Deciphering the Role of *Oryza sativa* Heat Shock Transcription Factors During Leaf Senescence.

DETAILED UGC-MRP PROJECT COMPLETION REPORT

UGC Reference No.: 41-512/2012
Period of Report: 17.7.2012 to 31.12.2015

Submitted by:
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Annexure -VIII

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

Final Report of the Work Done on the Major Research Project.

1. **Project report No.**: 2nd and Final

2. **UGC Reference No.:** F. No. 41-512/2012

3. **Period of report**: from 17.7.2012 to 31.12.2015

4. **Title of research project**: Deciphering the role of *Oryza sativa* heat shock transcription factors during leaf senescence.

5. **(a) Name of the Principal Investigator**: Dr. Surekha Katiyar Agarwal

(b) Department: Plant Molecular Biology

(c) University/College where work has progressed: University of Delhi South Campus

6**. Effective date of starting of the project**: 17.7.2012

7. **Grant approved, and expenditure incurred during the period of the report**:

a. Total amount approved: Rs. 10.33 lakhs

b. Total expenditure: Rs. 9,50,728/-

c. Report of the work done: Please see attached report.

i. Brief objective of the project:

1) To establish the staging of rice plant growth for natural or developmental senescence and optimize the conditions for induced leaf senescence in rice.
2) To perform expression profiling of different members of heat shock transcription factor family in rice during natural and induced leaf senescence.
3) To identify the rice HSFs which are differentially regulated during senescence.
4) To collate the expression data and determine the Hsfs, which are specifically induced during natural and induced senescence and also find out which Hsfs are common to both the pathways.
5) To generate transgenic lines for altered expression levels of selected HSF so as to understand the role of HSF during senescence.

ii. Work done so far and results achieved and publications, if any, resulting from the work:

Senescence is an age-dependent degeneration process leading to death of an organism. Leaf senescence is a highly orchestrated deterioration process that constitutes the final step in leaf development. It is clearly evident that leaf senescence is a critical regulator of overall fitness of the plants thus facilitating the optimal production of offspring. Like other plant developmental processes, leaf senescence is a genetically controlled process. Global gene expression analysis of leaf undergoing senescence identified more than 800 senescence associated genes (SAGs) in *Arabidopsis thalian*. Transcription factors are the master regulators of almost all the biological processes in a cell. Differential expression analysis studies have shown that around 100 genes encoding putative transcription factors are upregulated during leaf senescence, including NAC,

WRKY, C2H2 type zinc finger proteins, bZIP, CCAAT binding, MADS, MYB, kinase and HSF proteins.

In rice, 26 members comprise HSF family of transcription factors and they have been widely implicated in regulating abiotic stress response. However, there has been no evidence that these HSFs regulate flag leaf senescence in rice. With an aim to elucidate the role of Heat Shock Transcription Factors (HSF) in regulating senescence the objectives of this project were proposed. To begin with staging of senescence was established with two systems: natural senescence of flag leaves and induced senescence by dark in detached leaves. Chlorophyll content and expression of few SAGs was measured during the progression of senescence and different stages were marked for analyses. Quantitative PCR-based expression profiling showed that 17 and 13 HSFs exhibited significant change in transcript accumulation in at least one stage of flag leaf and dark-induced senescence. Comparative analysis showed that 10 rice HSFs were exclusively upregulated in flag leaf senescence, while no HSF was found to be specifically induced in dark-induced senescence. On the other hand, 4 and 7 HSFs were specifically downregulated in flag leaf and induced senescence. Interestingly, 3 HSFs and 4 HSFs were upregulated and downregulated in both types of senescence, respectively.

Comprehensive expression analyses indicated the possible role of HSFs in regulating leaf senescence in rice. Based on our analyses we selected two HSFs: OsHSFA7a and OsHSFC1b for understanding their role in senescence in rice using transgenic approach. Transgenic rice lines with alerted expression of both HSFs were generated followed by their molecular analyses. Constitutive overexpression of OsHSFA7a in rice resulted in stunted growth and impaired spikelet fertility. On the other hand, silencing of OsHSFA7a resulted in higher culm number and increased seed number. Constitutive overexpression of OsHSFC1b caused robust vegetative growth after active tillering phase of plant development in terms of increased culm length, leaf length and flag leaf length. While silencing of OsHSFC1b resulted in decline in all these morphological traits and retarded growth of rice. Changes in flag leaf senescence and dark-induced leaf senescence were observed due to altered expression of OsHSFC1b. Our results strongly suggest the role of OsHSFC1b as negative regulator of leaf senescence in rice. It would be worthwhile to dissect the exact role of OsHSFC1b by identifying the senescence-associated genes which are regulated by OsHSFC1b and find the protein interacting partners to provide an insight into its mechanism of action.

iii. Has the progress been according to original plan of work and towards achieving the objective: Yes

iv. Please indicate the difficulties, if any, experienced in implementing the project: None

v. If project has not been completed, please indicate the approximate time by which it is likely to be completed: Not Applicable (work completed)

vi. If the project has been completed, please enclose a summary of the findings of the study. *One bound copy of the final report of work done may also be sent to University Grants Commission:* Please see enclosed report.

vii. Any other information which would help in evaluation of work done on the project. At the completion of the project, the first report should indicate the output, such as:

(a) Manpower trained: One (Mr. Jyothish M.S.)

2012. Structural and Functional Diversity of Plant Heat Shock Factors. In: Pandey G. K. (Guest Ed.). Plant Stress: Stress mediated signaling in plants. Global Science Books Japan, pp898-96.]

A poster was presented in a national conference wherein the findings related to this project were included [Investigating the role of heat shock transcription factors in leaf senescence in rice. Presented at **National Conference on Plant Science Research**, organized by Society of Plant Research at Department of Botany, University of Delhi, India (February 5-7, **2016**). Jyothish Madambi, Balaji Mani, Manu Agarwal, Surekha Katiyar-Agarwal].

Since the project work involved generation of transgenic rice lines with modified expression of selected HSFs, efforts were made to generate homozygous lines of these lines which took almost 2 years. Detailed molecular and phenotypic analyses were carried out with these lines and the results are being compiled as a manuscript.

(d) Other impact, if any: Not applicable

Surekha

SIGNATURE OF THE PRINCIPAL INVESTIGATOR

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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:** Deciphering the role of *Oryza sativa* heat shock transcription factors during leaf senescence.

2. **Name and address of the principal investigator:** Dr. Surekha Katiyar Agarwal

3. **Name and address of the institution:** Department of Plant Molecular Biology, University of Delhi South Campus, Benito Juarez Road, New Delhi-110021.

4. **UGC approval letter no. and date:** F. No. 41-512/2012 dated 17.7.2012

5. **Date of implementation:** 17.7.2012

6. **Tenure of the project:** 3 years, 6 months (17.7.2012 to 31.12.2015)

7. **Total grant allocated:** Rs. 10.33 lakhs

8. **Total grant received:** Rs. 9.57 lakhs

9. **Final expenditure:** Rs. 9,50,728/-

10. **Title of the project:** Deciphering the role of *Oryza sativa* heat shock transcription factors during leaf senescence.

11. **Objectives of the project:**

1) To establish the staging of rice plant growth for natural or developmental senescence and optimize the conditions for induced leaf senescence in rice.
2) To perform expression profiling of different members of heat shock transcription factor family in rice during natural and induced leaf senescence.
3) To identify the rice HSFs which are differentially regulated during senescence.
4) To collate the expression data and determine the Hsfs, which are specifically induced during natural and induced senescence and also find out which Hsfs are common to both the pathways.

5) To generate transgenic lines for altered expression levels of selected HSF so as to understand the role of HSF during senescence.

12. **Whether objectives were achieved:** Yes, all the work pertaining to objectives proposed was completed. The objective-wise achievements are as follows:

1) *To establish the staging of rice plant growth for natural or developmental senescence and optimize the conditions for induced leaf senescence in rice*: Based on chlorophyll content, different stages of flag leaf senescence in field-grown plants were identified for the expression profiling of *HSFs*. Fully expanded flag leaves at panicle development stage were harvested and considered as unsenesced (US) control. The amount of chlorophyll present in the US leaves was considered as 100% and relative to it, flag leaves with 60-80%, 45-60% and 30-45% chlorophyll content were considered in early senescence (ES), mid senescence (MS) and late senescence (LS) stages, respectively. To validate the chlorophyll content-based staging of senescence, steady state levels of two senescence-associated genes, *RED CHLOROPHYLL CATABOLITE REDUCTASE* (*RCC*) and *WRKY6* was performed in the US, ES, MS and LS flag leaves. Transcripts of these marker genes were found to gradually accumulate with the progression of senescence in flag leaves, wherein RCC reductase exhibited higher level of induction at all the stages as compared to *WRKY6*. The inverse correlation was observed in content of chlorophyll and expression of SAGs.

   To artificially induce senescence, leaf pieces from the third leaf of seventeen days-old-seedlings were subjected to dark treatment for 24, 48 and 72 hours. Since chlorophyll degradation is the first visible morphological change during senescence, chlorophyll content was used as a parameter to confirm the initiation of senescence. Also, expression levels of senescence marker genes were checked to check the progression of senescence in dark-treated tissues. A gradual decrease and increase in chlorophyll content and SAGs expression levels was observed in the senescing tissues, respectively (**Figure 2**). Nonetheless the two SAGs used as markers, were highly induced as the senescence progressed.
2) *To perform expression profiling of different members of heat shock transcription factor family in rice during natural and induced leaf senescence*: Expression profiling of rice HSFs was performed by quantitative PCR. It was observed that 17 of 26 OsHSFs were differentially expressed during the flag leaf senescence in rice. On the other hand, during dark-induced senescence 10 HSFs were downregulated and three showed elevation in expression in at least one stage of senescence.
3) *To identify the rice HSFs which are differentially regulated during senescence*: Large number of HSFs were found to be differentially regulated during senescence, both natural and dark-induced. Seventeen and 13 rice HSFs exhibited significant changes in transcript accumulation in at least one stage of natural flag leaf and dark-induced detached leaf senescence, respectively.
4) *To collate the expression data and determine the Hsfs, which are specifically induced during natural and induced senescence and also find out which Hsfs are common to both the pathways*: Ten rice HSFs were exclusively upregulated in flag leaf senescence, while no HSF was found to be specifically induced in dark-induced senescence. On the other hand, four and seven HSFs were specifically downregulated in flag leaf and induced senescence. Interestingly, 3 HSFs and four HSFs were upregulated and downregulated in both types of senescence, respectively.

5) *To generate transgenic lines for altered expression levels of selected HSF so as to understand the role of HSF during senescence*: Based on the results obtained in expression profiling experiments, two HSFs (OsHSFA7a and OsHSFC1b) were selected for generating transgenic rice lines with their altered expression. Both overexpression and silencing transgenic rice lines were generated and their detailed molecular analysis were carried out. Phenotypic analysis for assessing progression of senescence were carried out and it was found that transgenic rice lines with altered expression of OsHSFC1b exhibited significant changes in senescence of flag leaves as well as dark-induced senescence of detached leaves. However, OsHSFA7a transgenic rice lines did not display any change in progression of senescence in flag leaf as well as detached leaves. Our results substantiate the role of OsHSFC1b as a negative regulator of natural and dark-induced senescence in rice.

13. **Achievements from the project:** The major achievements of the present project are:
    1) Comprehensive expression profiling of OsHSFs revealed that the transcript levels of several of HSFs is significantly altered during natural as well as dark-induced senescence.
    2) Alteration in expression of OsHSFC1b resulted in several morphological changes which indicate the possible role of OsHSFC1b in regulating plant development.
    3) Assessment of progression of senescence of flag leaves and detached leaves in HSFC1b transgenic rice lines showed that senescence is significantly altered in these lines. These results substantiate the role of HSFC1b as a negative regulator of natural and dark-induced senescence in rice.

14. **Summary of the findings:** Senescence is an age-dependent degeneration process leading to death of an organism. Leaf senescence is a highly orchestrated deterioration process that constitutes the final step in leaf development. It is clearly evident that leaf senescence is a critical regulator of overall fitness of the plants thus facilitating the optimal production of offspring. Like other plant developmental processes, leaf senescence is a genetically controlled process. Global gene expression analysis of leaf undergoing senescence identified more than 800 senescence associated genes (SAGs) in *Arabidopsis thalian*. Transcription factors are the master regulators of almost all the biological processes in a cell. Differential expression analysis studies have shown that around 100 genes encoding putative transcription factors are upregulated during leaf senescence, including NAC, WRKY, C2H2 type zinc finger proteins, bZIP, CCAAT binding, MADS, MYB, kinase and HSF proteins.

    In rice, 26 members comprise HSF family of transcription factors and they have been widely implicated in regulating abiotic stress response. However, there has been no evidence that these HSFs regulate flag leaf senescence in rice. With an aim to elucidate the role of Heat Shock Transcription Factors (HSF) in regulating senescence the objectives of this project were proposed. To begin with staging of senescence was established with two systems: natural senescence of flag leaves and induced senescence by dark in detached leaves. Chlorophyll content and expression of few SAGs was measured during the progression of senescence and different stages were marked for analyses. Quantitative PCR-based expression profiling showed that 17 and 13 HSFs exhibited significant change in transcript accumulation in at least one stage of flag leaf and dark-induced senescence. Comparative analysis showed that 10 rice HSFs were exclusively upregulated in flag leaf senescence, while no HSF was found to be specifically induced in dark-induced senescence. On the other hand, 4 and 7 HSFs were specifically downregulated in flag leaf and induced senescence. Interestingly, 3 HSFs and 4 HSFs were upregulated and downregulated in both types of senescence, respectively.

senescence and dark-induced leaf senescence were observed due to altered expression of OsHSFC1b. Our results strongly suggest the role of OsHSFC1b as negative regulator of leaf senescence in rice. It would be worthwhile to dissect the exact role of OsHSFC1b by identifying the senescence-associated genes which are regulated by OsHSFC1b and find the protein interacting partners to provide an insight into its mechanism of action.

15. **Contribution to the society:** The project related research work was carried out by a PhD student. The student was trained in basic and advanced molecular biology techniques and plant transformation methods along with assessment of transgenic rice lines. Training of skilled manpower was one of the contributions of this project to the society.

16. **Whether any Ph.D. enrolled/produced out of the project:** Yes, one Ph.D. student (Mr. Jyothish M.S.) worked on this project and reported the results obtained in his thesis as two chapters.

17. **No. of publications out of the project:** Since the project work involved generation of transgenic plants and their analyses, considerable time and efforts went for the generation of homozygous transgenic rice lines. Further detailed experiments were carried out for molecular as well as phenotypic analyses of these homozygous lines. A manuscript is being compiled based on the results obtained and shall be communicated soon. However, a chapter was contributed by the PI and collaborators, and a poster was presented by the student associated with this project, the details of which are:

a) Raxwal V, **Katiyar-Agarwal S,** Agarwal M. **2012**. Structural and Functional Diversity of Plant Heat Shock Factors. In: Pandey G. K. (Guest Ed.). Plant Stress: Stress mediated signaling in plants. Global Science Books Japan, pp898-96.

b) Investigating the role of heat shock transcription factors in leaf senescence in rice. Presented at **National Conference on Plant Science Research**, organized by Society of Plant Research at Department of Botany, University of Delhi, India (February 5-7, **2016**). Jyothish Madambi, Balaji Mani, Manu Agarwal, Surekha Katiyar-Agarwal.

