

Competitive Research Grant

Sub-Project Completion Report

on

**Introgression of heat tolerant QTL (*qHTSF4.1*) into
Bangladeshi mega rice varieties through marker-
assisted breeding**

Project Duration

May 2017 to September 2018

**Plant Physiology Division
Bangladesh Rice Research Institute
Gazipur**



**Submitted to
Project Implementation Unit-BARC, NATP-2
Bangladesh Agricultural Research Council
Farmgate, Dhaka-1215**



September 2018

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Citation

Introgression of heat tolerant QTL (*qHTS F4.1*) into Bangladeshi mega rice varieties through marker-assisted breeding

Project Implementation Unit

National Agricultural Technology Program-Phase II Project (NATP-2)

Bangladesh Agricultural Research Council (BARC)

New Airport Road, Farmgate, Dhaka – 1215

Bangladesh

Edited and Published by:

Project Implementation Unit

National Agricultural Technology Program-Phase II Project (NATP-2)

Bangladesh Agricultural Research Council (BARC)

New Airport Road, Farmgate, Dhaka – 1215, Bangladesh

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Acronyms

CSISA: Cereal Systems Initiative for South Asia
IRRI: International Rice Research Institute
MAS: Marker Assisted Selection
MABC: Marker Assisted Backcrossing
CAPS: Cleaved Amplified Polymorphic Sequences
N22: Nagina22
QTL: Quantitative Trait Loci
F₁: 1st Filial Generation
BC₁: Backcross One

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Executive Summary

A marker-assisted introgression of high temperature spikelet fertility QTL (*qHTSF4.1*) into BRR1 dhan28 and BRR1 dhan29 were started in 2013. For promotion of high temperature spikelet fertility QTL introgression lines in the background of BRR1 dhan28 and BRR1 dahn29 and introgression of *qHTSF4.1* in BRR1 dhan58 and BRR1 dhan48 weredone with NATP2 fund to cope the prevailing high temperature sterility problems in Bangladesh. Hybridization of BRR1 dhan58 and BRR1 dhan48 with Nagina22 (a heat tolerant Indian variety, N22) were carried out just in the beginning of the project. Therefore, during the reporting period (17months) F₁'s were developed from BRR1 dhan48/N22 and BRR1 dhan58/N22 andgenotyping of F₁'swere done using R4M30 marker. Out of 75 F₁'s, 28 from both cross combinations were confirmed and 1st backcrossing was carried out. A total of 1534BC₁F₁ seeds were produced for both cross combinations.Eighty progenies (because all the F1 seeds were not germinated) from each cross combination planted in the field for MAS and 2nd round backcrossing.MAS is completed with BC₁F₁ progenies. Twenty six plants in the background of BRR1 dhan48 and twenty plants in the background of BRR1 dhan58 were selected through use of marker for second round backcrossing.A total of 1311(590 +721) BC₂F₁ seeds were produced for both cross combinations by 2nd round backcrossing.Another experiment was started in 2013 to introgress high temperature spikelet fertility QTL in the background of BRR1 dhan28 and BRR1 dahn29. After the development BC₂F₂generation (in the background of BRR1 dhan28 and BRR1 dhan29) some material were advanced to BC₂F₅ through selfingduring 2014-2016. Some of these materials (BC₂F₂) were backcrossed with respective recurrent parent and advanced to BC₃F₃through selfingin 2016. These BC₂F₅ and BC₃F₃ materials were advanced with NATP-2 project fund. Eighty-eight lines from 20 BC₂F₄ progenies of high temperature spikelet fertility QTL introgression lines in the background of BRR1 dhan28 and BRR1 dahn29 were advanced to BC₂F₅for fixation of QTL allele and background traits through MAS and phenotypic selection. These 88 lines lines were advanced to BC₂F₆ generation and 760 lines (BRR1 dhan28:368 and BRR1 dhan29:392) having fixed QTL loci(*qHTSF4.1*)were reconfirmed by marker and selected phenotypically. These BC₂F₆fixed lines are ready for evaluation of phenotypic gain at high temperature in controlled condition. Two-hundred-sixty-four BC₃F₃ progenies of high temperature spikelet fertility QTL introgression lines in BRR1 dhan28 and BRR1 dahn29 background were also advanced for fixation of QTL allele through MAS. From BC₃F₃ generation 29 lines in BRR1 dhan28 background and 20 lines in BRR1 dhan29 background were selected phenotypically, later confirmed by marker and advanced to BC₃F₄ generation.Beside the BC₂F₁ seeds in the background of BRR1 dhan48 and BRR1 dhan58 we got BC₂F₆ fixed lines and also BC₃F₄breeding llines in the background of BRR1 dhan28 and BRR1 dhan29 through NATP 2.

