Annexure – IX

UNIVERSITY GRANTS COMMISSION
BAHADUR SHAH ZAFAR MARG
NEW DELHI – 110 002

PROFORMA FOR SUBMISSION OF INFORMATION AT THE TIME OF SENDING THE FINAL REPORT OF THE WORK DONE ON THE PROJECT

1. **Title of the Project**: Targeted identification of novel stress responsive gene copies in Brassica arisen through gene duplication.
2. **Name And Address Of The Principal Investigator**: Dr. MALAY DAS, Presidency University, 86/1 College Street, Kolkata-700073
3. **Name And Address Of The Institution**: Presidency University, 86/1 College Street, Kolkata-700073
4. **Ugc Approval Letter No. And Date**: 43-68/2014(SR), 17/08/2015
5. **Date Of Implementation**: 16/10/2015
6. **Tenure Of The Project**: 3 years w.e.f. 01/07/2015 to 30/06/2018
7. **Total Grant Allocated**: Rs 13,80,000
8. **Total Grant Received**: Rs 12,75,041
9. **Final expenditure :** 12,74,997.00
10. **Title of the project**: Targeted identification of novel stress responsive gene copies in Brassica arisen through gene duplication
11. **Objectives of the project:** Enclosed (Attachment 2)
12. **Whether objectives were achieved:** Enclosed (Attachment 2)
13. **Achievements from the project:** Enclosed (Attachment 3)
14. **Summary of the findings:** Enclosed (Attachment 4)
15. **Contribution to the society:** Enclosed (Attachment 3)
16. **Whether any ph.d. enrolled/produced out of the project: No**
17. **No. Of publications out of the project:** Attachment 5

Malay Das
(PRINCIPAL INVESTIGATOR)

Dr. Malay Das
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(CO-INVESTIGATOR)

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(Seal) Registrar Presidency University Kolkata

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TITLE OF THE PROJECT: Targeted identification of novel stress responsive gene copies in Brassica arisen through gene duplication

Principal Investigator: Dr. Malay Das, Presidency University, Kolkata

Objectives:

Objective 1: To study the consequence of whole genome duplication and triplication on the diversification of *Brassica rapa/B. napus* stress response genes.

Objective 2: To evaluate the role of dosage effect on the stress tolerance of amphidiploids *B. napus*.

Whether objectives were achieved or not:

Broadly speaking the major objective of this project have either been obtained or close to obtaining. The initial phase of the project observed hurdles with reference to seed germination and viability of *B. napus* germplasms. Therefore, additional experiments had to be conducted employing 100 *B. juncea* germplasms and assaying their salt stress sensitivity in the laboratory as well as field growth conditions to identify tolerant and susceptible genotypes to test the validity of the Bioinformatics predictions. Currently, real-time RTqPCR experiments are being undertaken and expected to be completed along with final data analysis by next few months.

Detailed Progress Report

1. Bioinformatics identification of duplicated gene copies in *Brassica rapa, B. juncea* and *Thellungiella halophila* (=*E. salsugineum*)

Thirteen important stress responsive *Arabidopsis thaliana* genes were selected to check their copy numbers in *B. rapa*, *B. juncea* and *T. halophila* genomes. BLAST-P analysis was performed in Phytozome v10.2 (https://phytozome.jgi.doe.gov/pz/portal.html) using *A. thaliana* proteins as query to check their copy numbers in *B. rapa* and *T. halophile* genomes. For *B. juncea* BLAST-p analysis was performed in BRAD genome database (http://brassicadb.org/brad/index.php). Cut-off values used for this analysis were identity ≥60% and e value $\leq e^{-5}$, average length coverage of target gene against query gene ≥50%. Out of the thirteen genes used as query, one-to-many situations were observed for all the genes except *NIG1* (Table 1). Therefore *NIG1* was excluded for any further analyses.

2. Comparison of gene model of *B. rapa, B. juncea* and *T. halophila* homologs to that of *A. thaliana*

Gene models of the duplicated gene copies were predicted using tools avalable in Phytozome and BRAD genome database and were compared to *A. thaliana* to predict whether they could point-out any major loss or gain of exon regions that may affect the overall functionality of the proteins (Table 1). For example, if there is an occurrence of non- functionalization, then premature insertion of stop codon would have been arisen. Similarly, in case of a neo-fuctionalization an addition or loss of an important functional domain is expected to occur. One such situation has been observed in case of *TH.HKT1.3* and *BJ.HKT1.4* where exon loss had led to loss of three conserved domains- $M1_D$, $F2_D$ and $M2_D$ (Figure 1).