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DETAILED UGC-MRP PROJECT COMPLETION REPORT

Deciphering the role of *Oryza sativa* heat shock transcription factors during leaf senescence.

Introduction

Senescence is an age-dependent degeneration process leading to death of an organism. Leaf senescence is a highly orchestrated deterioration process that constitutes the final step in leaf development. Although leaf senescence is generally associated with organismal death, it is a highly coordinated process involving enhanced catabolism and reduced anabolism. It is clearly evident that leaf senescence is a critical regulator of overall fitness of the plants thus facilitating the optimal production of offspring. Like other plant developmental processes, leaf senescence is a genetically controlled process. Global gene expression analysis of leaf undergoing senescence identified more than 800 senescence associated genes (SAGs) in *Arabidopsis thaliana* (Buchanan-wollaston et al., 2005; van der Graaff, 2006). These genes include different categories which control developmental processes or any other endogenous process, genes that affect response to environmental changes, regulatory genes, genes involved in degradation of regulatory factors associated with senescence ad genes involved in final execution of senescence process (Lim et al., 2003). Changes in gene expression are attributed to the transcriptional control, which are known to play important role in programming the senescence process (Balazadeh et al., 2008). A large number of genes have been identified as regulatory factors that are components of signal perception and transduction pathways during senescence. Transcription factors are the master regulators of almost all the biological processes in a cell. Genome sequence analysis of Arabidopsis reveals existence of at least 15000 transcription factor genes belonging to more than 30 families (Riechmann et al., 2000). Differential expression analysis studies have shown that around 100 genes encoding putative transcription factors are upregulated during leaf senescence, including NAC, WRKY, C2H2 type zinc finger proteins, bZIP, CCAAT binding, MADS, MYB, kinase and HSF proteins (Buchanan-wollaston et al., 2005; Balazadeh et al., 2008).

With an aim to elucidate the role of Heat Shock Transcription Factors (HSF) in regulating senescence the objectives of this project were proposed. Preliminary experiments were carried out to obtain an expression analysis of members of HSF family in Arabidopsis during natural senescence. Among the 21 members of HSFs in Arabidopsis, *HsfA2, HsfA6b, HsfA7a, HsfA7b, HsfA9, HsfB4,* and *HsfB1* showed high level of expression in senescing leaf (leaf 5) as compared to non-senescent leaf (i.e. leaf 6 harvested 40 days after emergence or DAE). On the contrary, *HsfA4a* and *HsfB2b* exhibited decline in expression during senescence. In our studies, a few numbers of Hsfs showed no significant alteration in expression during senescence including HsfB2a, *HsfB3, HsfA1d, HsfA1e, HsfA3, HsfA4c, HsfA5, HsfA6a* and *HsfA8.* All these observations do implicate the role of Hsfs in leaf senescence.

In rice, 26 members comprise HSF family of transcription factors and they have been widely implicated in regulating abiotic stress response (Mittal et al., 2009). However there has been no evidence that these HSFs regulate flag leaf senescence in rice. Since leaf senescence is also regulated by a number of environmental factors and hormones, it would be interesting to study the role of these HSFs in plants which when exposed to abiotic stress or exogenously applied hormone exhibit alteration in senescence pattern. In order to study natural leaf senescence in rice, flag leaf was included in this project. Flag leaf is one of the top three leaves which play important role in biomass production and grain yield in rice (Biswas and Choudhuri, 1978). Moreover, flag leaf has the longest life span, it being the last to senescence during rice development cycle. For induced senescence, dark-induced senescence of leaves was included.

Proposed Objectives of the Project:

1) To establish the staging of rice plant growth for natural or developmental senescence and optimize the conditions for induced leaf senescence in rice.
2) To perform expression profiling of different members of heat shock transcription factor family in rice during natural and induced leaf senescence.
3) To identify the rice HSFs which are differentially regulated during senescence.
4) To collate the expression data and determine the Hsfs, which are specifically induced during natural and induced senescence and also find out which Hsfs are common to both the pathways.
5) To generate transgenic lines for altered expression levels of selected HSF so as to understand the role of HSF during senescence.

Results:

A. Staging of Senescence in Rice

1. Staging of natural flag leaf senescence in rice

Flag leaf is the last leaf to senesce in a cereal plants. In rice, ~90% of the grain yield is contributed by flag leaf photosynthetic production. Based on chlorophyll content, different stages of flag leaf senescence in field-grown plants were identified for the expression profiling of *HSFs*. Fully expanded flag leaves at panicle development stage were harvested and considered as unsenesced (US) control. The amount of chlorophyll present in the US leaves was considered as 100% and relative to it, flag leaves with 60-80%, 45-60% and 30-45% chlorophyll content were considered in early senescence (ES), mid senescence (MS) and late senescence (LS) stages, respectively (**Figure 1 A and B**). To validate the chlorophyll content-based staging of senescence, steady state levels of two senescence-associated genes, *RED CHLOROPHYLL CATABOLITE REDUCTASE* (*RCC*) and *WRKY6* was performed in the US, ES, MS and LS flag leaves. Transcripts of these marker genes were found to gradually accumulate with the progression of senescence in flag leaves, wherein RCC reductase exhibited higher level of induction at all the stages as compared to *WRKY6* (**Figure 1 C, D**). The inverse correlation of chlorophyll content and SAGs

expression during the progression of senescence ratify the staging of natural flag leaf senescence in the present study.

2. Staging of dark-induced senescence in rice leaves

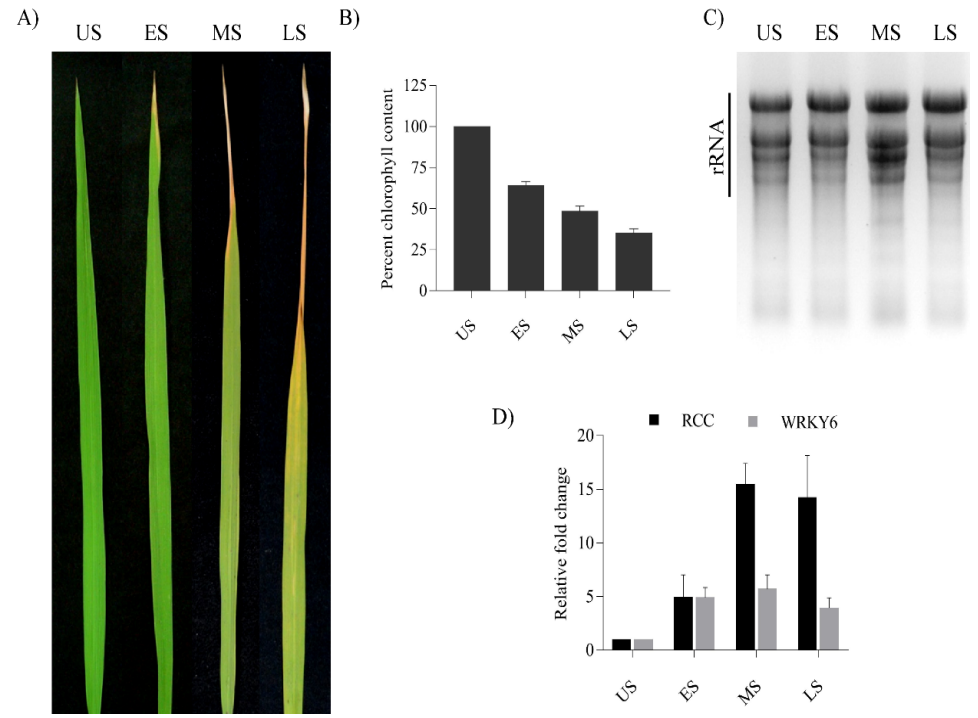


Figure 1: Staging of natural flag leaf senescence in rice. (**A**) Morphological appearance of flag leaf in field grown plants during different stages of leaf senescence. (**B**) Percent chlorophyll content in different stages of flag leaf senescence. (**C**) Qualitative analysis of total RNA using agarose gel electrophoresis. (**D**) Expression profile of senescence-associated genes (SAGs), *red chlorophyll catabolite reductase* (*RCC*) and *WRKY6* at different stages of flag leaf senescence. US: unsenesced flag leaf; ES: early senescence flag leaf; MS: mid-senescence flag leaf; LS: late senescence flag leaf.

Imposition of dark is often employed to induce uniform and rapid senescence in detached leaves of plants. In fact, dark-induced senescence is widely used as a model assay system to study changes associated with senescence (Weaver and Amasino, 2001). In a bid to map the HSFs that are regulated in a similar or dissimilar fashion in natural vs dark induced senescence, expression profiling of *HSF*s was performed in dark-induced detached leaf senescence in rice. To artificially induce senescence, leaf pieces from the third leaf of seventeen days-old-seedlings were subjected to dark treatment for 24, 48 and 72 hours. Since chlorophyll degradation is the first visible morphological change during senescence, chlorophyll content was used as a parameter to confirm the initiation of senescence. Also, expression levels of senescence marker genes were checked to endorse the progression of senescence in dark-treated tissues. A gradual decrease and

increase in chlorophyll content and SAGs expression levels was observed in the senescing tissues, respectively (**Figure 2**). Nonetheless the two SAGs used as markers, were highly induced as the senescence progressed.

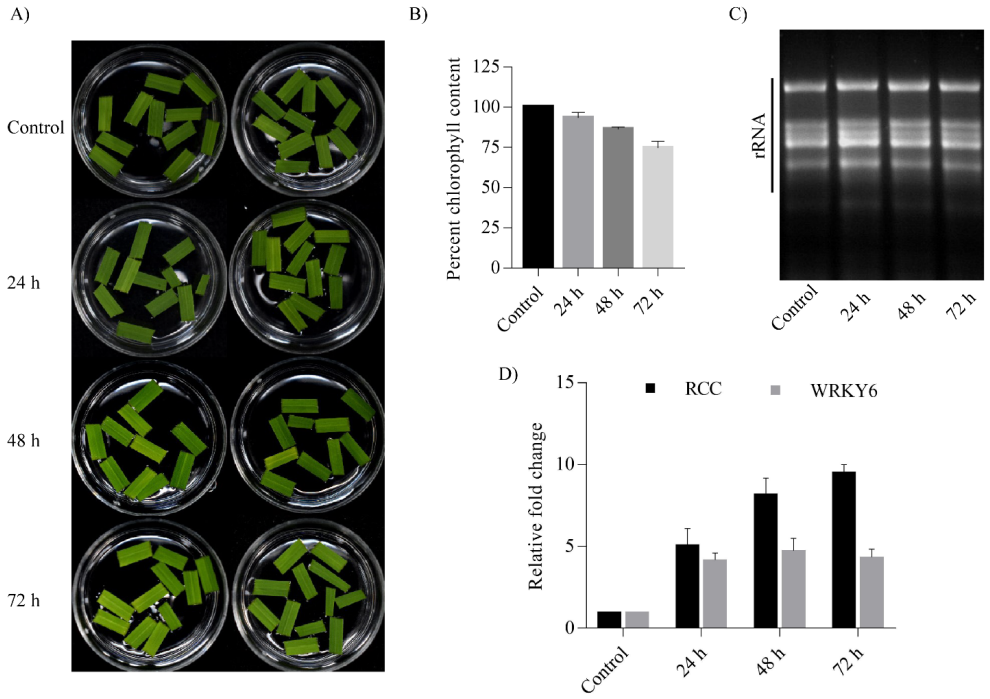


Figure 2: Dark-induced senescence of detached rice leaves. (**A**) Uniform-sized leaf pieces from 3rd leaf of 17 days-old seedlings were floated on water and incubated in dark conditions for 24 h, 48 h and 72 h. (**B**) Percent chlorophyll content at different time points of dark-induced senescence was measured. (**C**) Qualitative analysis of total RNA using agarose gel electrophoresis. (**D**) Expression profile of senescence-associated genes (SAGS), *red chlorophyll catabolite reductase (RCC)* and *WRKY6* during dark-induced leaf senescence.

B. Expression Profiling of *HSFs* During Senescence in Rice

1. Expression profiling of rice *HSFs* during flag leaf senescence

Expression profiling of rice HSFs was performed by quantitative PCR (qPCR). It was observed that 17 of 26 OsHSFs were differentially expressed during the flag leaf senescence in rice (**Figure 3**). Transcripts of two HSFs, *OsHSFA7b* and *OsHSFB2b*, could not be detected in the tissues included in this experiment. Among the upregulated HSF genes, *OsHSFB1a* was induced at early stage of senescence, whereas its levels declined at mid and late stages of senescence. Seven HSFs genes (*OsHSFA1a*, *OsHSFA2a*, *OsHSFA2b*, *OsHSFA4a*, *OsHSFB4a*, *OsHSFB4b* and *OsHSFC2a*) were specifically upregulated at mid stage of senescence. Although *OsHSFC1a* exhibited slight down-regulation in the early stage, the expression levels were significantly elevated in the mid and late stages of senescence. Five

HSFs (*OsHSFA7a*, *OsHSFB4c*, *OsHSFC1b*, *OsHSFC2b* and *Os06g22610*) exhibited upregulation at all the three stages of flag leaf senescence included in this study.

The transcript levels corresponding to three HSFs (*OsHSFA2e*, *OsHSFA3a* and *OsHSFA6b*) declined at all the three stages of senescence when compared with unsenesced flag leaves. It is believed that genes that show upregulation in expression during senescence may be actively participating in regulating the senescence programme. Based on these observations it is believed that several HSFs may be associated with senescence process in flag leaves.