RG Sub-Project Completion Report (PCR)

A. Sub-project Description

1. Title of the CRG sub-project: Introgression of heat tolerant QTL (*qHTSF4.1*) into Bangladeshi mega rice varieties through marker assisted breeding

2. Name and full address with phone, cell and E-mail of PI/Co-PI:

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3. Sub-project budget (Tk):

3.1 Total: 28,48,000.00

3.2 Revised (if any): 26,69,000.00

4. Duration of the sub-project:

4.1 Start date (based on LoA signed): 15 May 2017

4.2 End date: 30 September 2018

5. Justification of undertaking the sub-project:

In recent years high temperature at flowering stage becomes a critical factor in Boro rice production. In general, high temperature affects all the growth phases of rice plant but reproductive phase is the most vulnerable to high temperature. It affects anther dehiscence, pollination, and pollen germination, which then lead to spikelet sterility and yield loss (Yoshida *et al.*, 1981). Exposure at anthesis even for less than an hour at 33.7 °C may result in spikelet sterility (Jagadishet *et al.*, 2007), which will greatly increase at temperature above 35 °C (Matsui *et al.*, 1997). Increased severity of the problem in rice-growing areas in Asia is due to rising temperatures (Catherine *et al.*, 2012). Global temperatures are estimated to rise by 1.1 °C to 6.4 °C during the next century (IPCC, 2012), thereby threatening rice production. Higher temperatures can adversely affect rice yields through two principal pathways, namely (i) high maximum temperatures that cause-in combination with high humidity-spikelet sterility and adversely affect grain quality and (ii) increased night temperatures that may reduce assimilate accumulation (Wassmann *et al.*, 2009). Reproductive stage in rice is more sensitive to heat than vegetative stage (Yoshida *et al.*, 1981). Anthesis/flowering, identified with the appearance of the anthers, is the most sensitive process during reproductive stage to high temperature followed by microgametogenesis (Nakagawa *et al.*, 2002; Satake and Yoshida, 1978).

Temperature rises to maximum in April (>40 °C) throughout the country, however, it is slightly lowered in May but due to combination of high humidity the effects of high temperature remains greater at that period. Coinciding high temperature and high humidity dilemma at that time happening together of flowering and reproductive phase of long duration high yielding *boro* rice varieties (eg. BRRI dhan29 and BRRI dhan58); late boro (when farmer grow *boro* after potato harvest) and early cultivated *aus* rice face high temperature induced sterility. Amongst other factors, global warming would consequently increase occurrence of high temperature-induced floret sterility in rice. Several reports have been found from different regions of Bangladesh during the last 5 years regarding high temperature. Three *aus* cultivar (Nagina22, Dularand, Kachalath) have been identified as tolerant and moderately tolerant to heat stress, which can maintain high spikelet fertility at high temperature (>35°C) at flowering stage. Heat tolerance loci of Nagina22 (N22) have been mapped through SNP marker at IRRI. Based on the map information a marker assisted breeding was undertaken for improving the spikelet fertility of BRRI dhan28 and BRRI dhan29 by introgressing the spikelet fertility QTL (*qHTSF4.1*) from N22. The following marker-assisted introgression of *qHTSF4.1* into BRRI dhan28 and BRRI dhan29 was started 5 years back with the CSISA project funding from IRRI. The CSISA funding ended in 2015. Further promotion of introgressed lines of BRRI dhan28 and BRRI dhan29 and introgression of *qHTSF4.1* into other mega varieties such as another long duration boro variety (BRRI dhan58) and *aus* variety (BRRI dhan48) was necessary to cope the prevailing high temperature sterility problems in Bangladesh.