Table 1. Important *A. thaliana* osmotic stress response genes and identification of their multiplicated homologs in *B. rapa* and *T. halophila.*

Gene	Gene name	Identifier name	CDS length in amino acid
HKT1 (HIGH-AFFINITY K^+ TRANSPORTER 1)	*AT.HKT1*	*AT4G10310*	506
	BR.HKT1.1	*BR02G27530*	506
	BR.HKT1.2	*BR09G17490*	506
	BR.HKT1.3	*BR09G17470*	503
	BR.HKT1.4	*BR03G25550*	506
	BJ.HKT1.1	*BjuA007955*	504
	BJ.HKT1.2	*BjuB019475*	493
	BJ.HKT1.3	*BjuA034177*	493
	BJ.HKT1.4	*BjuA011236*	445
	TH.HKT1.1	*Thhalv10028595m*	505
	TH.HKT1.2	*Thhalv10028594m*	506
	TH.HKT1.3	*Thhalv10028767m*	354
SIZ1`	*AT.SIZ1*	*AT5G60410*	873
	BR.SIZ1.1	*BR10G13020*	873
	BR.SIZ1.2	*BR02G11620*	873
	BR.SIZ1.3	*BR06G18930*	870
	BJ.SIZ1.1	*BjuA038745*	839
	BJ.SIZ1.2	*BjuB034136*	835
	BJ.SIZ1.3	*BjuA006543*	845
	BJ.SIZ1.4	*BjuA022986*	852
	BJ.SIZ1.5	*BjuB018234*	1374
	TH.SIZ1	*Thhalv10012623m*	885
	TH.SIZ2	*Thhalv10002397m*	869
	TH.SIZ1.3	*Thhalv10012660m*	859
HARDY	*AT.HRD*	*AT2G36450*	184
	BR.HRD1.1	*BR04G21830*	179
	BR.HRD1.2	*BR03G18020*	187
	BJ.HRD1.1	*BjuA042562*	183
	BJ.HRD1.2	*BjuB016312*	183
	THHRD.1	*Thhalv10017537m*	190
ATOST1 (OPEN STOMATA 1)	*ATOST1*	*AT4G33950*	362
	BROST1.1	*BR01G04190*	362
	BROST1.2	*BR03G53840*	362
	BROST1.3	*BR07G11570*	357
	BJOST1.1	*BjuB020865*	362
	BJOST1.2	*BjuA011773*	362
	BJOST1.3	*BjuB038334*	319
	BJOST1.4	*BjuA010530*	361
	THOST1.1	*Thhalv10004469m*	361