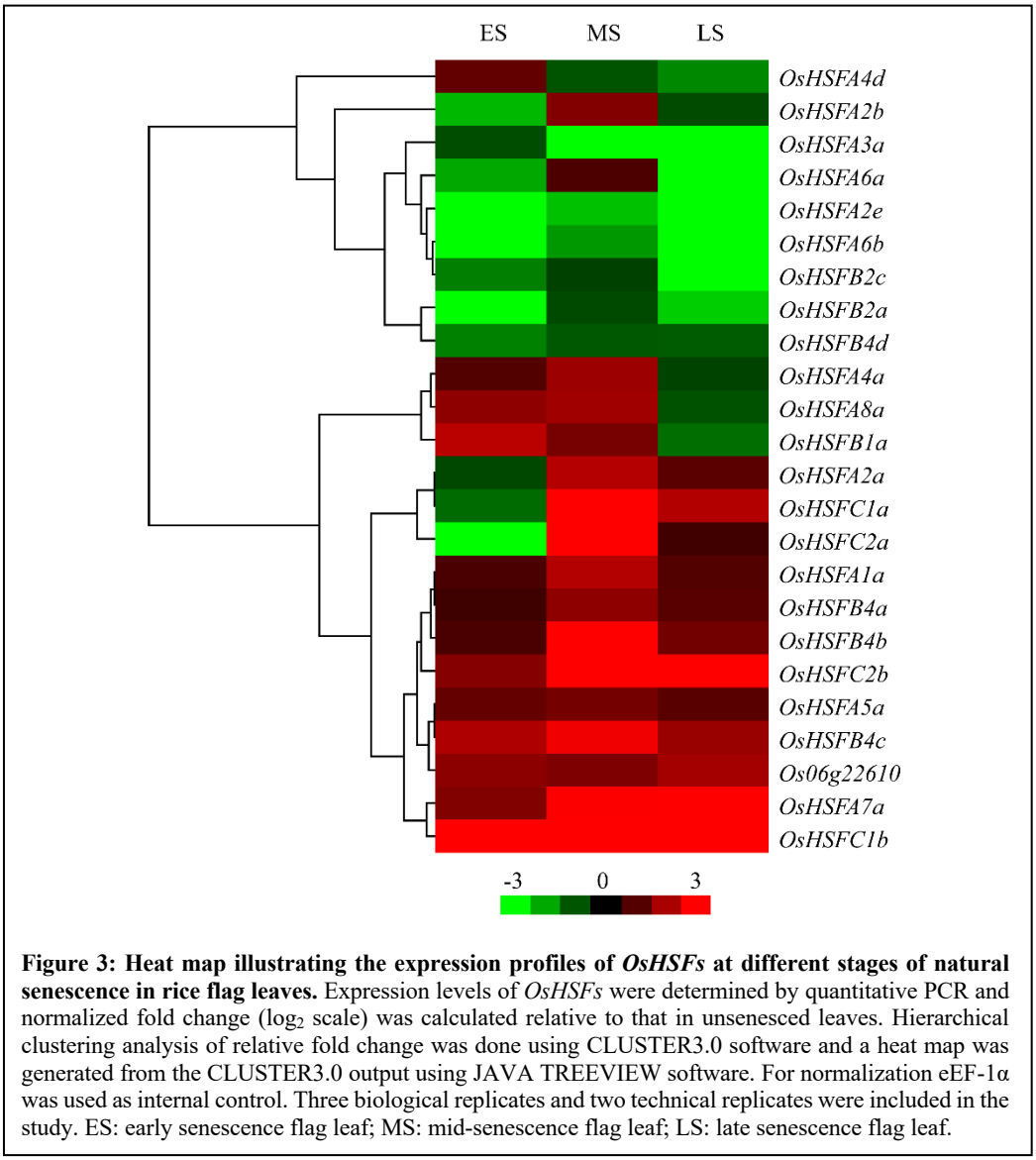


Figure 3: Heat map illustrating the expression profiles of *OsHSFs* at different stages of natural senescence in rice flag leaves. Expression levels of *OsHSFs* were determined by quantitative PCR and normalized fold change (log_2 scale) was calculated relative to that in unsenesced leaves. Hierarchical clustering analysis of relative fold change was done using CLUSTER3.0 software and a heat map was generated from the CLUSTER3.0 output using JAVA TREEVIEW software. For normalization eEF-1α was used as internal control. Three biological replicates and two technical replicates were included in the study. ES: early senescence flag leaf; MS: mid-senescence flag leaf; LS: late senescence flag leaf.

2. Expression profiling of rice *HSFs* during dark-induced senescence

Out of the 26 *HSF* genes tested in this study, five did not show any amplification

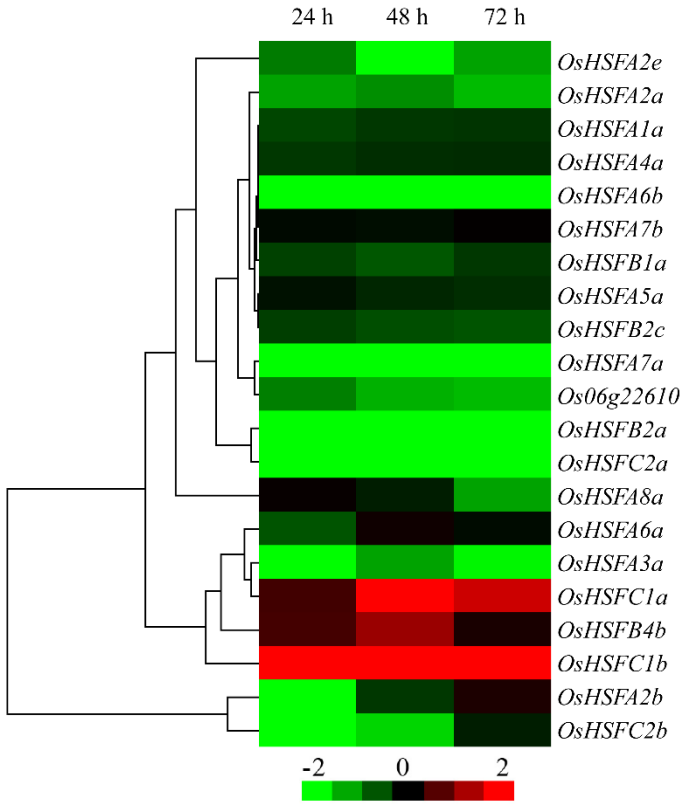


Figure 4: Heat map illustrating the expression profiles of *OsHSFs* during dark-induced senescence in detached rice leaves. Leaf pieces from 3rd leaf of 17 days-old seedlings were floated on water and incubated in dark conditions for 24 h, 48 h and 72 h. Expression levels of *OsHSFs* were determined by quantitative PCR and normalized fold change (log_2 scale) was calculated relative to that in control sample. Hierarchical clustering analysis of relative fold change was done using CLUSTER3.0 software and a heat map was generated from the CLUSTER3.0 output using JAVA TREEVIEW software. For normalization *eEF-1α* was used as internal control. Three biological replicates and two technical replicates were included in the study.

in the dark-induced senescence and therefore their analysis was not performed. However, 10 *OsHSF*s showed significant decline in expression levels at all the stages of dark-induced senescence (**Figure 4**). While the expression levels of *OsHSFB4a* were elevated at 48 h of dark treatment, *OsHSFC1a* exhibited decline in expression at 48 h and 72 h. Remarkably, *OsHSFC1b* exhibited high level of upregulation in expression at all the three stages of dark-induced senescence. In contrast to flag leaf senescence, more number of *HSFs* were down-regulated in dark-induced senescence. Moreover, two *HSF*s, *OsHSFA7a* and *OsHSFC2b,* which were up-regulated in flag leaf senescence were found to be down-regulated in dark-induced senescence. All these results strongly suggest the existence of distinct HSF-mediated gene regulatory mechanisms in natural and dark-induced senescence.

Once the expression of HSFs was accomplished, it was important to identify the HSFs that showed similar/dissimilar expression patterns across the two different senescing systems. Venn diagrams thus generated showed that ten *HSFs (OsHSFA1a, OsHSFA2a, OsHSFA4a, OsHSFA7a, OsHSFA8a, OsHSFB1a, OsHSFB4a, OsHSFB4c, OsHSFC2b,* and *Os06g22610)* were exclusively up-regulated in flag leaf senescence (**Figure 5**). Three *HSF*s (*OsHSFB4b, OsHSFC1a,* and *OsHSFC2b*) were found to be up-regulated in both flag leaf and dark-induced senescence. Interestingly, no *HSFs* were found

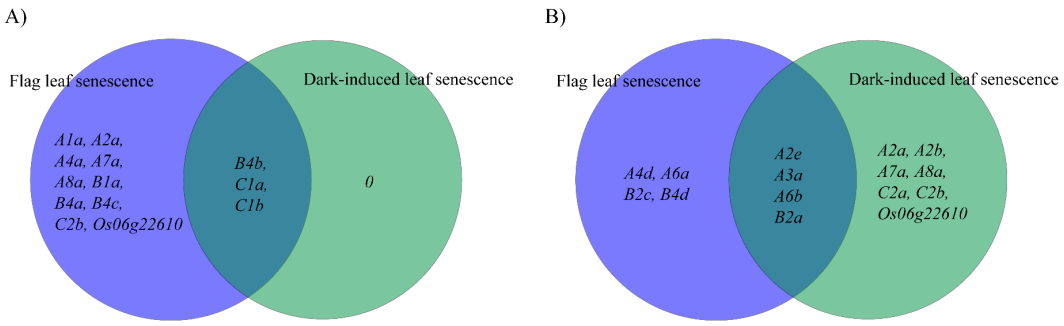


Figure 5: Overlap of rice *HSF* expression profiles in flag leaf senescence and dark-induced senescence. Venn diagram representing overlap of *OsHSFs* exhibiting significant change (log2 ±2-fold or more fold change in log2 scale) in expression in flag leaf, coleoptile and dark-induced senescence. Upregulated (**A**) and downregulated (**B**) genes are represented.

to be exclusively up-regulated in dark-induced senescence. Maximum number of *OsHSF* genes showed down-regulation in dark-induced senescence compared to flag leaf senescence. *OsHSFA4d, OsHSFA6a, OsHSFB2c* and *OsHSFB4d* displayed down-regulation exclusively in flag leaf senescence, while *OsHSFA2a, OsHSFA2b, OsHSFA7a, OsHSFA8a, OsHSFC2a, OsHSFC2b* and *Os06g22610* were specifically down-regulated in dark-induced senescence. Four *HSFs* (*OsHSFA2e, OsHSFA3a, OsHSFA6b,* and *OsHSFB2a*) were down-regulated in both flag leaf and dark-induced senescence.

Generation of transgenic lines for altered expression levels of selected HSFs

Plant HSFs are known for their regulatory role in plant growth and development. Despite their important regulatory role, information regarding the specific function of different HSFs in plants is still limited. The expression profiling study performed by us, indicated the role of *OsHSFA7a,* and *OsHSFC1b* in flag leaf and dark-induced senescence in rice. Since plant senescence is an important plant process that affects crop yield, we investigated the role of OsHSFA7a and OsHSFC1b in senescence by generating transgenic rice lines to manipulate levels of *OsHSFA7a* and *C1b*. For over-expression lines, selected HSFs was

expressed under the control of strong and constitutive maize ubiquitin 1 promoter. Artificial miRNA approach was employed for silencing the expression of *OsHSFA7a* and *C1b* in transgenic rice lines. The stable transgenic lines were monitored for developmental phenotypes and progression of senescence to gain an insight into the role of these HSFs in plant development and senescence.

Generation of transgenic lines over-expressing *OsHSFA7a* in rice

The amplified full-length coding sequence of *OsHSFA7a* was cloned in the Gateway entry vector, pENTR D-TOPO, followed by its transformation in *E. coli* (**Figure 6A**). Colonies were screened for the presence of desirable insert using colony PCR with gene-specific forward and vector-specific reverse primers (**Figure 6B**). Plasmid DNA was isolated from positive clones and presence of desired insert was confirmed by sequencing using M13 forward primer. The resultant positive entry clone was employed for LR recombination reaction for mobilization into destination plant expression vector, pUH-GW (**Figure 7A**). pUH-GW is a Gateway compatible plant expression vector containing maize ubiquitin promoter and NOS terminator. It contains hygromycin selectable marker for plants. Cloning and presence of *OsHSFA7a* in expression vector were confirmed by employing colony PCR and sequencing (**Figure 7B**). The destination vector containing *OsHSFA7a* was mobilized into *Agrobacterium* and colony PCR was used to screen for the presence of plasmid (**Figure 7C**). Further, *Agrobacterium* harboring the expression vector was used for

the genetic transformation of scutellum-derived calli to generate over-expression transgenic lines (Toki et al., 2006).

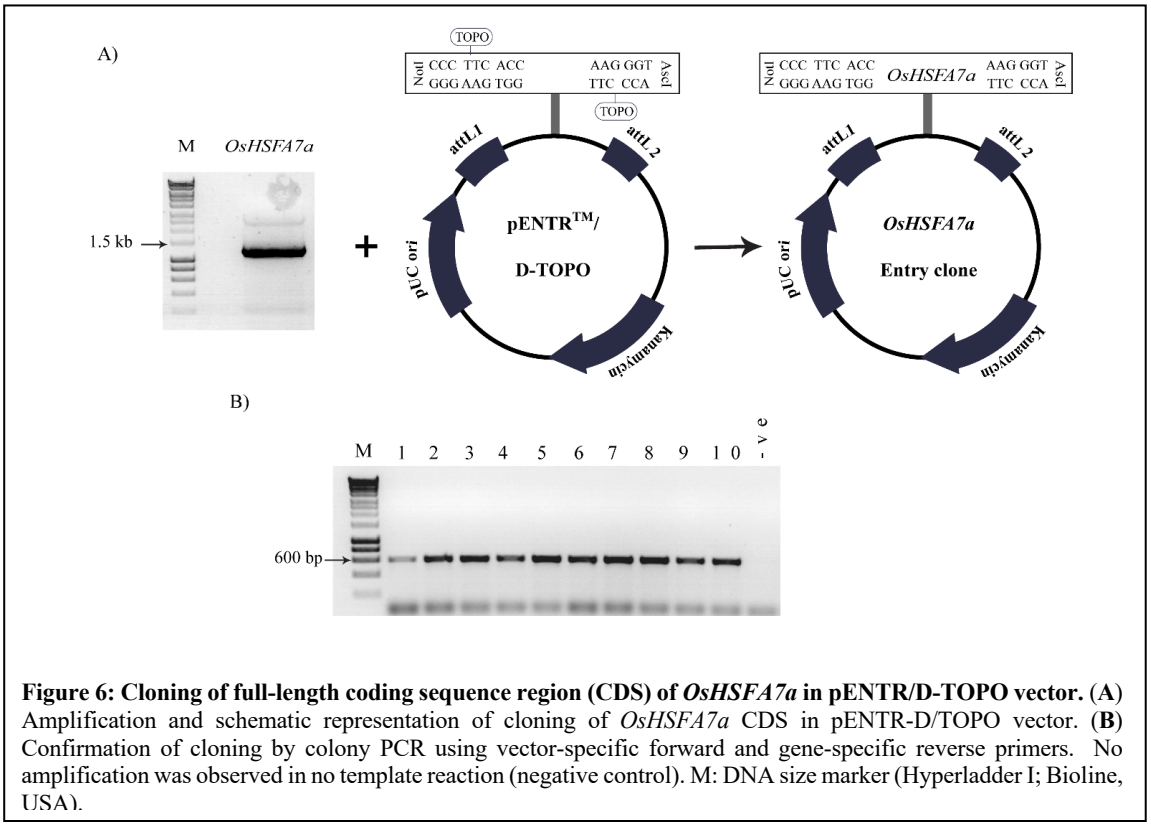


Figure 6: Cloning of full-length coding sequence region (CDS) of *OsHSFA7a* in pENTR/D-TOPO vector. (**A**) Amplification and schematic representation of cloning of *OsHSFA7a* CDS in pENTR-D/TOPO vector. (**B**) Confirmation of cloning by colony PCR using vector-specific forward and gene-specific reverse primers. No amplification was observed in no template reaction (negative control). M: DNA size marker (Hyperladder I; Bioline, USA).