6. Sub-project goal: Development of high yielding high temperature tolerant rice variety

7. Sub-project objectives:

- i) Introgress heat tolerant QTL (*qHTSF4.1*) into Bangladeshi mega rice varieties.
- ii) Promote fixed introgression lines to proposed variety trial.
- iii) Evaluate phenotypic gain of heat tolerant QTL introgression lines against high temperature.

8. Implementing location: Plant Physiology Division, BRRI, Gazipur-1701

9. Methodology in brief:

Two farmers' popular mega variety (BRRI dhan28 and BRRI dhan29) as recipient variety and heat tolerant N22 as donor were used in 2013.

Marker-Assisted Backcrossing (MABC) scheme: Marker aided foreground selection followed by stringent phenotypic selection at the background were carried out for all 3 Backcross Generation (BC₁-BC₃) for both population.

In 2017 two farmers' popular high yielding variety (BRRI dhan48 and BRRI dhan58) were used as recipient variety and heat tolerant N22 as donor. Introgression of high temperature spikelet fertility QTL (*qHTSF4.1*) into BRRI dhan58 and BRRI dhan48 were completed through hybridization followed by marker assisted selection (MAS). After F₁'s confirmation, backcrossing was carried out with respective recurrent parents followed by MAS. Ten F₁ progenies (BRRI dhan48 × N22) and 18 F₁ progenies (BRRI dhan58 × N22) were selected for backcrossing. Based on the map information (Ye, et

al. 2012), an InDel (R4M30) and a CAPS marker was designed and validated. By using these markers, the marker-assisted introgression of *qHTSF4.1* in the background of BRRi dhan48 and BRRi dhan58 was carried out. At least 3 cycles of backcrossing and two selfing generation were targeted for backcrossing work. A stringent phenotypic selection was carried out for maximum recovery of the recurrent phenome.

10. Results and discussion:

Activity 1, 2, 3: Marker assisted introgression of spikelet fertility loci (*qHTSF4.1*) from N22 into BRRi dhan48 and BRRi dhan58

For introgression of high temperature spikelet fertility QTL (*qHTSF4.1*) in to BRRi dhan48 and BRRi dhan58 hybridization was completed with Nagina22 (a heat tolerant Indian variety, N22) (Photograph 1). During the first 6 months of the project F₁'s were developed from BR58/N22 and BR48/N22 and confirmed through InDelmarkerR4M30. Out of 75 F₁ progeny, 28 F₁'s from both cross combinations were confirmed and first round of backcrossing was carried out with confirmed progenies (Photograph 2, 3 and 4). A total of 1534 BC₁F₁ seeds were produced for both cross combinations by backcrossing with respective recipient parents (Photograph 5). Now BC₁F₁ progenies from both cross combinations are grown in the field (Photograph 6) for marker-assisted selection and subsequent second round of backcrossing. From BC₁F₁ progeny 26 plants in the background of BRRi dhan48 and 20 plants in the background of BRRi dhan58 were selected with the aid of InDelmarkerR4M30 (Photograph 7, 8). After second backcrossing a total of 1311(590 in the background of BRRi dhan48 and 721 in the background of BRRi dhan58) BC₂F₁ seeds were collected for both cross combinations with respective recipient parents. The basis of MABC strategy is to transfer a specific allele at the target locus from a donor line to a recipient line while selecting against donor introgressions across the rest of the genomes (Neeraja, *et al.* 2007). The use of molecular markers, which permit the genetic dissection of the progeny at each generation, increases the speed of the selection process, thus increasing genetic gain per unit time (Hospital, 2003). The main advantages of MABC are (1) efficient foreground selection for the target locus, (2) efficient background selection for the recurrent parent genome, (3) minimization of linkage drag surrounding the locus being introgressed, and (4) rapid breeding of new genotypes with favorable traits. The effectiveness of MABC depends on the availability of closely linked markers and/or flanking markers for the target locus, the size of the population, the number of backcrosses, and the position and number of markers for background selection (Frisch and Melchinger, 2005). Molecular breeding technologies have been widely applied in countries all over the world. It provides powerful tool for

development of stress tolerant varieties that can deal with the adverse effects from climate change.

