential antioxidative responses to cadmium in roots and leaves of pea (*Pisum sativum* L. cv. Azad). J Exp Bot. 52: 1101-1109.

Drapeau G R (1976) Protease from *Staphylococcus aureus*. Methods Enzymol. 45: 469-75.

Erel O (2005) A new automated colorimetric method for measuring total oxidant status. Clin Biochem., 38: 1103-1111.

Fadzillah NM, Gill V, Finch RP, Burdon RH (1996) Chilling, oxidative stress and antioxidant responses in shoot cultures of rice. Planta. 199: 552-556.

Giannopolitis CN, Ries SK (1977) Superoxide dismutases I. Occurrence in higher plants. Plant Physiol. 59: 309-314.

Hall AE (2011) Breeding cowpea for future climates. In: Yadav SS, Redden R, Hatfield JL, Lotze-Campen H, Hall AJW (eds) Crop adaptation to climate change, John Wiley & Sons.

Hameed A, Goher M, Iqbal N (2012) Heat stress-induced cell death, changes in antioxidants, lipid peroxidation, and protease activity in wheat leaves. J Plant Growth Regul. 31: 283-291.

Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplasts I. kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys. 125: 189-198.

Howarth CJ (2005) Genetic improvement of growth and survival at high temperature. In: Ashraf M, Harris PJC (eds) Abiotic stresses: Plant resistance through breeding and molecular approaches, Haworth Press Inc, New York.

IPCC (2013) Working group I Contribution to the IPCC fifth assessment report climate change: The physical science basis, summary for policy makers. Available on line at http://www.Climatechange 2013.Org/ images/ report/WG1AR5_SPM_FINAL.pdf

Iqbal N, Farooq S, Arshad R, Hameed A (2010) Differential accumulation of high and low molecular weight heat shock proteins in basmati rice (*Oryza sativa* L.) cultivars. Genet Resour Crop Evol. 57: 65-70.

Johnson DE (1998) Applied multivariate methods for data. Johnson/Duxbury Press, New York.

Jumiatun, Junaedi A, Lubis I, Chozin MA, Miyazaki A (2016) Morphological, physiological and yield responses of some rice varieties (*Oryza sativa* L.) as exposed under high temperature in Indonesia. American J Plant Physiol. 11: 33-41.

Kakani VG, Reddy KR, Koti S, Wallace TP, Prasad PVV, Reddy VR (2005) Differences in *in vitro* pollen germination and pollen tube growth of cotton cultivars in response to high temperature. Ann Bot. 96: 59-67.

Kang HM, Saltveit ME (2002) Antioxidant enzymes and dpph-radical scavenging activity in chilled and heat-shocked rice (*Oryza Sativa* L.) seedlings radicles. J Agric Food Chem. 50**:** 513-518.

Kumar N, Kumar N, Shukla A, Shankhdhar SC, Shankhdhar D (2015) Impact of terminal heat stress on pollen viability and yield attributes of rice (*Oryza sativa* L.). Cereal Res Comm. 43(4): 616-626.

Liu Z, Yuan YL, Liu SQ, Yu XN, Rao LQ (2006) Screening for high-temperature tolerant cotton cultivars by testing *in vitro* pollen germination, pollen tube growth and boll retention. J Integ Plant Biol. 48: 706-714.

Liu QH, Wu X, Li T, Ma JQ, Zhou XB (2013) Effects of elevated air temperature on physiological characteristics of flag leaves and grain yield in rice. Chilean J Agric Res. 73: 85-89.

Maestri E1, Klueva N, Perrotta C, Gulli M, Nguyen HT, Marmiroli N (2002) Molecular genetics of heat tolerance and heat shock proteins in cereals. Plant Mol Biol. 48(5-6): 667-81.

Mahloch JL (1974) Multivariate technique for water quality analysis. J Environ Eng Div. Amer. Soc. Civil Eng. 100(EE5): 1119-1124.

Mazlum N. 1994. Multivariate analysis of water quality data. M. Sc. thesis submitted to Dokuz Eylul University, Graduate School of National and Applied Sciences, Bornova, Izmir.

Mazlum N, Ozer A, Mazlum S (1999) Interpretation of water quality data by principal component analysis. Tr J Eng Environ Sci. 23: 19-26.

Mohammed AR, Tarpley L (2009) Impact of high nighttime temperature on respiration, membrane stability, antioxidant capacity, and yield of rice plants. Crop Sci. 49: 313-322.

Mohammed AR, Tarpley L (2011) Effects of night temperature, spikelet position and salicylic acid on yield and yield-related parameters of rice (*Oryza sativa* L.) plants. J Agron Crop Sci. 197: 40-49.

Ohe I, Saitoh K, Kuroda T (2007) Effects of high temperature on growth, yield and dry-matter production of rice grown in the paddy field. Plant Prod. Sci. 10: 412-422.