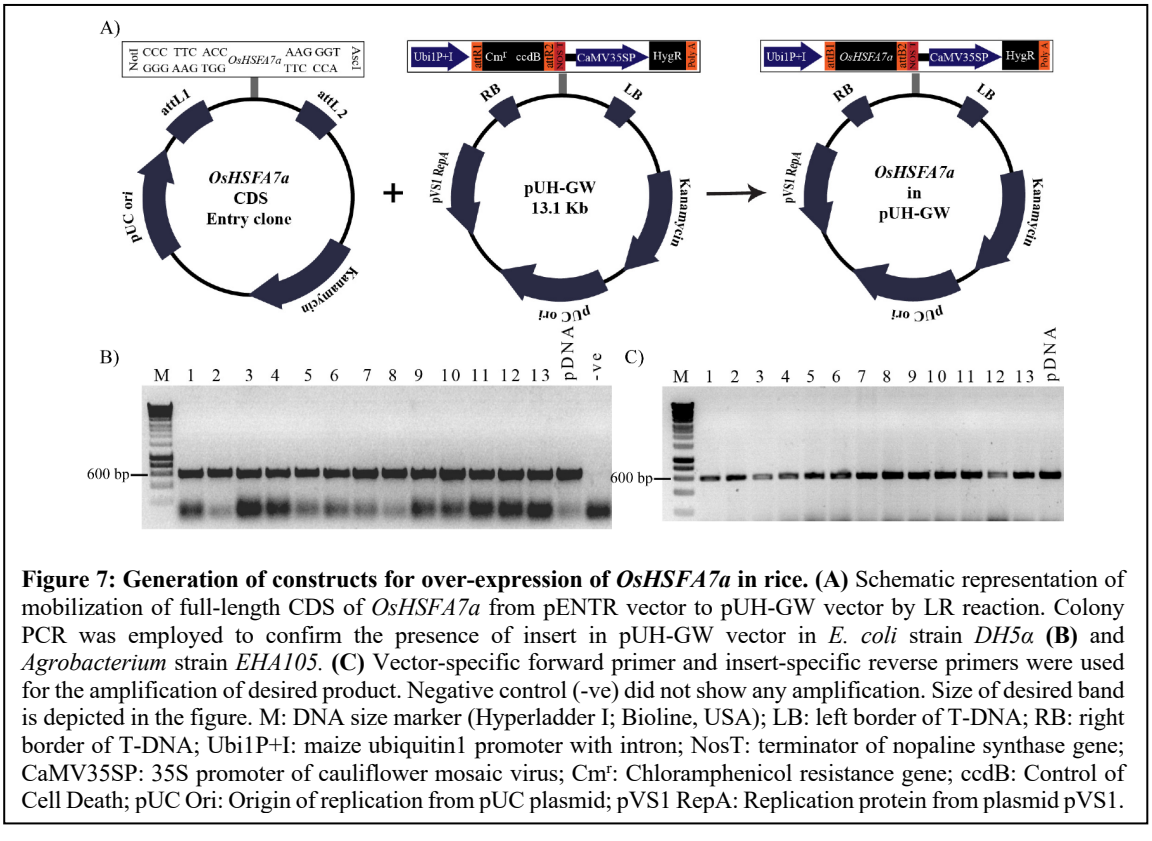


Figure 7: Generation of constructs for over-expression of *OsHSFA7a* in rice. (A) Schematic representation of mobilization of full-length CDS of *OsHSFA7a* from pENTR vector to pUH-GW vector by LR reaction. Colony PCR was employed to confirm the presence of insert in pUH-GW vector in *E. coli* strain *DH5α* **(B)** and *Agrobacterium* strain *EHA105*. **(C)** Vector-specific forward primer and insert-specific reverse primers were used for the amplification of desired product. Negative control (-ve) did not show any amplification. Size of desired band is depicted in the figure. M: DNA size marker (Hyperladder I; Bioline, USA); LB: left border of T-DNA; RB: right border of T-DNA; Ubi1P+I: maize ubiquitin1 promoter with intron; NosT: terminator of nopaline synthase gene; CaMV35SP: 35S promoter of cauliflower mosaic virus; Cm^r: Chloramphenicol resistance gene; ccdB: Control of Cell Death; pUC Ori: Origin of replication from pUC plasmid; pVS1 RepA: Replication protein from plasmid pVS1.

Molecular analysis of transgenic rice plants for overexpression of *OsHSFA7a*

The plantlets obtained after transformation of scutellum-derived rice calli were checked for transgene integration by PCR with gene-specific forward and NOS terminator-specific reverse primers (**Figure 8A**). Thirteen transgenic lines were found to contain the transgene cassette. The authenticity of PCR was confirmed by absence of transgene amplification in the untransformed plants (UT) and negative control. Further, quantitative PCR (qPCR) was employed to check the relative expression of *OsHSFA7a* in these transgenic lines. Four

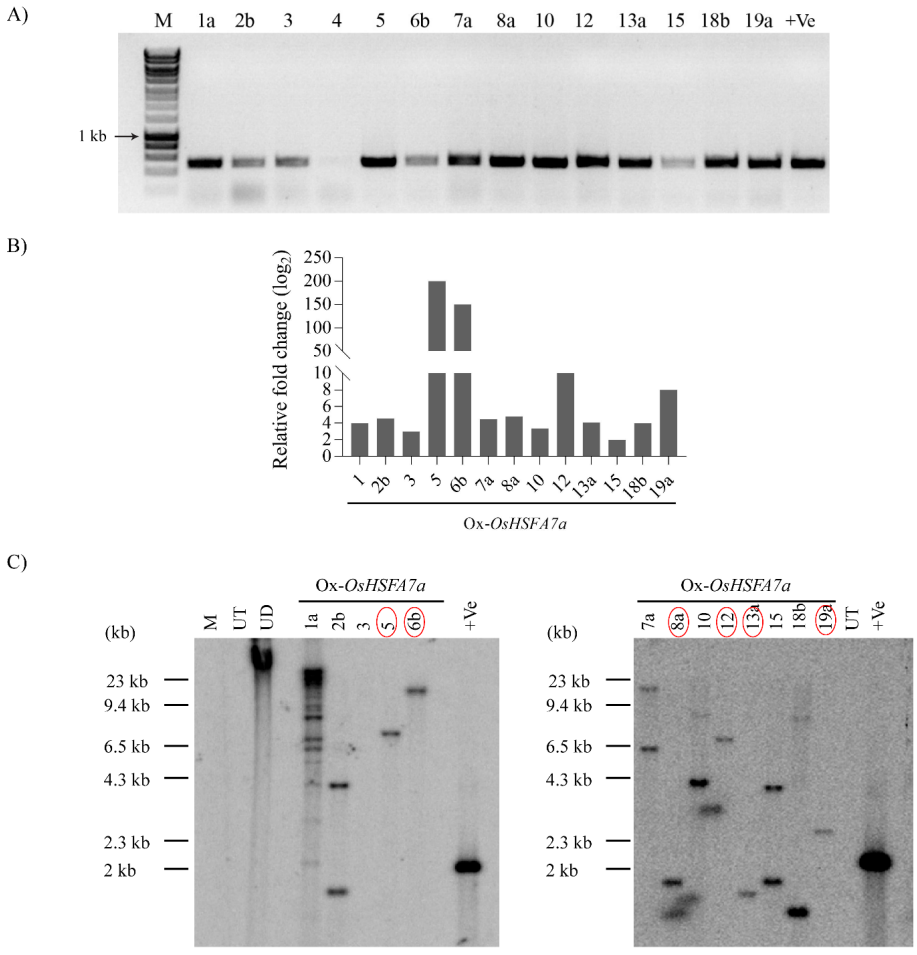


Figure 8: Molecular analysis of transgenic rice plants for overexpression of *OsHSFA7a* (OX-*OsHSFA7a*). (A) Confirmation of T_0 rice transgenic plants by genomic DNA PCR. Gene-specific forward and NOS termintaor-specific reverse primers were used for the amplification of desired product. Untransformed (UT) plants did not show any amplification. Size of desired band is depicted in the figure. **(B)** Expression levels of *OsHSFA7a* in transgenic plants were determined by quantitative PCR and normalized fold change (log_2 scale) was calculated relative to that in untransformed (UT) plants. For normalization *eEF-1α* was used as internal control. **(C)** Southern blot analysis of T_0 transgenic rice plants for confirming integration and determination of copy number of T-DNA. 10 µg of genomic DNA was digested with *Bgl*II and hybridized with a radiolabeled fragment corresponding to ubiquitin promoter. 2 kb fragment corresponding to ubiquitin promoter was used as a positive control. The numbers indicated above the blot denote independent transgenic rice lines. Transgenic rice lines with single copy transgene insertions are highlighted in red color. kb: kilobases; UD: undigested transgenic line DNA; +Ve: positive control DNA.

lines (5, 6b, 12 and 19a) showed high transgene expression with respect to the UT plants

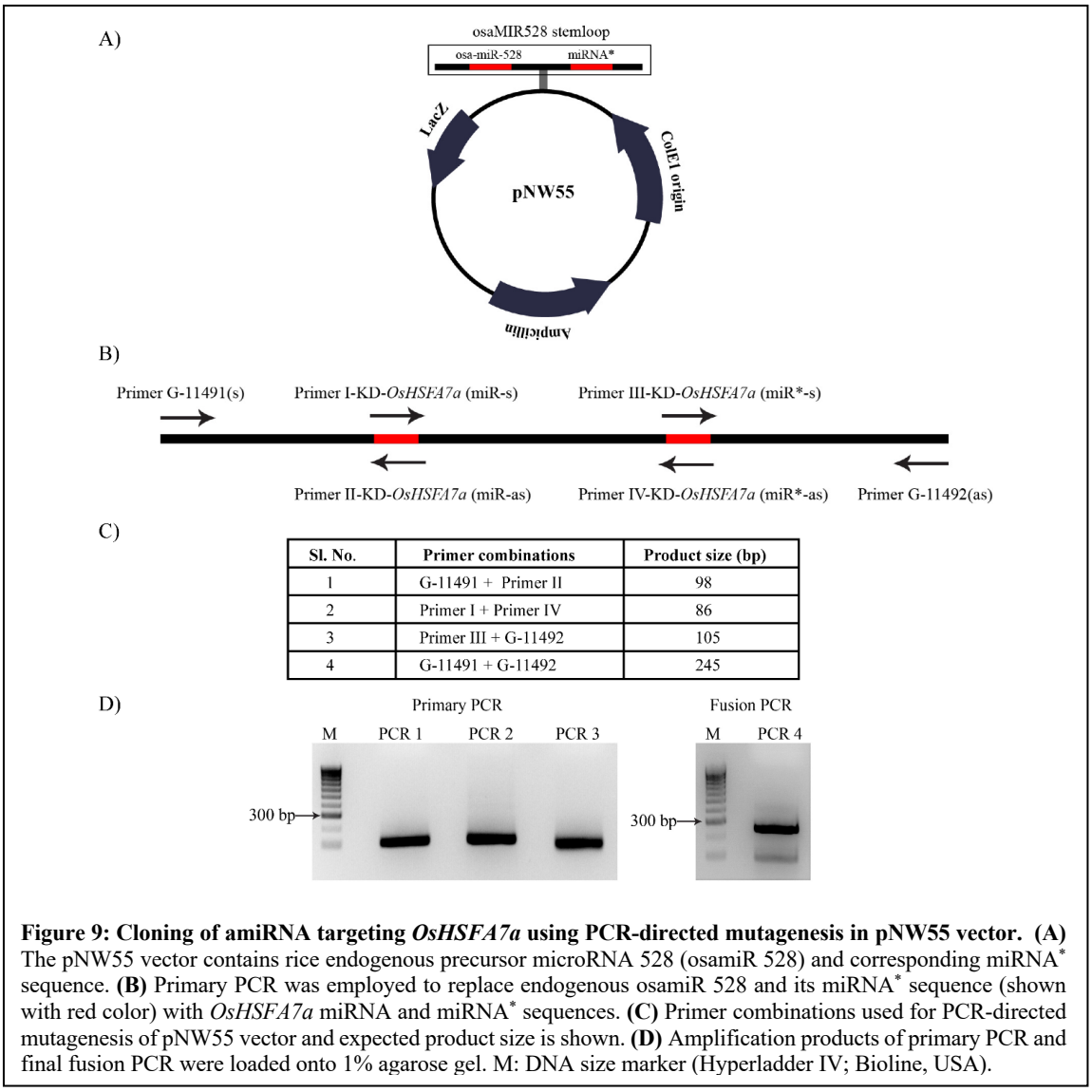
(**Figure 8B**). Among the four high expression lines line, 5 and 6b had exceptionally high expression of the transgene (>100-fold change on a log2 scale).

For confirming the transgene integration and to determine the copy number of the transgene in transgenic lines Southern blot analysis was performed. An equal quantity of genomic DNA (10 µg) isolated from thirteen transgenic rice lines and UT was digested with *Bgl*II, followed by electrophoresis on 1% agarose gel and subsequent capillary transfer to charged Nylon membrane. The membrane was probed with radiolabeled ubiquitin promoter-specific probe. Out of the thirteen transgenic lines analyzed, six lines (5, 6b, 8a, 12, 13a and 19a) had insertion of single copy of the transgene (**Figure 8C**). No band was detected in the UT plants. The pattern of the bands obtained by Southern analysis suggested that transgene integration occurred at different positions in different transgenic lines and therefore, they were considered as independent transgenic events. Based on Southern analysis and gene expression studies two transgenic rice lines were selected as they exhibited insertion of single copy of the transgene along with its high expression and they were named as OX-5 and OX-6b. These two lines were further propagated and screening for homozygous lines for transgene insertion was carried out with T1 seeds. However, no homozygous lines could be obtained for overexpression of *OsHSFA7a*. Heterozygous lines were further propagated and T2 seeds were again screened for obtaining homozygous lines but with no success as only heterozygous and UT plants were obtained. The morphological comparisons were performed with heterozygous lines of OX-*OsHSFA7a* whose seeds were first germinated on selection medium and only hygromycin-resistant plants were included in the analysis.