However, application of molecular breeding such as MABC had only initiated sporadically in Bangladesh. Hence, the attempt of this study was to develop heat-tolerant version of the widely grown BRR1 dhan48 and BRR1 dhan58 by applying the MABC method.

Activity 4: Advancement of fixed heat introgression BC₂F₄ lines in the background of BRR1 dhan28 and BRR1 dhan29

A marker-assisted introgression of *qHTSF4.1* in to BRR1 dhan28 and BRR1 dhan29 was started 5 years back (2013) with the funding of CSISA project from IRRI. Eighty-eight (88) lines were phenotypically selected from 20 fixed high temperature spikelet fertility QTL (*qHTSF4.1*) introgression lines at BC₂F₄ generation of BRR1 dhan28 and BRR1 dhan29 background using CSISA funding. These eighty-eight phenotypically selected lines at BC₂F₅ generation of BRR1 dhan28 and BRR1 dhan29 background were grown in the field as late Boro (2017-18) for further selection under NATP2 project. All the lines were confirmed for fixed QTL (homozygous at QTL marker allele) (Photograph 9). A head to row phenotypic selection was done to fix background traits (Photograph 10). Eighty-eight lines were advanced to BC₂F₆ generation and 760 lines (BRR1 dhan28:368 and BRR1 dhan29:392) having fixed QTL loci (*qHTSF4.1*) reconfirmed by marker and selected phenotypically. DNA analysis was done for bulk sample. Gel picture showed that the alleles are N22 type. Phenotypic selection was done on the basis of similarity with recurrent parent.

Activity 5: Generation advance and selection of progenies of spikelet fertility introgression lines at BC₃F₂ stage of BRR1 dhan28 and BRR1 dhan29 background

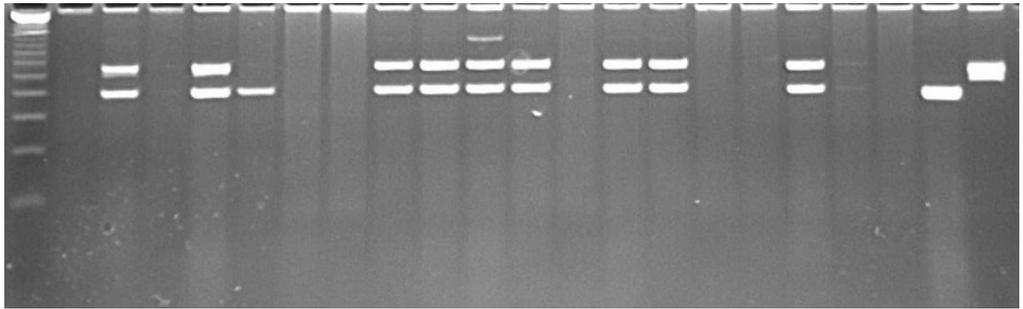
Under CSISA project thirteen (13) high temperature spikelet fertility QTL (*qHTSF4.1*) introgression lines at BC₃F₂ of BRR1 dhan28 and BRR1 dhan29 were advanced under field condition. An additional backcrossing was carried out to increase the genome content of the recurrent parent in the introgression lines. In addition to Activity 4, for fixation of high temperature spikelet fertility QTL (*qHTSF4.1*) at BC₃F₃, 264 introgression lines were planted in the field (Photograph 11) and MAS was done through InDelR4M30 marker under NATP 2. The selected lines were advanced to BC₃F₄ for fixing other background traits. From BC₃F₃ generation 29 lines in the background of BRR1 dhan28 and 20 lines in the background of BRR1 dhan29 selected phenotypically, confirmed by marker and advanced to BC₃F₄ generation. After phenotypic selection marker assisted selection was done for donor parent allele. Different sub-project activities are illustrated in photographs 1-11 cited below.