	THOST1.2	*Thhalv10025907m*	287
	THOST1.3	*Thhalv10004481m*	287
ATCBL10 (CALCINEURIN B-LIKE PROTEIN 10)	*ATCBL10*	*AT4G33000*	256
	BRCBL10.1	*BR08G14810*	246
	BRCBL10.2	*BR03G53140*	258
	BRCBL10.3	*BR01G04930*	211
	BJCBL10.1	*BjuA003297*	195
	BJCBL10.2	*BjuA016230*	189
	THCBL10.1	*Thhalv10026019m*	259
	THCBL10.2	*Thhalv10028908m*	251
APX2	*ATAPX2*	*AT5G63980*	407
	BRAPX2.1	*BR06G22010*	349
	BRAPX2.2	*BR09G06670*	358
	BJAPX2.1	*BjuB020593*	406
	BJAPX2.2	*BjuB039601*	406
	THAPX2.1	*Thhalv10004312m*	412
ATSAT32 (SALT-TOLERANCE 32)	*ATSAT32*	*AT1G27760*	441
	BRSAT32.1	*BR09G18370*	442
	BRSAT32.2	*BR08G22160*	421
	BJSAT32.1	*BjuA034971*	442
	THSAT32.1	*Thhalv10007663m*	438
	THSAT32.2	*Thhalv10009506m*	408
	THSAT32.3	*Thhalv10001793m*	429
	THSAT32.4	*Thhalv10017670m*	416
	THSAT32.5	*Thhalv10017549m*	418
ATLSM5	*ATLSM5*	*AT5G48870*	88
	BRLSM5.1	*BR02G35090*	89
	BRLSM5.2	*BR09G04750*	89
	BJLSM5.1	*BjuA008359*	89
	BJLSM5.2	*BjuB007714*	53
	THLSM5.1	*Thhalv10005202m*	88
439ABI5 (ABA INSENSITIVE 5)	*ATABI5*	*AT2G36270*	442
	BRABI5.1	*BR05G08750*	438
	BRABI5.2	*BR04G21670*	396
	BJABI5.1	*BjuA018972*	442
	BJABI5.2	*BjuB041699*	439
	BJABI5.3	*BjuO002525*	439
	THABI5.1	*Thhalv10016668m*	439
	THABI5.2	*Thhalv10016671m*	439
ABA1 (ABA DEFICIENT 1)	*ATABA1*	*AT5G67030*	667
	BRABA1.1	*BR07G11430*	664
	BRABA1.2	*BR09G08410*	654
	BJABA1.1	*BjuB020853*	723
	BJABA1.2	*BjuA011762*	668

	THABA1.1	*Thhalv10003769m*	667
ABI1 (ABA INSENSITIVE 1)	*ATABI1*	*AT4G26080*	434
	BRABI1.1	*BR01G15890*	425
	BRABI1.2	*BR08G17450*	422
	BRABI1.3	*BR03G48970*	413
	BRABI1.4	*BR10G10210*	422
	BJABI1.1	*BjuA004393*	415
	BJABI1.2	*BjuA013512*	411
	THABI1.1	*Thhalv10025224m*	439
	THABI1.2	*Thhalv10013611m*	430
ATMYB15 (MYB DOMAIN PROTEIN 15)	*ATMYB15*	*AT3G23250*	285
	BRMYB15.1	*BR03G37520*	286
	BRMYB15.2	*BR07G05310*	285
	BRMYB15.3	*BR01G27980*	281
	BRMYB15.1	*BjuA012756*	286
	BRMYB15.2	*BjuA025680*	279
	THMYB15.1	*Thhalv10022110m*	285

Table 2. Morphological and biochemical parameters studied to assess the sensitivity of 100 *Brassica juncea* germplasms towards salt stress using field growth conditions.

Sr.no	Parameters	Tissue stages used	Procedures used
1	Fresh shoot biomass	Shoot	Fresh weight of the harvested above ground shoot tissues
2	Shoot dry biomass	Shoot	Harvested above ground shoot tissues were oven-dried at 70°C overnight prior to obtaining their weight.
3	Shoot length	Shoot	The length of 21 days old seedlings
4	chlorophyll SPAD value	Leaf tissue	Chlorophyll SPAD measured using 21 days old seedlings
5	Number of leaves	Leaf tissue	counted manually
6	Total number of seeds per silique valve	Flowering stage	counted manually
7	Total number of silique per plant	Flowering stage	counted manually
8	Silique length	Flowering stage	counted manually
9	Number of branches	Flowering stage	counted manually
10	Proline	Vegetative leaf tissue	As per the procedures of Bates et al(1973)

11	Estimation of Na^+ and K^+ content	Leaf tissue	1 ml of sap was diluted with deionized water to a volume of 5ml. After calibrating the LAQUAtwin Potassium Ion meter according to manufacturer's instructions, 1ml of the diluted sap were placed into the sensor of LAQUAtwin Potassium Ion meter to determine the K^+ concentration. The Na^+ estimation was also done using similar procedures.