Generation of transgenic rice plants for silencing of *OsHSFA7a*

Artificial microRNA (amiRNA) approach was used for silencing the endogenous expression levels of *OsHSFA7a*. Web MicroRNA Designer server tool (WMD3 server; http://wmd3.weigelworld.org/cgi-bin/webapp.cgi? page=Home; project=stdwmd) was employed to design an amiRNA which specifically targets *OsHSFA7a*, with no predicted off- targets in rice. The miRNA528 sequence and the corresponding miRNA* sequences in the amiRNA cloning vector pNW55 were replaced with the *OsHSFA7a* specific amiRNA and its antisense sequence using overlapping PCRs (**Figure 9A, B, D**). Primers designed from WMD3 server which were specific to the *OsHSFA7a* amiRNA was used in the overlapping PCRs (**Figure 9C**). The PCR amplified *OsHSFA7a*^{amiR} product was cloned in pENTR/D-Topo vector (**Figure 10**). Colonies were screened for the presence of desirable

insert using colony PCR with insert-specific forward and vector specific reverse primers. Positive plasmids which contained *OsHSFA7a*^{amiR} were confirmed with sequencing and



Sl. No.	Primer combinations	Product size (bp)
1	G-11491 + Primer II	98
2	Primer I + Primer IV	86
3	Primer III + G-11492	105
4	G-11491 + G-11492	245

Figure 9: Cloning of amiRNA targeting *OsHSFA7a* using PCR-directed mutagenesis in pNW55 vector. (A) The pNW55 vector contains rice endogenous precursor microRNA 528 (osamiR 528) and corresponding miRNA* sequence. **(B)** Primary PCR was employed to replace endogenous osamiR 528 and its miRNA* sequence (shown with red color) with *OsHSFA7a* miRNA and miRNA* sequences. **(C)** Primer combinations used for PCR-directed mutagenesis of pNW55 vector and expected product size is shown. **(D)** Amplification products of primary PCR and final fusion PCR were loaded onto 1% agarose gel. M: DNA size marker (Hyperladder IV; Bioline, USA).

subsequently used for the recombination reaction with destination vector pUH-GW (**Figure 11A**).

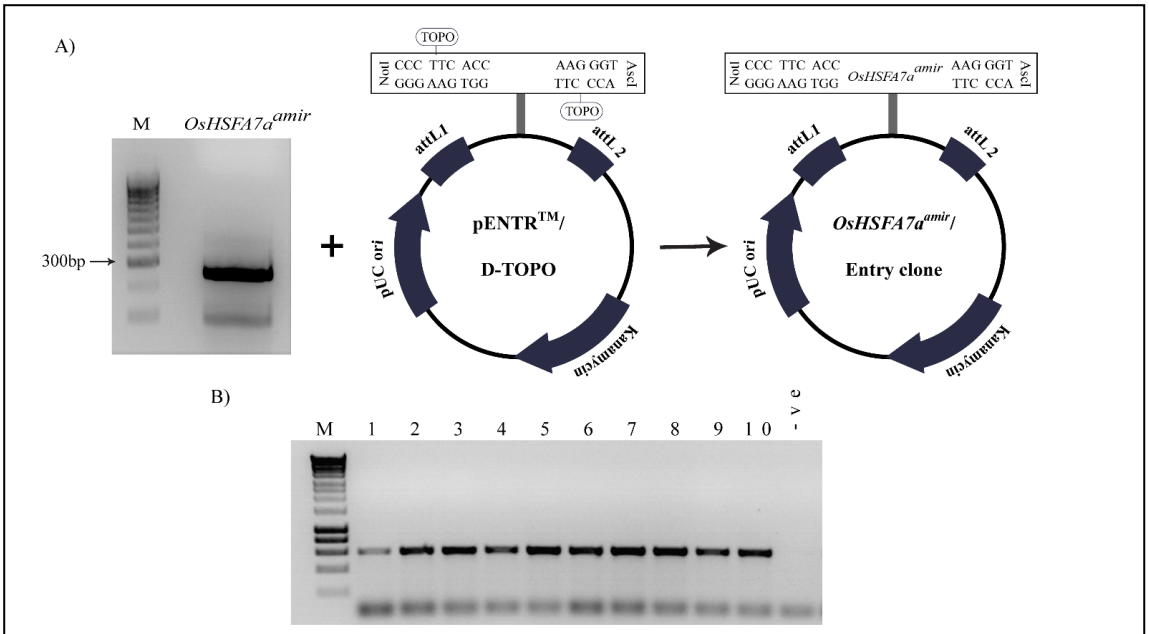


Figure 10: Cloning of *OsHSFA7a*^{amiR} in pENTR vector. (A) Schematic representation of cloning of amiRNA targeting *OsHSFA7a* (*OsHSFA7a*^{amiR}) into pENTR/D-TOPO vector. **(B)** Colony PCR was employed to confirm the presence of insert in pENTR/D-TOPO vector. Insert-specific forward and vector-specific reverse primers were used for the amplification of desired product. Negative control (-ve) did not show any amplification. Size of desired band is depicted in the figure. M: DNA size marker (Hyperladder I; Bioline, USA).

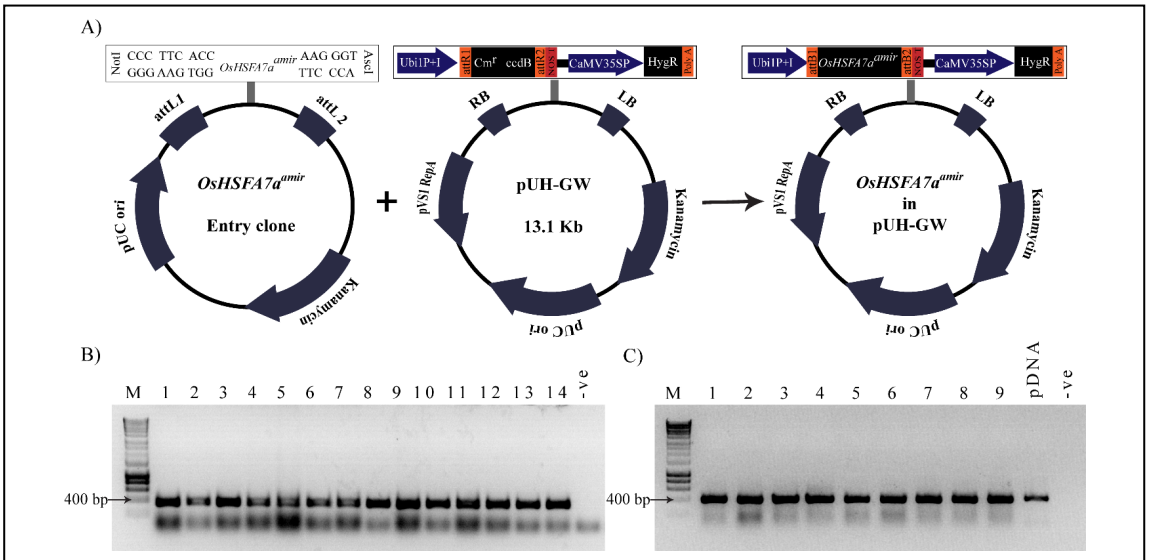


Figure 11: Generation of plasmid constructs for silencing of *OsHSFA7a* in rice. (A) Schematic representation of mobilization of *OsHSFA7a*^{amiR} from pENTR vector to pUH-GW vector by LR reaction. Colony PCR was employed to confirm the presence of insert in pUH-GW vector in *E. coli* strain *DH5α* **(B)** and *Agrobacterium tumefaciens* strain *EHA105*. **(C)** Vector-specific forward and gene-specific reverse primers were used for the amplification of desired product. Negative control (-ve) did not show any amplification. Size of desired band is depicted in the figure. M: DNA size marker (Hyperladder I; Bioline, USA), LB: left border of T-DNA, RB: right border of T-DNA, Ubi1P+I: maize ubiquitin1 promoter with intron, NosT: terminator of nopaline synthase gene, CaMV35SP: 35S promoter of cauliflower mosaic virus, Cm^r: chloramphenicol resistance gene, ccdB: control of cell death gene, pUC Ori: origin of replication from pUC plasmid, pVS1 RepA: replication protein from plasmid pVS1.

This cloning resulted in the constitutive expression of hairpin structure producing amiRNA precursor under the control of maize ubiquitin promoter. Cloning and presence of

OsHSFA7a^{amiR} in expression vector were confirmed by employing colony PCR and sequencing (**Figure 11B**). The positive destination vector containing *OsHSFA7a*^{amiR} was mobilized into *Agrobacterium* and further screened for the presence of desired insert using colony PCR (**Figure 11C**). Further, *Agrobacterium* harboring the expression vector was used for transforming scutellum-derived rice calli.

Molecular analysis of transgenic rice plants for silencing of *OsHSFA7a*

Genomic PCR was employed for checking the integration of *OsHSFA7a*^{amiR} expression cassette in transgenic plants. The reliability of PCR was confirmed by the absence of amplification in the untransformed (UT) plants and negative control (**Figure 12A**). Out of the seventeen plants checked, thirteen plants showed the integration of *OsHSFA7a*^{amiR}

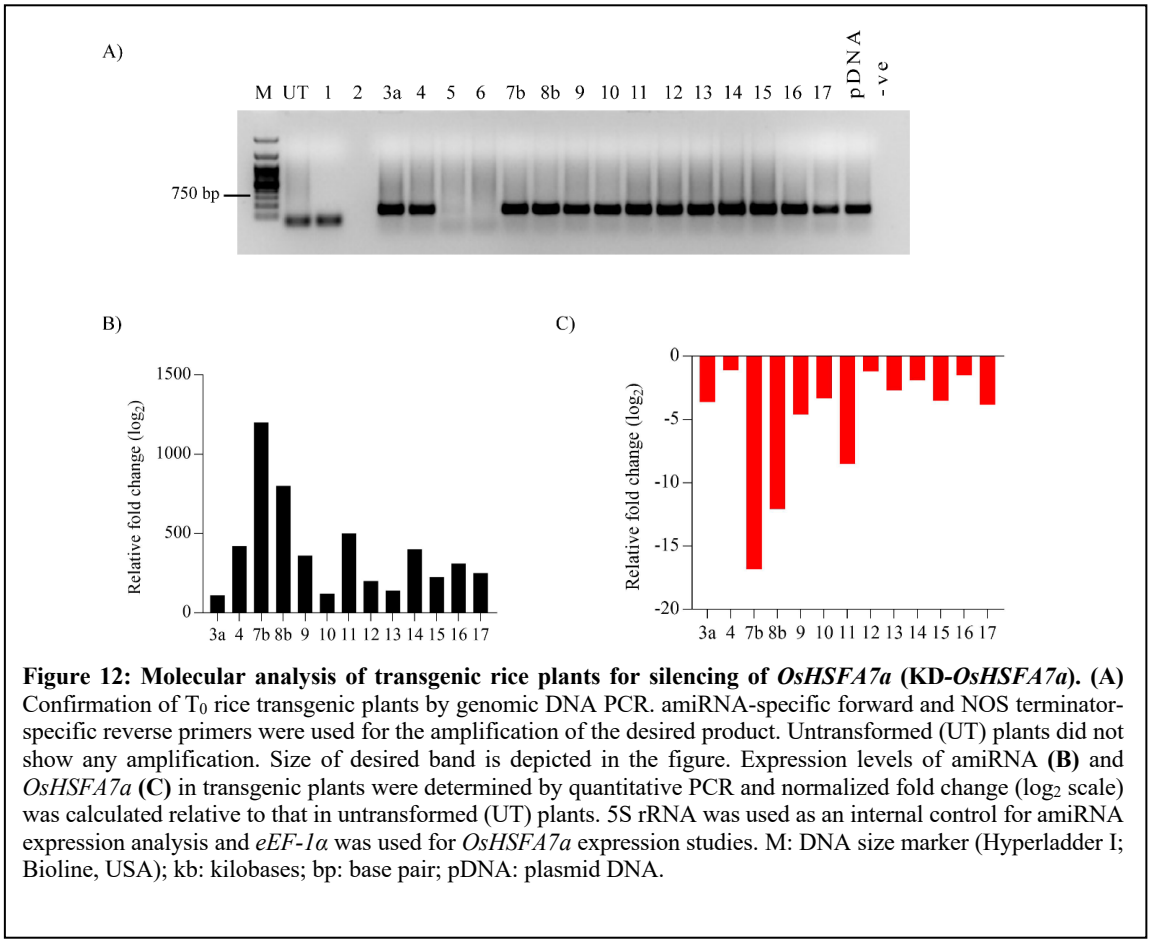


Figure 12: Molecular analysis of transgenic rice plants for silencing of *OsHSFA7a* (KD-*OsHSFA7a*). (A) Confirmation of T_0 rice transgenic plants by genomic DNA PCR. amiRNA-specific forward and NOS terminator-specific reverse primers were used for the amplification of the desired product. Untransformed (UT) plants did not show any amplification. Size of desired band is depicted in the figure. Expression levels of amiRNA **(B)** and *OsHSFA7a* **(C)** in transgenic plants were determined by quantitative PCR and normalized fold change (log_2 scale) was calculated relative to that in untransformed (UT) plants. 5S rRNA was used as an internal control for amiRNA expression analysis and *eEF-1α* was used for *OsHSFA7a* expression studies. M: DNA size marker (Hyperladder I; Bioline, USA); kb: kilobases; bp: base pair; pDNA: plasmid DNA.

expression cassette. Expression levels of amiRNA and its target *OsHSFA7a* were checked by qPCR. Eight lines showed high expression levels of amiRNA (>250-fold change) with respect to the UT plants (**Figure 12B**). As anticipated reduced expression levels of *OsHSFA7a* were detected in these lines (**Figure 12C**). Among the analyzed silencing lines (KD lines), KD-7b and KD-8b showed very high expression level of amiRNA with

concomitant decline in levels of *OsHSFA7a* and therefore were selected for propagation. The homozygous plants were obtained for these two lines and were employed for morphometric analyses.

Phenotypic analysis of *OsHSFA7a* transgenic rice plants

Expression profiling of *OsHSFA7a* at different developmental stages of rice revealed that *OsHSFA7a* is specifically expressed in seeds. To further investigate the functional role of *OsHSFA7a* in rice, we performed the phenotypic analysis of transgenic rice plants with altered expression of *OsHSFA7a.* Seeds of transgenic and UT plants were germinated on half-strength MS medium and transferred to liquid RGM for 14 days. Morphometric parameters like shoot length and root length of three-week-old seedlings were analyzed. The *OsHSFA7a* over-expression transgenic lines (OX-*OsHSFA7a*) exhibited dwarf phenotype, while silencing lines (KD-*OsHSFA7a*) showed similar phenotype as UT plants

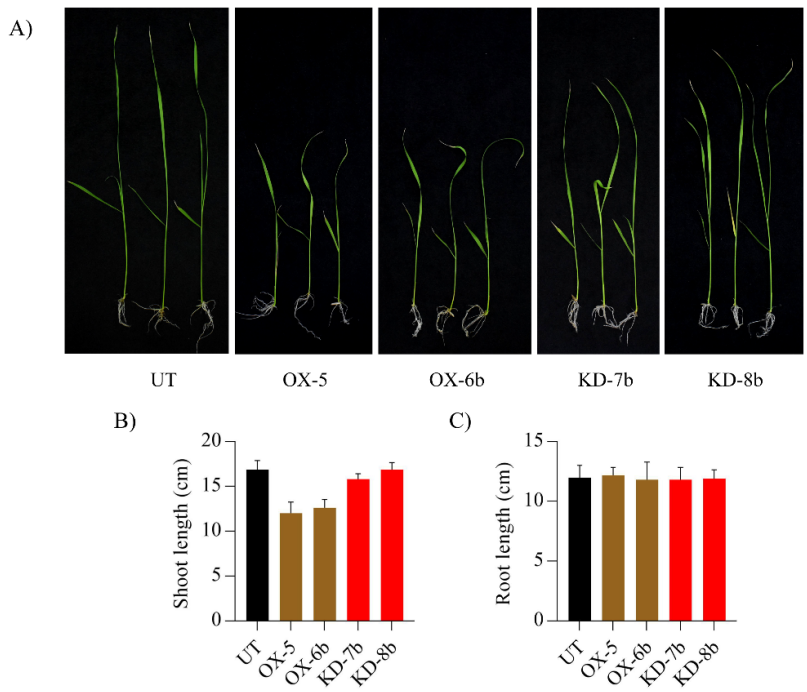


Figure 13: Phenotypic analysis of transgenic rice seedlings with altered expression of *OsHSFA7a*. (A) Photographs of 14-days-old seedlings of untransformed (UT), two overexpression lines (OX-*OsHSFA7a*) and two silencing lines (KD-*OsHSFA7a*). Bar graphs depict the seedling shoot length **(B)**, seedling root length **(C)**. Data shown is mean of 10 seedlings ± SD.