Photograph 1. Hybridization of BRRi dhan48 and BRRi dhan58 with donor N22



Photograph 2. F₁ progenies at field



Photograph 3. PAGE (8%) for F₁ Confirmation by R4M30 Marker



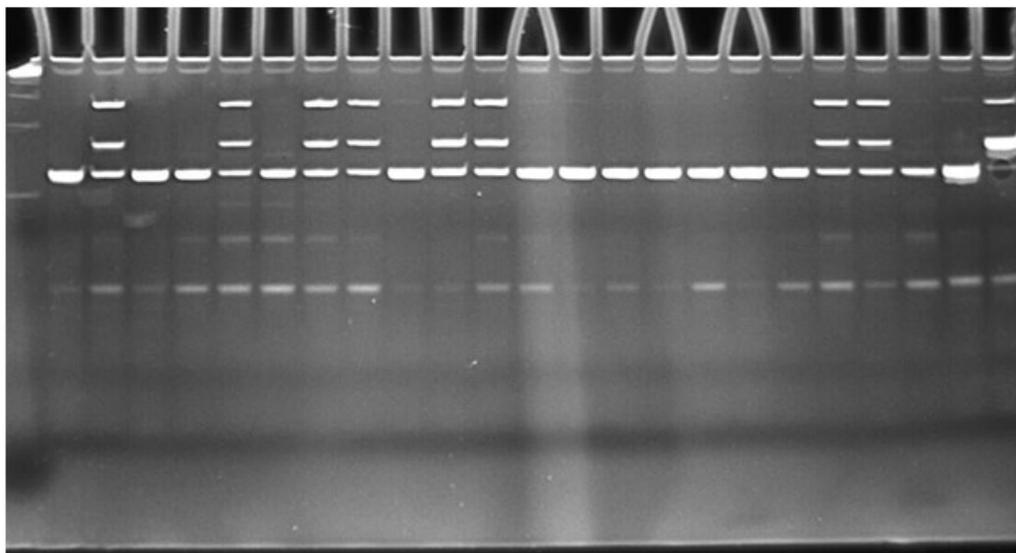
Photograph 4. Tagging of selected F₁ progenies



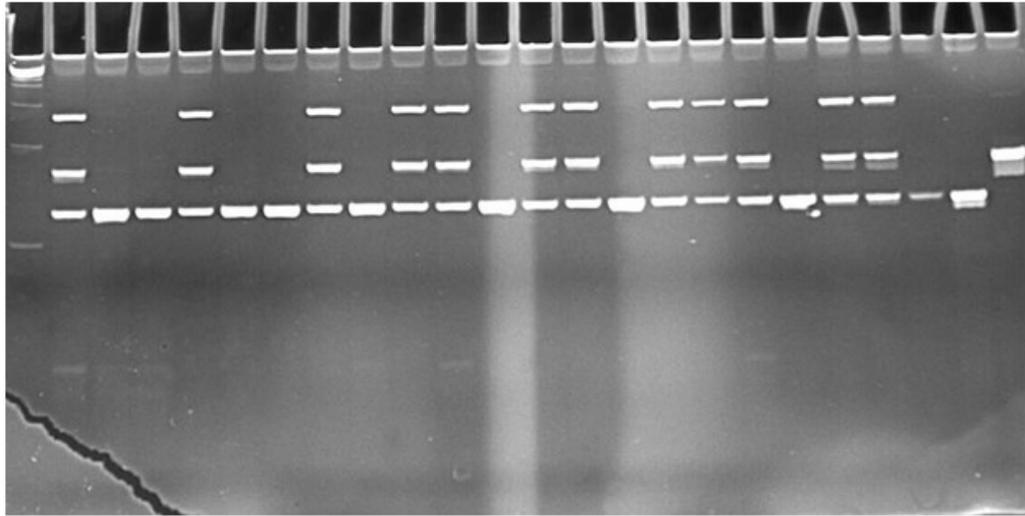
Photograph 5. Dusting of pollen during first backcrossing by BRRI dhan48 and BRRI dhan58