Prasanth VV, Chakravarthi DVN, Kiran TV, Rao YV, Panigrahy M, Mangrauthia SK, Viraktamath BC, Subrahmanyam D, Voleti SR, Sarla N (2012) Evaluation of rice germplasm and introgression lines for heat tolerance. Annals Biol Res. 3(11): 5060-5068.

Ramesh R, Ramesh T, Rao PRV, Shankar G. Bhave MHV (2017) High temperature stress effected the biochemical parameters of rice (*Oryza sativa* L.) varieties and hybrids. Int J Pure App Biosci. 5(4): 1478-1490.

Ray DK, Gerber JS, MacDonald GK, West PC (2015) Climate variation explains a third of global crop yield variability. Nature commun. 6.

Sailaja B, Anjum N, Prasanth VV, Voleti SR, Subrahmanyam D, Sarla N (2014) Comparative study of susceptible and tolerant genotype reveals efficient recovery and root system contributes to heat stress tolerance in rice. Plant Mol Biol Rep. 32: 1228-1240.

Sailaja B, Subrahmanyam D, Neelamraju S, Vishnukiran T, Rao YV, Vijayalakshmi P (2015) Integrated physiological, biochemical, and molecular analysis identifies important traits and mechanisms associated with differential response of rice genotypes to elevated temperature. Front Plant Sci. 6: 1-13.

Shah F, Huang J, Cu IK, Nie L, Shah T, Chen C, Wang K (2011) Impact of high temperature stress on rice plant and its traits related to tolerance. J. Agr. Sci. 149: 545-556.

Shah F, Nie L, Cui K, Shah T, Wu W, Huang J (2014) Rice grain yield and component responses to near 2 ^{o}C of warming. Field Crops Res. 157: 98-110.

Shalata A, Neumann PM (2001) Exogenous ascorbic acid (vitamin C) increases resistance to salt stress and reduces lipid peroxidation. J Exp Bot. 52: 2207-2211.

Shi W, Ishimaru T, Gannaban RB, Oane W, Jagadish SVK (2014) Popular rice (*Oryza sativa* L.) cultivars show contrasting responses to heat stress at gametogenesis and anthesis. Crop Sci. 55: 589-596.

Tayade R, Nguyen TD, Oh SA, Hwang YS, Yoon IS, Deshmuk R, Jung KH, Park SK (2018) Effective strategies for enhancing tolerance to high-temperature stress in rice during the reproductive and ripening stages. Plant Breed Biotech. 6(1): 1-18.

Van Oort, PAJ, Zwart SJ (2018) Impacts of climate change on rice production in Africa and causes of simulated yield changes. Glob Chang. Biol. 24: 1029-1045.

Venkateswarlu B, Visperas RM (1987) Solar radiation and rice productivity. Res Paper Sers No. 129. IRRI, Manila, Philippines.

Wahid A, Gelani S, Ashraf M, Foolad MR (2007) Heat tolerance in plants: An overview. Environ Exp Bot. 61: 199-223.

Wassmann R, Jagadish SVK, Heuer S, Ismail A, Redona E, Serraj R, Singh RK, Howell G, Pathak H, Sumfleth K (2009) Climate change affecting rice production: the physiological and agronomic basis for possible adaptation strategies. Adv. Agron. 101: 59-122.

Xia MY, Qi HX (2004) Effects of high temperature on the seed setting percent of hybrid rice bred with four male sterile lines. Hubei Agric Sci. 2: 21-22.

Yang HC, Huang ZQ, Jiang ZY, Wang XW (2004) High temperature damage and its protective technologies of early and middle season rice in Anhui province. J Anhui Agric Sci. 32: 3-4.

Yang Z, Zhang Z, Zhang T, Fahad S, Cui K, Nie L, Peng S, Huang J (2017) The effect of season-long temperature increases on rice cultivars grown in the central and southern regions of china. Front Plant Sci. 8: 1-15.

Zafar SA, Hameed A, Khan AS, Ashraf M (2017) Heat shock induced morpho-physiological response in indica rice (*Oryza sativa* L.) at early seedling stage. Pak J Bot. 49(2): 453-463.

Zhang GZ, Chen LY, Zhang ST, Xiao YH, He ZZ, Lei DY (2006) Effect of high temperature stress on protective enzyme activities and membrane permeability of flag leaf in rice. Acta Agron Sin. 32: 1306-1310.

Zhang G, Chen L, Zhang S, Zheng H, Liu G (2009) Effects of high temperature stress on microscopic and ultrastructural characteristics of mesophyll cells in flag leaves of rice. Rice Sci. 16: 65-71.

Zhou WH, Xue DW, Zhang G (2012) Identification and physiological characterization of thermo-tolerant rice genotypes. J Zhejiang Univ. 38: 1-9.

Zhu CL, Xiao YH, Wang CM, Jiang L, Zhai HQ, Wan JM (2005) Mapping QTLs for heat tolerance during grain filling in rice. China J Rice Sci. 19: 117-121.

CONTENT