(**Figure 13A**). The dwarf phenotype of OX-*OsHSFA7a* lines was evident from the reduced

shoot length (**Figure 13B**). However, no discernible change in root length was observed in both the OX and KD lines (**Figure 13C**).

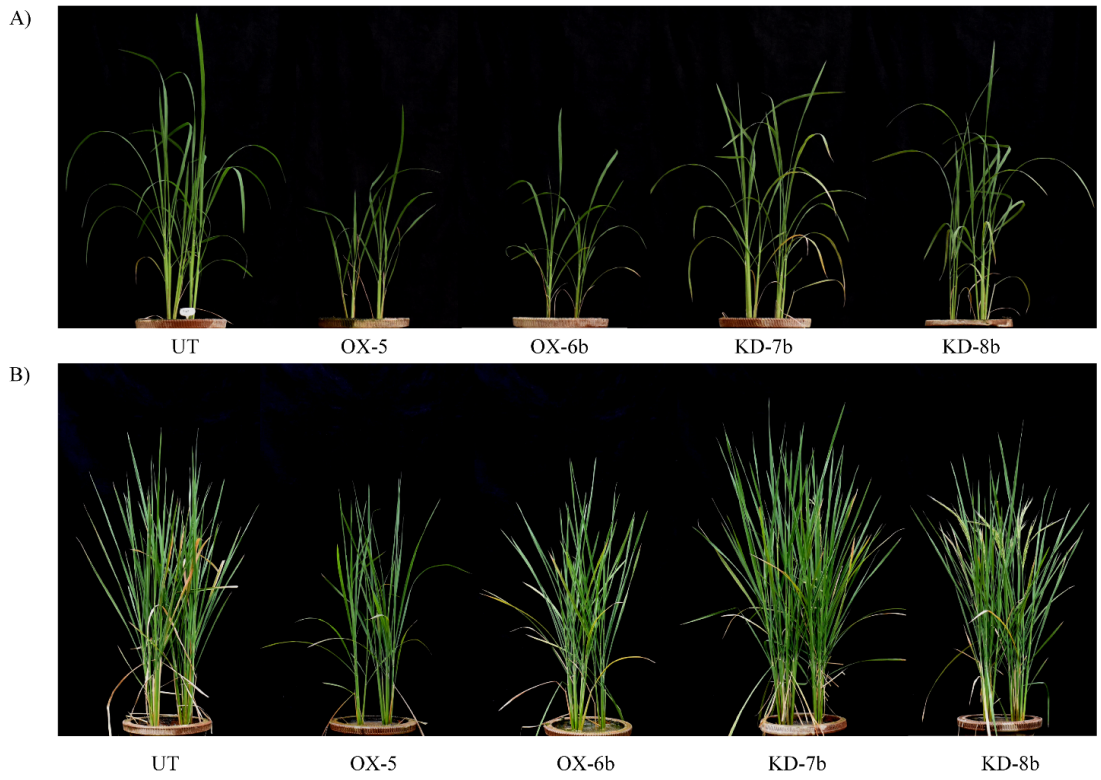


Figure 14: Phenotypic analysis of transgenic rice plants with altered expression of *OsHSFA7a* at different developmental stages. Phenotype of mature transgenic rice plants at 55 days post germination (**A**) and 110 days post germination **(B)** of untransformed (UT), overexpression lines (OX-*OsHSFA7a*) and silencing lines (KD-*OsHSFA7a*).

For phenotypic analysis of mature plants, transgenic rice seedlings along with UT plants were transferred to pots and grown under controlled conditions in the greenhouse. The developmental phenotype was monitored throughout the life cycle of transgenic rice plants. As seen with seedlings, a similar dwarf phenotype was observed in over-expression lines at late stages of development (**Figure 14**).

Analysis of *OsHSFA7a* transgenic plants during flag leaf and dark-induced senescence

Since *OsHSFA7a* showed higher accumulation of transcripts during flag leaf senescence, we examined whether manipulation of *OsHSFA7a* levels also affected the time required for flag leaf senescence in transgenic lines. Fully expanded flag leaves were tagged and monitored till the mid-senescence stage (chlorophyll content 45-60% of unsenesced flag leaf). Unexpectedly, both the overexpression and knock down *OsHSFA7a* transgenic lines

did not show any significant difference in flag leaf senescence compared to UT plants **(Figure 15)**. However, the reason for no discernible change in the pattern of flag leaf senescence in transgenic rice lines is not known. The expression levels of SAGs may be analyzed in these lines followed by checking the senescence in other plant tissues.

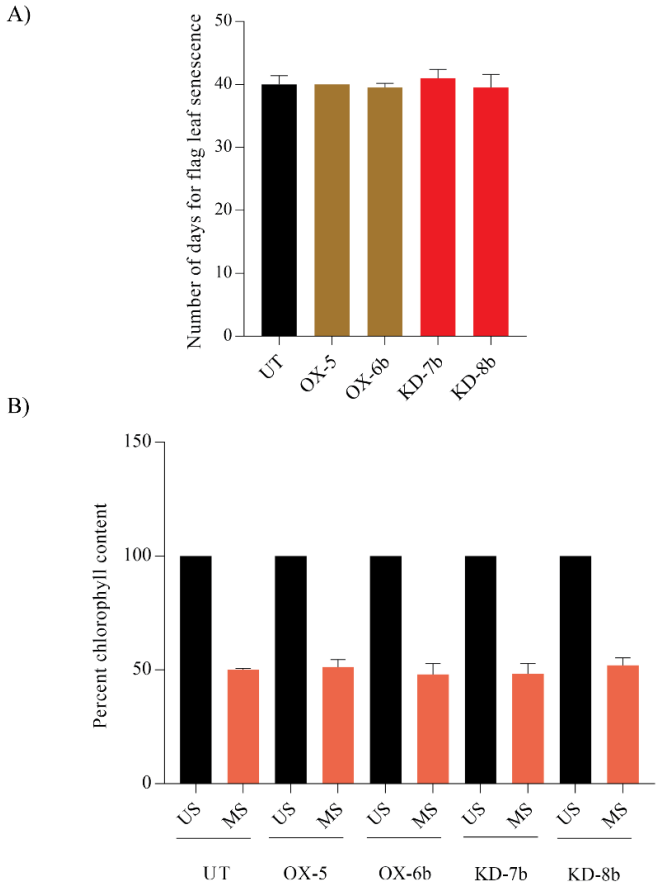


Figure 15: Flag leaf senescence in *OsHSFA7a* transgenic rice plants. (A) Mature unsenesced flag leaves (US) were monitored till the mid-senescence stage (MS) and the number of days to reach MS stage (45-60% of unsenesced stage) was noted for the UT, OX-*OsHSFA7a* and KD-*OsHSFA7a* lines. (**B**) Percentage chlorophyll content in senesced leaves as compared to fully expanded mature flag leaves of each line.

Although the expression levels of OsHSFA7a were downregulated during dark-induced senescence, we checked the phenotype of *OsHSFA7a* transgenic leaves incubated in dark conditions. Leaf bits from second leaves of *OsHSFA7a* transgenic and UT plants were floated on water and incubated in dark for three days. OX-*OsHSFA7a* and KD-*OsHSFA7a* lines exhibited delayed senescence phenotype during dark-induced senescence **(Figure 16A)**. The delayed senescence phenotype was supported with the higher chlorophyll content in *OsHSFA7a* transgenics as compared to UT after dark treatment **(Figure 16B)**.

The exact reason for the delay in senescence in both the overexpression and silencing lines of *OsHSFA7a* is not clear at present.

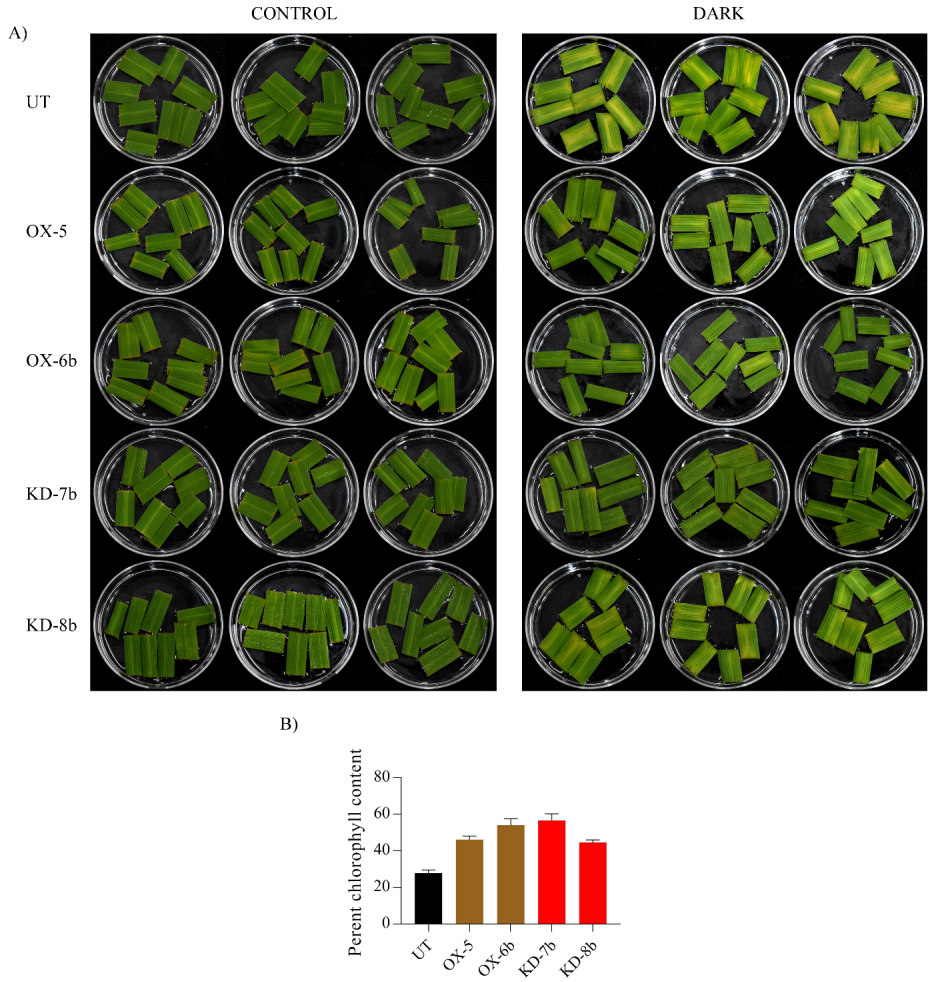


Figure 16: Dark-induced senescence in detached leaves of transgenic rice plants with altered expression of *OsHSFA7a*. Detached leaf senescence assay was performed to analyze the response of overexpression (OX) and silenced (KD) transgenic plants in dark. Detached leaf pieces were floated on water (control) and kept in dark to study progression of senescence. **(A)** Photographs of detached leaf pieces of UT, OX-*OsHSFA7a* and KD-*OsHSFA7a* after 3 days of incubation in dark. **(B)** Percentage chlorophyll content was estimated after 3 days of dark treatment with respect to the control samples. Error bars represent standard deviation (SD) of three biological replicates.

On the basis of all these observations it is concluded that although the transcript levels of *OsHSFA7a* are induced during natural senescence in rice, manipulation of its levels in transgenic rice lines alter the development of plant but no obvious changes were observed with the progression of senescence in flag leaves. It seems that the expression level of *OsHSFA7a* need to be critically maintained and possibly the perturbation in expression levels in overexpression or silencing lines disturbs the stoichiometry of interactions with other protein partners which may regulate plant development in rice. Efforts should be made to analyze the senescence of other leaves in transgenic rice lines.

Generation of transgenic rice lines for overexpression of *OsHSFC1b*

Full-length CDS of *OsHSFC1b* cloned in the entry vector was mobilized into destination plant expression vector, pUH-GW, by LR recombination reaction. Cloning and presence of *OsHSFC1b* in expression vector were confirmed by employing colony PCR and sequencing. The positive destination vector containing *OsHSFC1b* was mobilized into *Agrobacterium* and further screened for the presence of desired insert using colony PCR. Finally, *Agrobacterium* harbouring the expression vector was used for the transformation of scutellum-derived rice calli to generate overexpression transgenic lines.

Molecular analysis of transgenic rice plants for over-expression of *OsHSFC1b*

The tissue culture raised plants were checked for transgene integration by using genomic PCR with gene-specific forward and NOS terminator-specific reverse primers (**Figure 17A**). Sixteen rice lines were found to be contain the transgene.