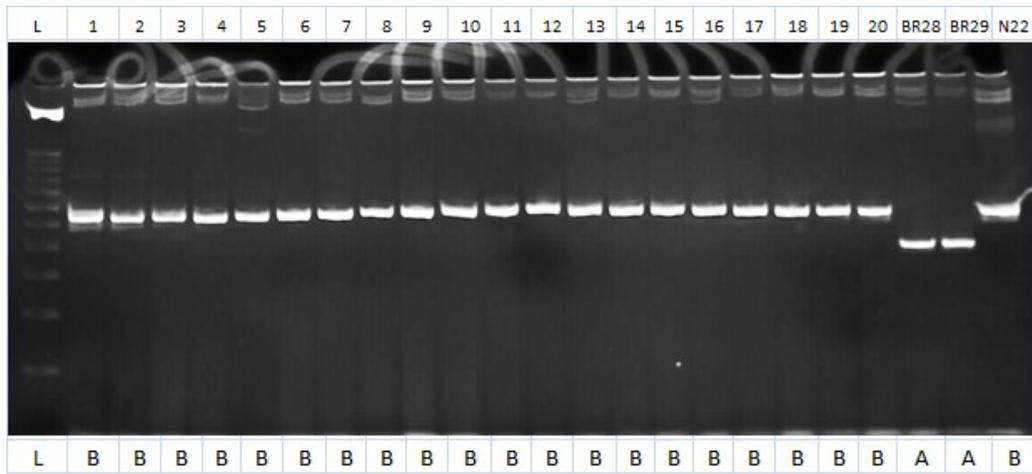
Photograph 6: BC₁F₁ progeny of BRRI dhan48 and BRRI dhan58 background planted in the field for MAS and 2nd round of backcrossing



Photograph 7. PAGE (8%) for BC₁F₁ confirmation by R4M30 marker in BRRI dhan48 background



Photograph 8. PAGE (8%) for BC₁F₁ confirmation by R4M30 marker in BRRi dhan58 background



Photograph 9. PAGE (8%) of BC₂F₅ fixed QTL lines at the marker R4M30 (Lane 1-10 = Progenies of BRRi dhan28, 11-20= progenies of BRRi dhan29, BR28, BR29, N22 allele)



Photograph 10. A team with Plant Breeders and Plant Physiologists visited field during selection of BC₂F₅ advanced progenies of BRRi dhan28 and BRRi dhan29 background in May 2018



Photograph 11. BC₃F₃ progenies in the background of BRRi dhan28 and BRRi dhan29 in May 2018.

11. Research highlight/findings (Bullet point – max 10 nos.):

- Hybridization for introgression of *qHTSF4.1* QTL into BRR1 dhan48 and BRR1 dhan58 was successful and 75 F₁ seeds were produced
- Genotyping of F₁'s completed through use of marker and 28 F₁'s were confirmed
- First backcrossing carried out in the confirmed progenies of BRR1 dhan48 and BRR1 dhan58 successfully and 1534 BC₁F₁ seeds were produced
- Genotyping of BC₁F₁ progenies through marker and second backcrossing of BRR1 dhan48 and BRR1 dhan58 progenies carried out with the confirmed progenies
- Eighty eight BC₂F₃ lines having fixed QTL loci (*qHTSF4.1*) of BRR1 dhan28 and BRR1 dhan29 were advanced to BC₂F₆ generation and 760 lines (BRR1 dhan28:368 and BRR1 dhan29:392) having fixed QTL loci (*qHTSF4.1*) reconfirmed by marker.
- From BC₃F₃ generation 29 lines in the background of BRR1 dhan28 and 20 lines in the background of BRR1 dhan29 were selected phenotypically, confirmed by marker and advanced to BC₃F₄ generation