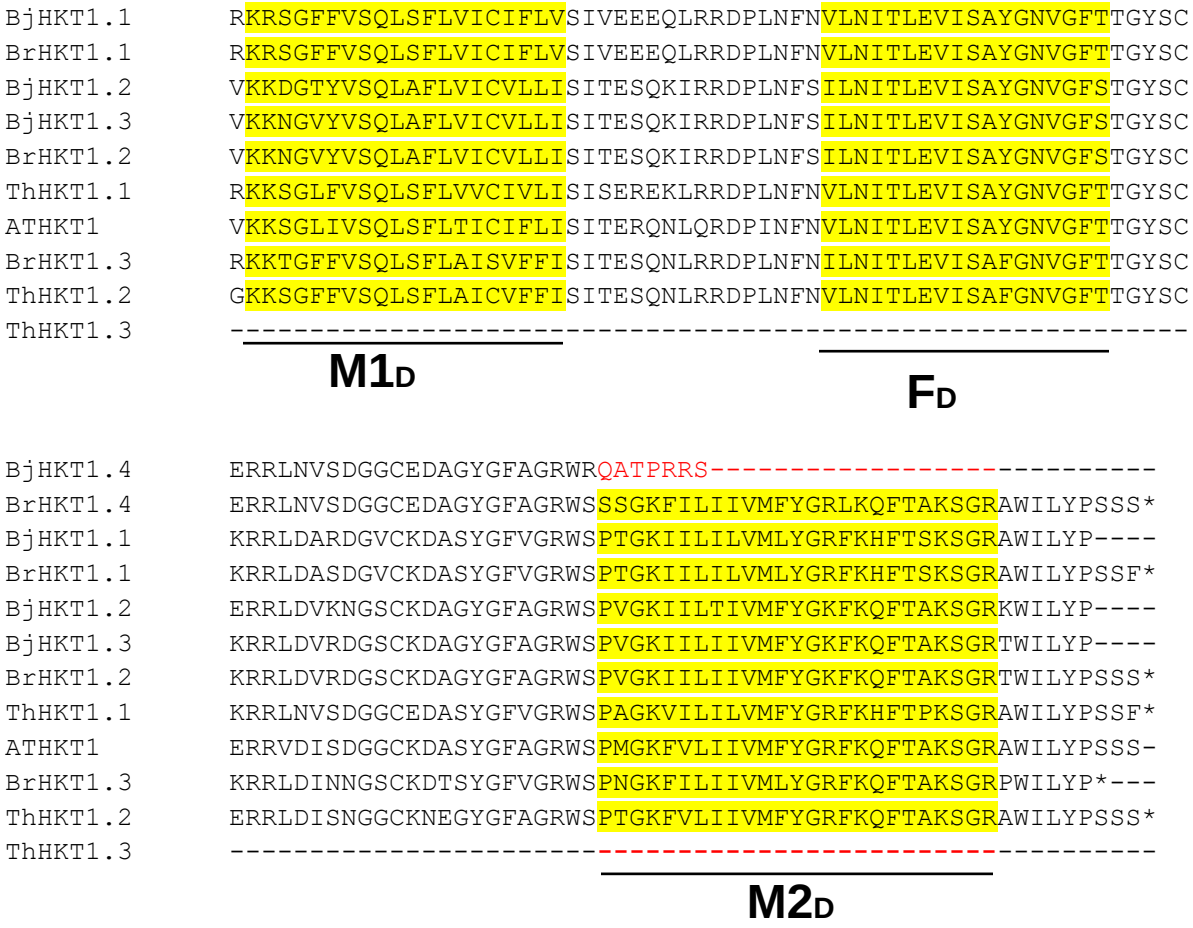

Figure 1. Loss of conserved domains ($M1_D$, F_D and $M2_D$) in *TH.HKT3* and *BjHKT1.4 homolog*.

3. Cis-elements analyses for the duplicated copies of *B. rapa, B.juncea* and *T. halophila*

Cis-element prediction was done using the Plant care database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). Approximately 1000 bp upstream region from ATG was downloaded from Phytozome and BRAD genome databases and were used as query sequences in the Plant Care database. Cis-elements which had proven functional role specifically towards abiotic stresses were selected. Major cis-elements selected for this analyses were abscisic acid responsive element (*ABRE*), heat shock elements (*HSE*), TC rich repeat which is involved in defense and stress response and MYB binding site (MBS), which is a drought inducible element. The total number of such cis elements present in the promoter region of a homolog was recorded to conclude on the enrichment of cis elements among various closely related hpmologs. For example in TH.HKT1.2, a total of 11 cis elements were found, while in case of *AT.HKT1* it was only 3. This gain of cis elements may

provide enhanced stress tolerance to *T. halophila*. However, further confirmation of this hypothesis can be done using qRT-PCR analyses.