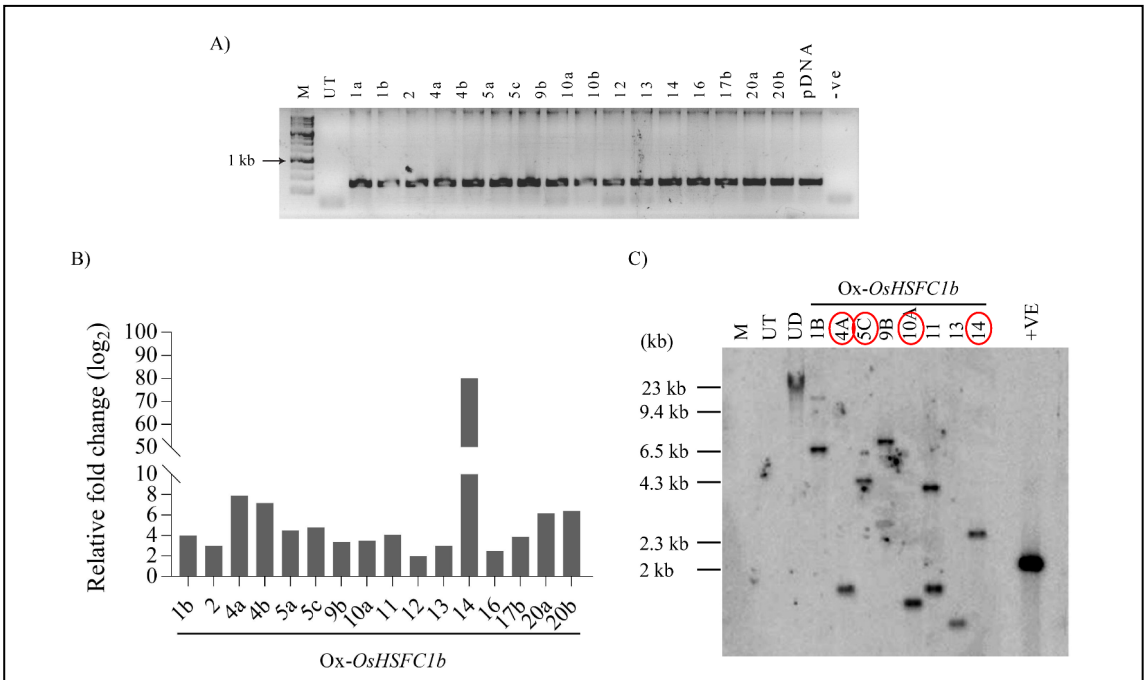


Figure 17: Molecular analysis of transgenic rice plants for overexpression of *OsHSFC1b* (OX-*OsHSFC1b*). **(A)** Confirmation of T_0 transgenic rice plants by genomic DNA PCR. Gene-specific forward and NOS terminator-specific reverse primers were used for the amplification of desired product. Untransformed (UT) plants did not show any amplification. Size of the desired band is depicted in the figure. **(B)** Expression levels of *OsHSFC1b* in transgenic plants were determined by quantitative PCR and normalized fold change (log_2 scale) was calculated relative to that in untransformed (UT) plants. For normalization *eEF-1α* was used as internal control. **(C)** Southern blot analysis of T_0 transgenic rice plants for confirming integration and determination of copy number of T-DNA. 10 µg of genomic DNA was digested with *Bgl*II and hybridized with a radiolabeled fragment of ubiquitin promoter. 2 kb fragment corresponding to ubiquitin promoter was used as a positive control. The numbers indicated above the blot denote independent transgenic lines. Transgenic lines with single copy transgene insertions are highlighted in red color. kb: kilobases; UD: undigested transgenic line DNA; pDNA: plasmid control; +VE: positive control DNA.

Further, qPCR was employed to check the relative expression of *OsHSFC1b* in these transgenic lines. Three independent lines showed high expression of the transgene (ranging from 6-fold to 70-fold change on a log2 scale) (**Figure 17B**). Further, the copy number of the transgene in transgenic plants was assessed using Southern blot analysis. An equal quantity of genomic DNA (10 µg) from transgenic lines and UT was digested with *Bgl*II and electrophoresed on 1% agarose gel. The resolved digested DNA were subsequently capillary transferred onto a charged Nylon membrane. The membrane was hybridized with radiolabelled ubiquitin promoter-specific probe (**Figure 17C**). The bands obtained after hybridization were analysed. Based on the number and pattern of band distribution it was concluded that four transgenic rice lines (OX-4a, 5c, 10a and 14) harboured single copy of the transgene and that they were obtained by independent transformation events.

Generation of transgenic rice lines for silencing of *OsHSFC1b*

To knock down the endogenous levels of *OsHSFC1b*, artificial microRNA (amiR) was employed as described earlier. Artificial miRNA specifically targeting *OsHSFC1b* was designed by using WMD3 server tool (Web MicroRNA Designer server tool; http://wmd3.weigelworld.org/cgi-bin/webapp.cgi?page=Home; project= stdwmd). By using overlapping PCRs, miR528 sequence and the corresponding miRNA* sequence in the amiR vector pNW55 were replaced with the *OsHSFC1b* specific amiRNA and its antisense sequence. The final PCR product, *OsHSFC1b*-specific amiRNA precursor, was cloned in pENTR/D-Topo vector and subsequently transformed in *E. coli* cells. Colonies were screened for the presence of desirable insert using colony PCR with insert-specific forward and vector specific reverse primers. PCR-confirmed clones which contained *OsHSFC1b*^{amiR} were checked with sequencing and subsequently used for the recombination reaction with destination vector pUH-GW. Cloning and presence of *OsHSFC1b*^{amiR} in expression vector were confirmed by employing colony PCR and sequencing. The final destination vector containing *OsHSFC1b*^{amiR} was mobilized into *Agrobacterium* and further screened for the presence of desired insert using colony PCR. Further, *Agrobacterium* harbouring the expression vector was used for transforming scutellum-derived rice calli.

Molecular analysis of transgenic rice plants for silencing of *OsHSFC1b*

Genomic PCR was employed for checking the integration of *OsHSFC1b*^{amiR} expression cassette in transgenic plants. The authenticity of PCR was confirmed by the absence of amplification in the untransformed (UT) plants and negative control (**Figure 18A**). Out of thirteen transgenic lines checked, eight lines showed the integration of *OsHSFC1b*^{amiR}.

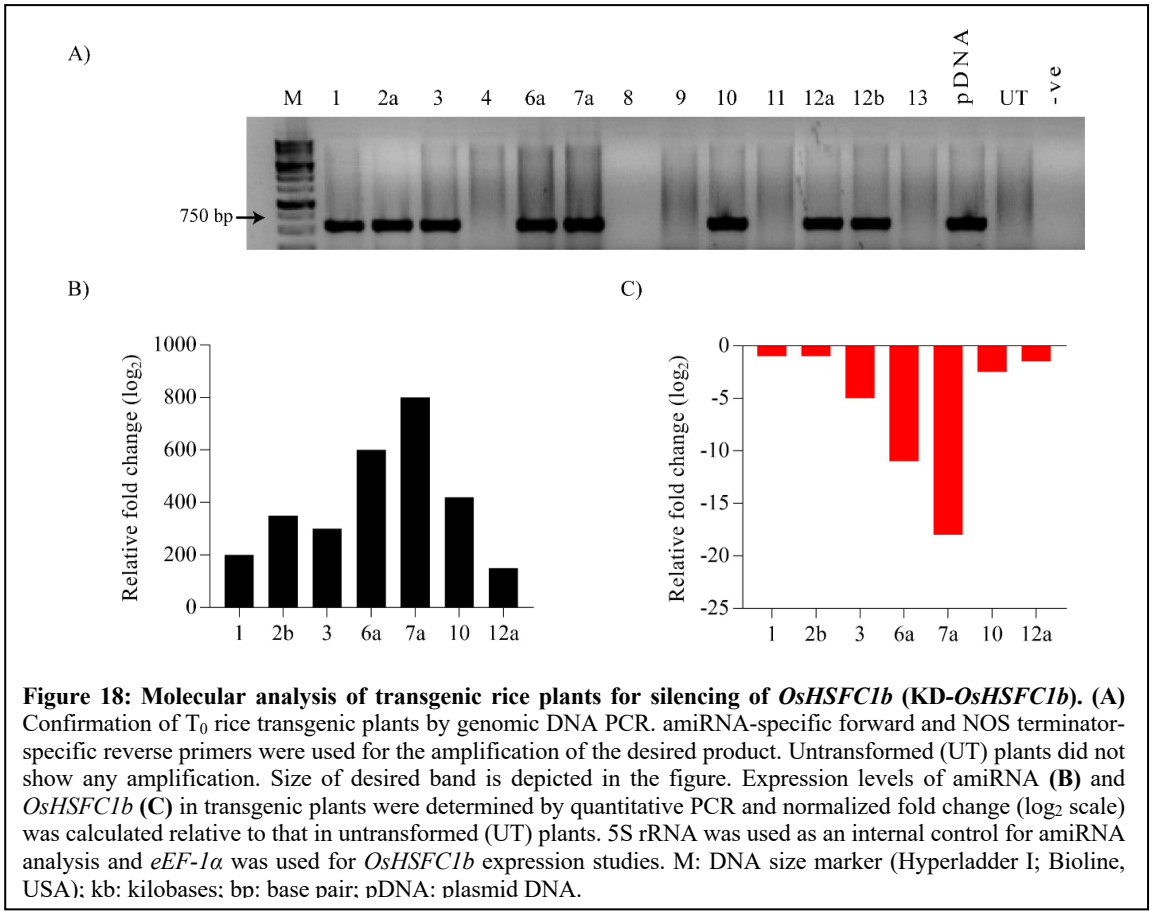


Figure 18: Molecular analysis of transgenic rice plants for silencing of *OsHSFC1b* (KD-*OsHSFC1b*). (A) Confirmation of T_0 rice transgenic plants by genomic DNA PCR. amiRNA-specific forward and NOS terminator-specific reverse primers were used for the amplification of the desired product. Untransformed (UT) plants did not show any amplification. Size of desired band is depicted in the figure. Expression levels of amiRNA **(B)** and *OsHSFC1b* **(C)** in transgenic plants were determined by quantitative PCR and normalized fold change (log_2 scale) was calculated relative to that in untransformed (UT) plants. 5S rRNA was used as an internal control for amiRNA analysis and *eEF-1α* was used for *OsHSFC1b* expression studies. M: DNA size marker (Hyperladder I; Bioline, USA); kb: kilobases; bp: base pair; pDNA: plasmid DNA.

Further, expression levels of *OsHSFC1b*^{amiR} were assessed in these transgenic lines by employing qPCR. Five KD-*OsHSFC1b* transgenic lines displayed high expression of amiRNA (>200-fold change) with respect to the UT plants (**Figure 18B**). Expression of the target gene OsHSFC1b was also analysed in KD-*OsHSFC1b* lines and it was found that two lines, KD-6a and KD-7a, exhibited more than 10-fold decline in expression level of *OsHSFC1b* (**Figure 18C**). Therefore, these two transgenic rice lines were selected for further analysis.

Phenotypic analysis of *OsHSFC1b* transgenic rice plants

The expression profiling data showed *OsHSFC1b* was expressed at high levels in leaves of active tillering phase and mature seeds. Therefore, to elucidate the functional role of OsHSFC1b in rice growth and development, we performed phenotypic analysis of the selected overexpression and silencing transgenic rice lines. No significant difference in

phenotype was observed in the seedlings of OX-*OsHSFC1b* and KD-*OsHSFC1b* lines when compared to UT plants (**Figure 19A**). These results were confirmed by analysing the morphometric parameters, including shoot length and root length (**Figure 19B and C**). In contrast to our observations, Schmidt et al. (2012) reported retarded growth in amiRNA

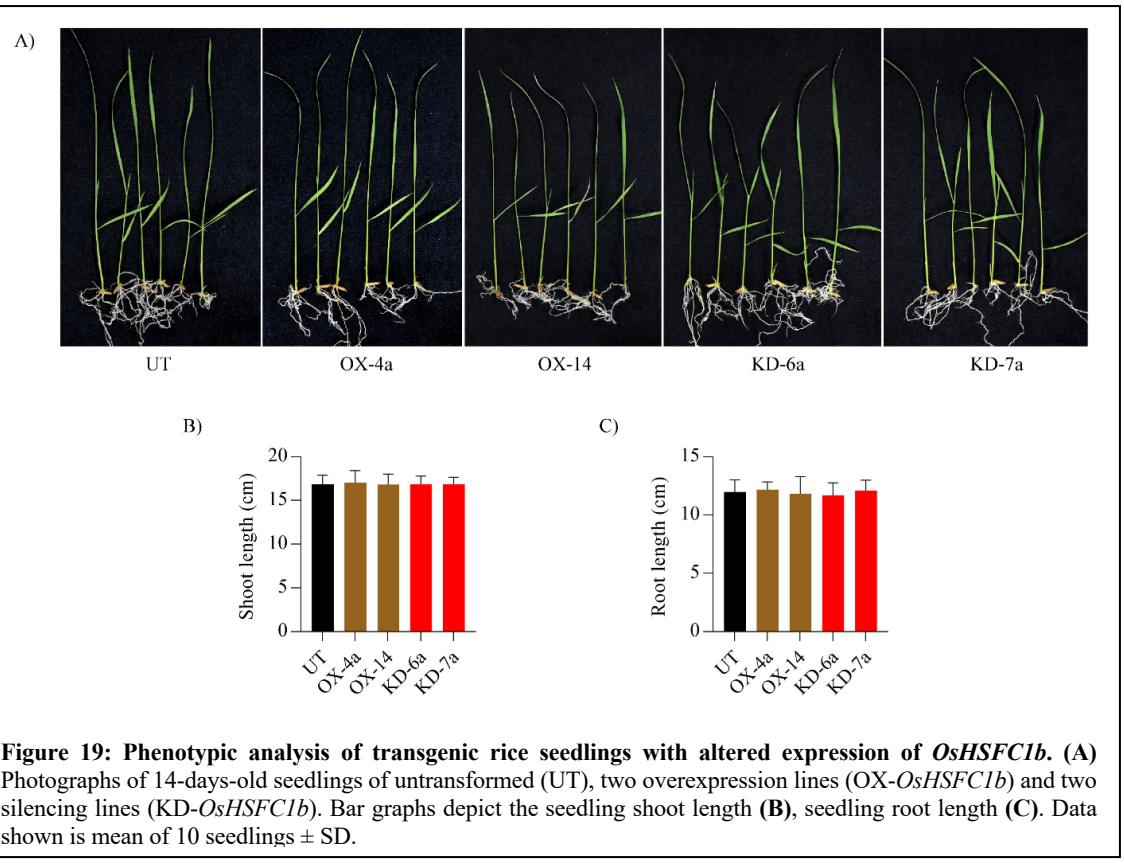


Figure 19: Phenotypic analysis of transgenic rice seedlings with altered expression of *OsHSFC1b*. (A) Photographs of 14-days-old seedlings of untransformed (UT), two overexpression lines (OX-*OsHSFC1b*) and two silencing lines (KD-*OsHSFC1b*). Bar graphs depict the seedling shoot length **(B)**, seedling root length **(C)**. Data shown is mean of 10 seedlings ± SD.

lines and T-DNA mutants of *OsHSFC1b* at 4-week-old seedling stage. This variability in phenotypes could be attributed to the extent of suppression of the expression levels of *OsHSFC1b* and the stage at which analyses were performed.