B. Implementation Position

1. Procurement:

Description of equipment and capital items	PP Target		Achievement		Remarks
	Phy (#)	Fin (Tk)	Phy (#)	Fin (Tk)	
(a) Office equipment	1) Laptop - one 2) Laser Printer- one	60,000 20,000	1) Laptop - one 2) Laser Printer - one	65,000 18,000	
(b) Lab & field equipment	1) Dehumidifier– one 2) Infra-Red Temp. meter- one 3) Wall mount temp/RH/CO ₂ meter - one	1,50,000 20,000 30,000	1) Dehumidifier– one 2) Infra-Red Temp. meter- one 3) Wall mount temp/RH/CO ₂ meter -one	1,43,000 30,800 22,000	
(c) Other capital items	none				

2. Establishment/renovation facilities: not applicable

Description of facilities	Newly established		Upgraded/refurbished		Remarks
	PP Target	Achievement	PP Target	Achievement	

3. Training/study tour/ seminar/workshop/conference organized: not applicable

Description	Number of participant			Duration (Days/weeks/ months)	Remarks
	Male	Female	Total		
(a) Training					
(b) Workshop					

C. Financial and physical progress

Fig in Tk

Items of expenditure/activities	Total approved budget	Fund received	Actual expenditure	Balance/ unspent	Physical progress (%)	Reasons for deviation
A. Contractual staff salary	535273	546036	535273	10763	100	Excess money returned to NATP2
B. Field research/lab expenses and supplies	1569365	1543284	1543284	0	100	
C. Operating expenses	104562	103287	101569	1718	100	Remaining in bank to keep alive the account
D. Vehicle hire and fuel, oil & maintenance	21000	19425	19425	0	100	
E. Training/workshop/seminar etc.	0	0	0	0	0	
F. Publications and printing	100000	85000	0	85000	0	Returned to NATP for PCR printing
G. Miscellaneous	60,000	59465	57965	1500	100	Remaining in bank to keep alive the account
H. Capital expenses	2,78,800	2,78,800	2,78,800	0	100	

D. Achievement of Sub-project by objectives: (Tangible form)

Specific objectives of the sub-project	Major technical activities performed in respect of the set objectives	Output(i.e. product obtained, visible, measurable)	Outcome(short term effect of the research)
1. Introgress heat tolerant QTL (<i>qHTSF4.1</i>) in to Bangladeshi mega rice varieties.	Hybridization for introgression of <i>qHTSF4.1</i> QTL into BRR1 dhan48 and BRR1 dhan58	32 F ₁ seed (background BRR1 dhan48) and 43 F ₁ seed (background BRR1 dhan58) were produced	Hybridization successful
	F ₁ Confirmation through marker of BRR1 dhan48/N22 and BRR1 dhan58/N22	10 F ₁ (background BRR1 dhan48) and 18F ₁ (background BRR1 dhan58) seeds were produced	F1 confirmation done
	First backcrossing carried out in the confirmed progenies of BRR1 dhan48 and BRR1 dhan58	1534 (613 + 921)BC ₁ F ₁ seed got	First backcrossing successful