4. Stress sensitivity assay of *B. juncea* germplasms in the laboratory and field conditions

In order to evaluate the role of dosage effect, the amphidiploid plant proposed for this study was *B. napus*. However, multiple experimental hurdles such as asynchronous germination of seeds or no germination arose while working on *B. napus* germplasms. Therefore, as an alternative 100 *B. juncea* germplasms, which is also an amphidiploid *Brassica* was obtained from ICAR Central Soil Salinity Research Institute, Karnal and ICAR-Directorate of Rapeseed Mustard Research, Bharatpur. Many of these germplasms previously demonstrated contrasting phenotype with respect to their sensitivity to salt stress (Kumar et al., 2009). However, since stress sensitivity is always subjective to the local environmental conditions, it was imperative for us to perform an independent salt stress sensitivity assay both under laboratory as well as field settings.

In order to perform assay in the laboratory conditions, seeds were surface sterilized in 70% ethyl alcohol, stratification was done for two days and then placed on solid media for germination. Different treatments for both salinity and drought were used. For salinity assay, 75 mM and 150mM NaCl was supplemented with MS media, while for drought assays 100 mM and 200 mM mannitol was added to MS media. Approximately 20 seeds/plate were used for this assay. Data were obtained after 10 days of germination. Measurement of Root and Hypocotyl length was done using the IMAGE J software. The field assay was carried out during December 2017 to April 2018. The saline field was located in Gosaba (22.1652° N, 88.8079° E), while the control, non-saline field was located in Baruipur (22.3597° N, 88.4318° E). After 3 weeks of germination, vegetative tissues were collected to assay 11 vegetative parameters such as fresh and dry biomass, biochemical parameters such as proline, sodium and potassium content and reproductive parameters such as number of siliques per plant and total number of seeds were obtained (Table 2).

Tolerant genotypes predicted on the basis of one parameter did not overlap to that of the other predictions. For example B. juncea RH-781 was predicted as the most tolerant genotype based on the analyses on sodium and potassium content of shoot tissues, while it was B. juncea NC1-jhumka on the basis of proline content in shoots, B. juncea NPJ-200 for dry biomass of shoot tissues, B. juncea RW-46-6-3 for total number of seeds per plant and B. juncea Sitara- Srinagar on the basis of number of siliques per plant (Figure 2, Das Laha et al., 2018).

5. Stress experiment for tissue harvesting of *B. rapa and B. juncea* for assaying expression pattrens of the targeted gene copies

All the selected genotypes plants were grown hydroponically in plant growth room at 21^{O}C temperature, 50-55% of humidity and 70-80 $umolm^{-2}$ sec^{-1} light intensity (Das et al., 2016). Two to three weeks old plants were subjected to 100 mM and 150 mM NaCl as well as 200 mM mannitol treatments. Shoot and root tissues were harvested separately after 8h and 24h post treatments from both the treated as well as untreated plants. Three sets of biological replicates have been taken by pooling 5 plants each for one biological set.

6. Primer designing, total RNA extraction and real-time RT-qPCR analyses

Gene specific primers for all the twelve duplicated genes, have already been designed and synthesized. Primers were designed with the help of Primer 3 WEB (http://bioinfo.ut.ee/primer3/). PCR ability of these primers have already been done by amplifying them using genomic DNA as template. *ACTIN 2* and *GAPC* genes were selected as reference genes for *A. thaliana* and *T. halophila* (Das et al., 2016; Gong et al., 2005). *GAPDH* and *UBQ10* were selected as reference genes for *B.*

rapa (Qi et al., 2010). Isolation of total RNA and performing real-time RTqPCR analyses will be performed as per the procedure established in the laboratory (Das et al., 2016; Dutta et al., 2018).