To further assess the phenotype of *OsHSFC1b* transgenic lines at maturity, seedlings of OX-*OsHSFC1b*, KD-*OsHSFC1b* and UT plants were transplanted to pots and grown in the field during the rice-growing season. Plants were photographed at different stages of development. No visible phenotypes were observed between two OX and two KD lines till the active tillering phase (55 days post germination or DPG) (**Figure 20A**). Interestingly, a gradual upsurge in plant growth was observed during and after the active tillering phase in OX-*OsHSFC1b* plants compared to the UT plants (**Figure 20**). Even though *OsHSFC1b* expression levels were constitutively high at all the stages of plant development, the robust growth was observed at active tillering phase. A plausible explanation for this stage-specific phenotype could be that the availability of OsHSFC1b

interactors or putative targets at active tillering phase. It would be worthwhile to perform genome-wide transcriptome analysis of *OsHSFC1b* transgenic lines at active tillering phase in order to understand the changes in gene expression due to overexpression of OsHSFC1b which could have contributed to the robust growth of OX plants. The robustness in OX-*OsHSFC1b* plant growth became significantly evident at 110 days post germination (**Figure 20B**). In contrary to the robust growth exhibited by OX-*OsHSFC1b* lines, KD-*OsHSFC1b* lines displayed retarded growth at 110 DPG. Also, *OsHSFC1b* transgenic plants showed significant variation in flowering time. KD-*OsHSFC1b* flowered at least ten

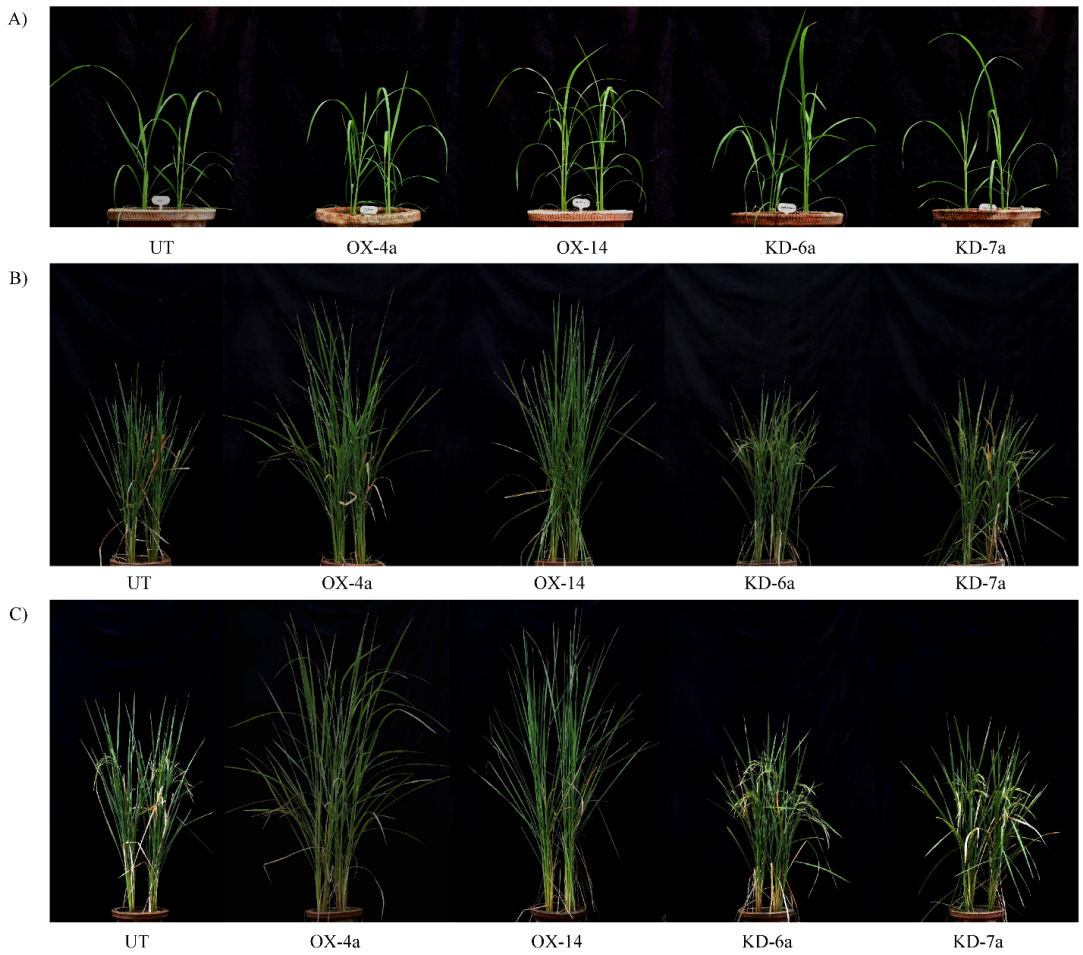


Figure 20: Phenotypic analysis of transgenic rice plants with altered expression of *OsHSFC1b* at different developmental stages. Phenotype of UT, OX-*OsHSFC1b* and KD-*OsHSFC1b* mature transgenic rice plants at 55 days post germination (DPG) **(A)**, 110 DPG **(B)** and 140 DPG **(C)**.

days before UT plants and showed an early flowering phenotype (**Figure 20C**). On the other hand, OX-*OsHSFC1b* lines exhibited a delayed flowering phenotype (~20 days later than UT plants). These results imply that *OsHSFC1b* is possibly involved in regulating the growth and development of rice plants, especially the stature of the plant and transition from vegetative to reproductive phase.

Analysis of natural flag leaf and dark-induced senescence in *OsHSFC1b* transgenic plants

The transcript of *OsHSFC1b* was significantly upregulated during senescence in flag leaves and coleoptile and during dark-induced senescence in detached leaves of rice (**Figure 21**). This prompted us to monitor flag leaf senescence in *OsHSFC1b* transgenic rice lines. Fully expanded flag leaves were tagged and observed till the mid-senescence stage (chlorophyll content 45-60% of unsenesced flag leaf). The number of days were calculated for flag leaves to senesce to the MS stage. Remarkably, flag leaves of OX-O*sHSFC1b* lines exhibited delayed senescence phenotype (**Figure 21**). A 10-12 days delay in flag leaf senescence was observed in OX-*OsHSFC1b* lines, while flag leaves of KD-*OsHSFC1b* lines senesced early (9-10 days) than UT plants. It is possible that the photosynthates

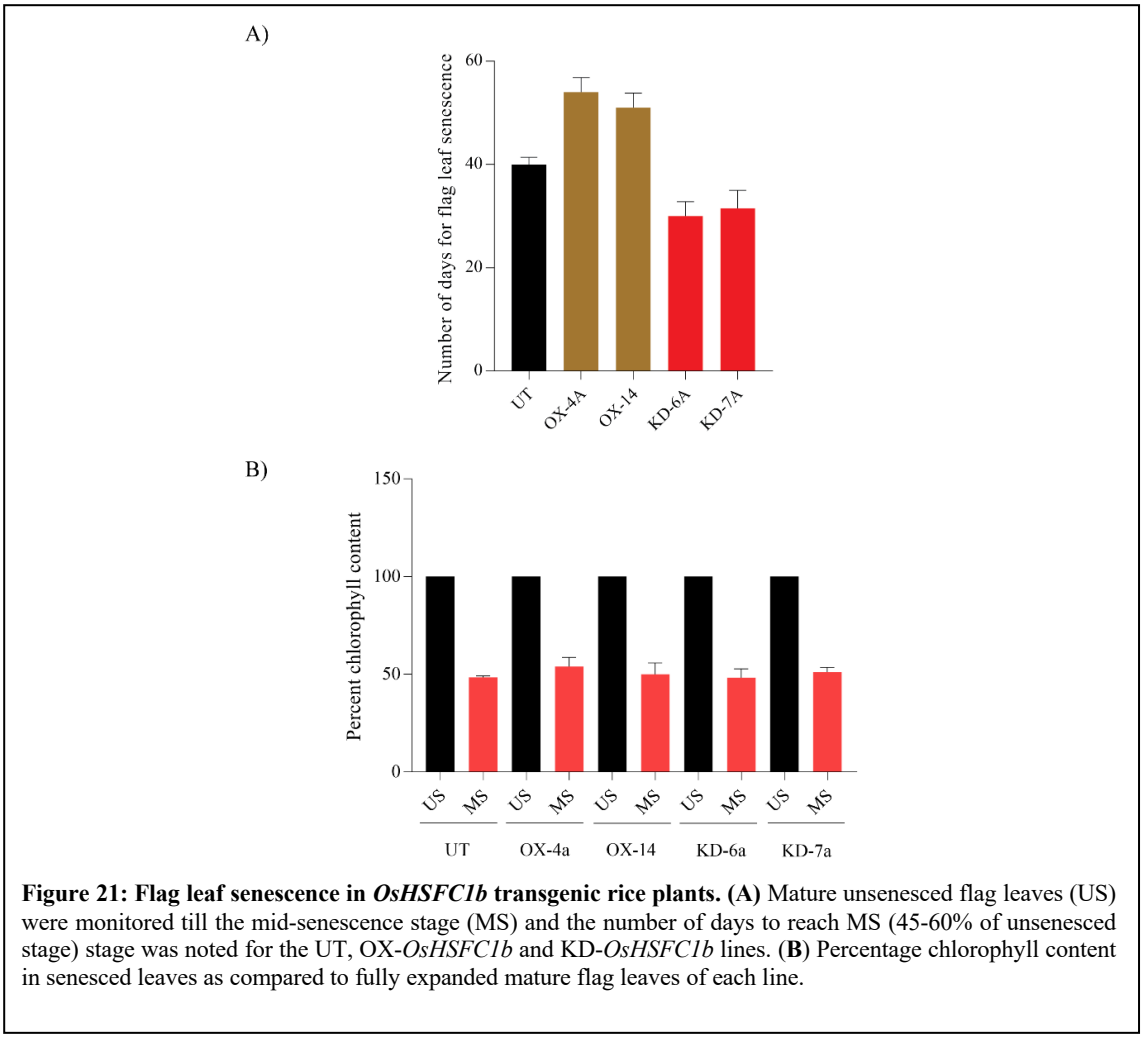


Figure 21: Flag leaf senescence in *OsHSFC1b* transgenic rice plants. (A) Mature unsenesced flag leaves (US) were monitored till the mid-senescence stage (MS) and the number of days to reach MS (45-60% of unsenesced stage) stage was noted for the UT, OX-*OsHSFC1b* and KD-*OsHSFC1b* lines. (**B**) Percentage chlorophyll content in senesced leaves as compared to fully expanded mature flag leaves of each line.

produced during this prolonged lifespan of flag leaves could have contributed to seed weight even with the reduced spikelet fertility in OX-*OsHSFC1b* lines.

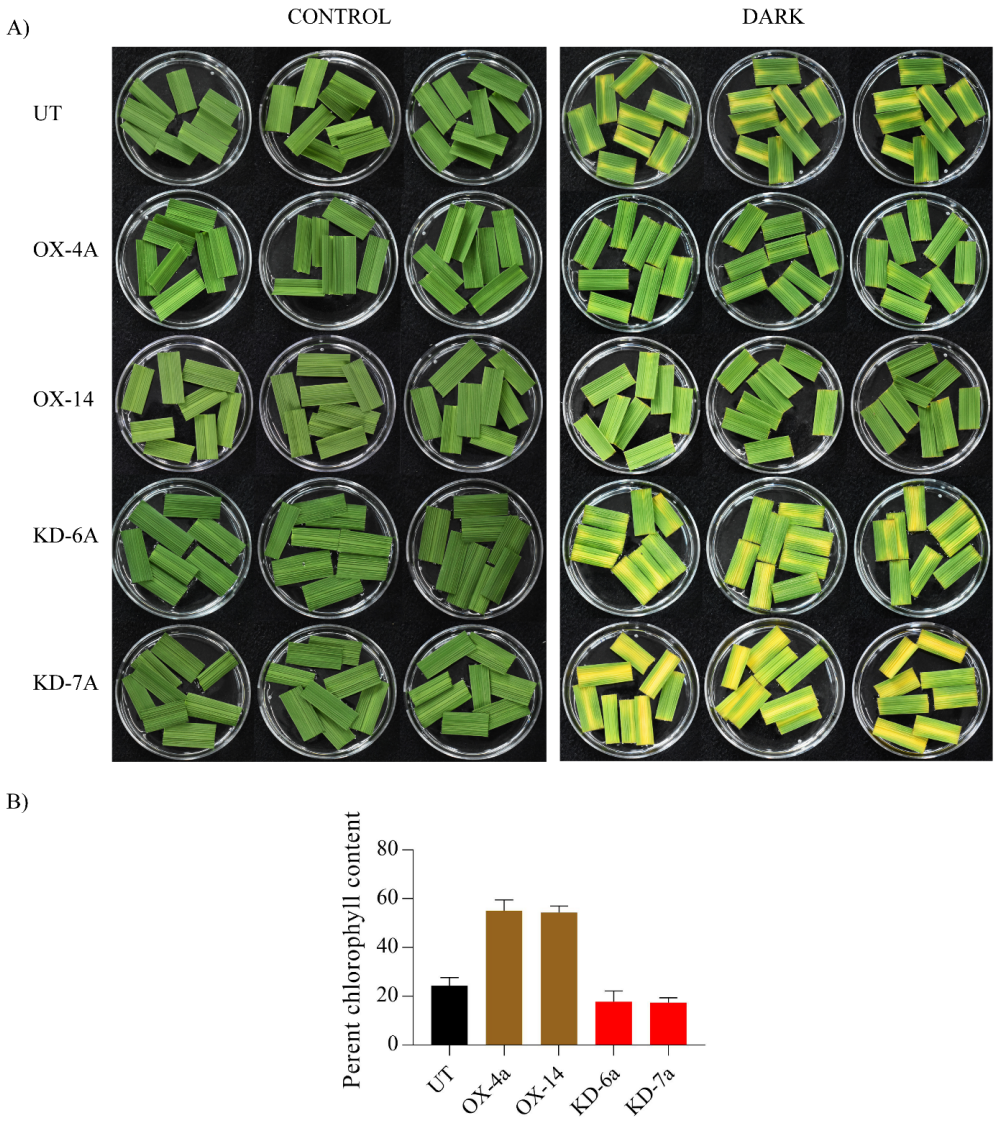


Figure 22: Dark-induced senescence in detached leaves of transgenic rice plants with altered expression of *OsHSFC1b*. Detached leaf senescence assay was performed to analyze the response of overexpression (OX-*OsHSFC1b*) and silencing (KD-*OsHSFC1b*) transgenic plants in dark. Detached leaf pieces were floated on water (control) and kept in dark to study progression of senescence. **(A)** Photograph of detached leaf pieces of UT, OX-*OsHSFC1b* and KD-*OsHSFC1b* after 3 days of incubation in dark. **(B)** Percentage chlorophyll content was estimated after 3 days of dark treatment with respect to the control samples. Error bars represent standard deviation (SD) of three biological replicates.

Dark-induced senescence is a widely employed method for assaying leaf senescence in plants. For confirming the role of *OsHSFC1b* in the senescence process, we monitored the senescence in detached leaves of OX-*OsHSFC1b*, KD-O*sHSFC1b* and UT plants under dark conditions. Leaf pieces of OX, KD and UT plants were floated on water and incubated in dark condition for three days and photographs were taken. In agreement with the results obtained with flag leaf senescence assay, OX-*OsHSFC1b* lines exhibited

delayed senescence phenotype, while KD-*OsHSFC1b* lines displayed accelerated senescence (**Figure 22A**). These results were supported with retention of higher chlorophyll content in OX lines as compared to UT and KD lines (**Figure 22B**). All these results substantiate the role of OsHSFC1b as a negative regulator of natural and dark-induced senescence in rice.

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