Specific objectives of the sub-project	Major technical activities performed in respect of the set objectives	Output(i.e. product obtained, visible, measurable)	Outcome(short term effect of the research)
	BC ₁ F ₁ progenies (BRRIdhan48:N22) were grown in the field and 26 BC ₁ F ₁ progeny selected through marker for second backcrossing BC ₁ F ₁ progenies (BRRIdhan58:N22) grown in the field and 20 BC ₁ F ₁ progeny (BRRIdhan48:N22) selected through marker for second backcrossing	26 progeny selected through marker 20 progeny selected through marker	
	Second Backcrossing was done	590 BC ₂ F ₁ seeds were produced from BRRIdhan48:N22 721 BC ₂ F ₁ seeds were produced from BRRIdhan58:N22	Breeding lines developed which need to be backcrossed with parent for BC ₃ F ₁ and advancement of the lines is necessary to develop fixed heat tolerant line
2. Develop high temperature tolerant lines potential for proposed variety trial 3. Evaluate phenotypic gain of heat tolerant QTL introgression lines against high temperature	From 88 (BC ₂ F ₅)lines 760 lines (BRRIdhan28:368 and BRRIdhan29:392) having fixed QTL loci (<i>qHTSF4.1</i>) were selected phenotypically, QTL reconfirmed by marker and advanced to BC ₂ F ₆ generation BC ₂ F ₆ generation grown for phenotypic evaluation at high temperature.	BC ₂ F ₆ fixed lines developed in the background of BRRIdhan28 and BRRIdhan29	Fixed lines need to be screened out to identify heat tolerant high yielding advanced lines
	Generation advance and selection of progenies of spikelet fertility introgression lines at BC ₃ F ₃ stage of BRRIdhan28 and BRRIdhan29 background	29 lines in the background of BRRIdhan28 and 20 lines in the background of BRRIdhan29 selected phenotypically, confirmed by marker and advanced to BC ₃ F ₄ generation	Further generation advancement is needed to get fixed lines

E. Materials Development/Publication made under the Sub-project:

Publication	Number of publication		Remarks (e.g. paper title, name of journal, conference name, etc.)
	Under preparation	Completed and published	
Technology bulletin/ booklet/leaflet/flyer etc.			
Journal publication			
Information development			
Other publications, if any		2 (two) Report	1) Introgression of heat tolerant QTL (<i>qHTSF4.1</i>)

			<p>into Bangladeshi mega rice varieties through marker-assisted breeding</p> <p>BRRi Annual Report 2017-18 and Annual Research Review 2017-18</p> <p>2) Marker assisted introgression of spikelet fertility loci (<i>qHTSF4.1</i>) from N22 in to two Bangladeshi mega rice variety BRRi dhan28 and BRRi dhan29</p> <p>BRRi Annual Report 2017-18 and BRRi Annual Research Review 2017-18</p>
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F. Technology/Knowledge generation/Policy Support (as applied):

i. Generation of technology (Commodity & Non-commodity)

Commodity, high temperature tolerant rice breeding lines developed need to advance through further backcross followed by selfing.

ii. Generation of new knowledge that help in developing more technology in future

Breeding lines were developed which need further generation advancement

iii. Technology transferred that help increased agricultural productivity and farmers' income

Not yet. It will take time to develop a proposed rice variety tolerant to high temperature

iv. Policy Support

Present achievement is not helpful directly in policy support, but once heat tolerant rice variety is developed it would help policy makers to draw a strategy heading to sustainable food security

G. Information regarding Desk and Field Monitoring

i) Desk Monitoring (description & output of consultation meeting, monitoring workshops/seminars etc.):

Monitoring team	Date(s) of visit	Total visit till date (No.)	Remarks
Desk monitoring by Crops Division, BARC	28 February 2018	one	Progress is satisfactory
Internal Monitoring BRRRI DG, DA, DR, Head FMD, Head TD, Head Agronomy and Focal point	16 April 2018	one	Progress is satisfactory

ii) Field Monitoring (time& No. of visit, Team visit and output):

Monitoring team	Date(s) of visit	Total visit till date (No.)	Remarks
Technical Division/ Unit, BARC	14 May 2018	one	Research work and progress appreciable and necessary to continue
Internal Monitoring by Director General, BRRRI, Director (Administration) BRRRI	29 May 2018	one	Progress is satisfactory
Others Visitors (if any) Head, CSO and PSO of Plant Breeding Division, Head and all scientists of Plant Physiology Division, BRRRI	22 May, 2018	one	CSO and Head (Plant Breeding) suggested to advance the breeding lines for developing fixed lines

H. Lesson Learned/Challenges (if any)

Lesson Learned

- i)
- ii)
- iii)

Challenges

To continue the remaining research work to develop heat tolerant fixed line of rice.

Signature of the Principal Investigator

Date

Seal

Counter signature of the Head of the organization/authorized representative

Date

Seal

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