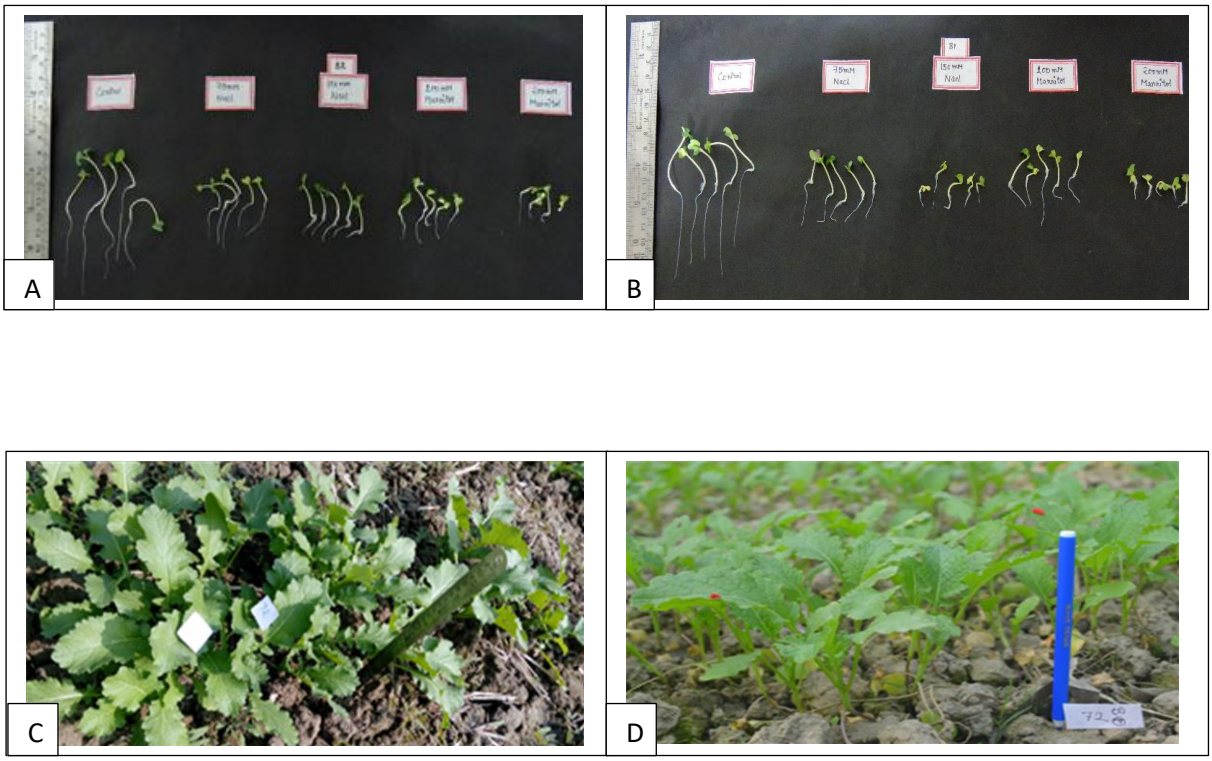

Figure 2. Screening of *B. juncea* in laboratory conditions (A, B), Field conditions (C, D).

References

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Das M, Reichman JR, Haberer G, Welzl G, Aceituno FF, Mader MT, Watrud LS, Pfleeger TG, Gutiérrez R, Schäffner AR and Olszyk D (2010) A composite transcriptional signature differentiates responses towards closely related herbicides in Arabidopsis thaliana and Brassica napus. Plant Molecular Biology 72(4-5): 545-56.

Das M, Haberer G, Panda A, Das Laha S, Ghosh T C, Schäffner A R (2016) Expression pattern similarities support the prediction of orthologs retaining common functions after gene duplication events. Plant Physiol 171(4): 2343-2357.

Das Laha S, Naskar AJ, Sarkar T, Guha S, Mondal HA, Das M (2018) Field based stress phenotyping of crops and the future scopes of high throughput plant phenotyping to redefine research in this area (submitted).

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Gong Q, Li P, Ma S, Rupassara, SI, Bohnert JH (2005) Salinity stress adaptation competence in the extremophile *Thellungiella halophila* in comparison with its relative *Arabidopsis thaliana.* The Plant Journal 44: 826–839.

Xi J, Yu S, Zhang F, Shen X, Zhao Y, Yu Y, Zhang D (2010) Reference Gene Selection for Real-Time Quantitative Polymerase Chain Reaction of mRNA Transcript Levels in Chinese Cabbage (*Brassica rapa* L. ssp. pekinensis). Plant Mol Biol Rep 28:597–604.

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Achievements from the project:

1. Man Power Development: One project fellow (Ms. Shayani Das Laha) was trained in this project, obtained knowledge in the field of bioinformatics and plant genomics and is currently pursuing PhD thesis studies.

2. Publications: Two research publications (one published and one submitted)

Das M, Haberer G, Panda A, Das Laha S, Ghosh T C, Schäffner A R (2016) Expression pattern similarities support the prediction of orthologs retaining common functions after gene duplication events. **Plant Physiology** 171(4): 2343-2357.

Das Laha S, Naskar AJ, Sarkar T, Guha S, Mondal HA, Das M (2018) Field based stress phenotyping of crops and the future scopes of high throughput plant phenotyping to redefine research in this area (Under review), Invited contribution to a book entitled "**Intelligent Image Analysis for Plant Phenotyping**" to be edited by A. Samal, and S. Das Choudhury, published by CRC Press/Taylor & Francis Group

3. Identification of salt stress tolerant germplasms: Comparison of the overall performance of 104 germplasms of *Brassica juncea* in the saline and non-saline soil growth conditions had identified *B. juncea* B-85 (Seeta), RW-351 (Bhagarathi) and CS 58 as the putatively tolerant germplasms.

4: Identification of a few novel allelic variants conferring putative tolerance to salt stress.

Contribution to the society:

Brassicaceae consists of the worlds most economically important crops and is of high importance for India. Identification of the putatively tolerant germplasms and Understanding the evolutionary fate of stress response genes in *Brassica juncea* could help us to identify novel genes that could potentially be utilized for genetic crop improvement in future

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Attachment 4

Summary of the findings

Abiotic stress severely affects agricultural crop productivity world-wide. Since our objective was to identify the effects of gene duplication in abiotic stress tolerance in Brassica, a reference tolerant Brassica genotype for assessment of stress tolerance mechanism was required . As *Brassica. juncea* is an economically important crop and is also know to contain various tolerant genotypes. Therefore, a cumulative study which included study in both laboratory and field settings was conducted on 104 *Brassica juncea* and their responses to salt stress was assessed using diverse morphological and biochemical parameters. The finding identifies the germplasms selected to show maximum tolerance for different parameters. For example *B. juncea*RH-781 showed highest tolerance for Sodium and potassium content of shoot tissues, *B. juncea*NC1-jhumka showed maximum tolerance for proline content in shoot tissues,*B. juncea* NPJ-200 for dry biomass of shoot tissues, *B. juncea*RW-46-6-3 for total number of seeds per plant ,*B. juncea*Sitara- Srinagar showed best tolerance in case of number of siliques per plant. In the *insilico* experiments comparison of gene model of homologs *of B. rapa, B.juncea and T.halophila* to that of *A. thaliana* predicted out many major loss or gain of exon regions that may affect the overall functionality of the proteins. For example in case of *TH.HKT1.3* and *BJ.HKT1.4* exon loss had led to loss of three conserved domains- $M1_D$, $F2_D$ and $M2_D$. Study of the major cis-elements responsive to abiotic stress (ABRE, HSE,MYB, MBS) also provides major cues which helped in the identification of important gene copy responsible for providing stress tolerance. For example in TH.HKT1.2 total of 11 cis elements were found in comparison with 3 in *AT.HKT1*. This gain of cis elements possible could provide stress tolerance to *T. halophila*.

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Attachment 5

Number of publications

1. Das M, Haberer G, Panda A, Das Laha S, Ghosh T C, Schäffner A R (2016) Expression pattern similarities support the prediction of orthologs retaining common functions after gene duplication events. **Plant Physiology** 171(4): 2343-2357.

2. Das Laha S, Naskar AJ, Sarkar T, Guha S, Mondal HA, Das M (2018) Field based stress phenotyping of crops and the future scopes of high throughput plant phenotyping to redefine research in this area (Under review), Invited contribution to a book entitled "**Intelligent Image Analysis for Plant Phenotyping**" to be edited by A. Samal, and S. Das Choudhury, published by CRC Press/Taylor & Francis Group

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